Analysis of RNAs that Interact with Herpes Simplex Virus Type 1 Immediate Early Protein ICP27

by

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Abstract

ICP27 is an HSV-1 immediate early protein required for the switch from early to late gene expression. It is a multifunctional, 63kDa protein that has homologues throughout the *Herpesviridae,* reflecting the importance of this protein to the virus. This may also indicate that ICP27's regulatory role is maintained in the identified homologues. Deleting ICP27 from the HSV-1 genome severely affects virus production, and, although not absolutely required for viral DNA replication, ICP27 enhances the levels of replicated DNA by up to 10-fold. Regulatory roles of this protein include effects on transcription, RNA stability, splicing, export and translation. ICP27 binds viral and cellular RNAs, and recently a yeast three-hybrid (Y3-H) analysis has identified an array of viral RNA sequences that interact with ICP27.

Presented here are analyses of functional assays using a selection of the Y3-H identified RNA sequences inserted into the 5' untranslated region (UTR) of a chloramphenicol acetyl transferase (CAT) reporter plasmid. A set of plasmids was transfected into baby hamster kidney (BHK) cells and CAT assays carried out to analyse the effects of the sequences on gene expression. Results indicated that expression was increased when ICP27-binding sequences were present even though no viral proteins were present. Comparison of sequences revealed that no common activation code or RNA structure was present that could be responsible for the increase in CAT gene expression. The levels of expression were further determined in the presence of wild type (wt), ICP27-null or ICP27 mutant HSV-1 infection to investigate whether ICP27 had any affect on CAT expression when ICP27-binding sequences were present. Interestingly, enhanced expression was observed during wt HSV-1–infection when ICP27-binding sequences were present, whereas little to no enhancement was observed during ICP27-null or mutant virus infections. However, a higher fold increase in CAT gene expression was observed during a HSV-1 infection when ICP27-binding sequences were not present. This indicated that an inhibitory effect on CAT expression observed during wt HSV-1 infection when ICP27-binding sequences were present was ICP27-dependent. As a control, a noncoding, protein binding regulatory HPV RNA sequence was inserted into the CAT reporter plasmid, transfected into BHK cells and then infected with wt HSV-1, ICP27-null or ICP27 mutant viruses. Surprisingly, CAT expression was increased,

albeit to only a limited extent, indicating that the previously observed increase in gene expression was not HSV-1 sequence specific. However, upon transfection of plasmids with the HPV control sequence inserted in the reverse orientation and a subsequent infection of cells no increase in CAT expression was observed.

Analysis of the control sequence in the reverse orientation identified a shortage in G residues, which led to the construction of CAT reporter plasmids containing homopolymer sequences inserted into the 5'UTR. A series of transfections and subsequent mock, wt HSV-1 or ICP27-null virus infections were carried out using this set of constructs. CAT assay analysis revealed an increase in CAT expression, to levels similar to those observed when the HSV-1 sequences were present, when poly(G) homopolymers were used as inserts during wt HSV-1 infection, whereas poly(A), (C) and (T) gave low levels of expression. Little to no enhanced gene expression was observed during ICP27-null virus infection when homopolymer sequences were present indicating firstly a preference during an HSV-1 infection for G-rich sequences and secondly that enhanced gene expression observed during infection was ICP27-dependent. This was a significant finding as the HSV-1 genome is 68% GC-rich, indicating a preference for ICP27 to enhance expression of viral genes during infection.

Preliminary experiments were undertaken to identify the role of ICP27 when CAT gene expression was enhanced. Reporter plasmids containing HSV-1 sequence inserts were transfected into BHK cells and infected with wt HSV-1 or ICP27 mutant viruses. Total RNA was isolated from these cells and CAT RNA was quantified using quantitative reverse transcription and polymerase chain reaction (Q-RT PCR). No significant change in levels of CAT RNA was detected, irrespective of whether the RNA had been isolated from cells that had been infected with wt HSV-1 or ICP27 mutant viruses, indicating that the increase in CAT gene expression was not at the level of transcription. In further experiments nuclear and cytoplasmic fractions were isolated after a CAT reporter plasmid with an ICP27-binding sequence insert was transfected into BHK cells that were subsequently either mock or wt HSV-1 infected. CAT RNA levels were detected using Q-RT PCR and preliminary results identified no significant change in the levels of RNA whether the cells were mock or HSV-1 infected, indicating that enhanced CAT

expression occurs at the level of translation. These results, alongside recent publications linking ICP27 to translation machinery, support the hypothesis that ICP27 enhances the expression of G-rich sequences at the level of translation.

This is dedicated to the memory of

John Barklie Clements

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List of Figures and Tables

Abbreviations

A	adenine
aa	amino acid
ab	antibody
APS	ammonium persulphate
ARE	AU-rich element
ARE-BP	ARE-binding proteins
ASF/SF2	alternative splicing factor/splicing factor 2
α-TIF	alpha trans-inducing factor
ATP	adenosine triphosphate
BHK	baby hamster kidney
BHV-1	bovine herpesevirus type 1
BPS	branch point site
BSA	bovine serum albumen
C	carboxy (-terminal end of protein)
CAT	Chloramphenicol acetyltransferase
CF	cleavage factor
Ci	Curie
CIP	calf intestinal alkaline phosphatase
CK2	Casein Kinase II
CNS	central nervous system
CPSF	cleavage and polyadenylation specificity factor
CRM 1	chromosome region maintenance 1
CstF	cleavage stimulation factor
CTE	constitutive transport element
DAS	downstream activation signal
DICE	differentiation control element
DNA	deoxyribonucleic acid
DRB	5,6-dichloro-1-B-D-ribofuranosylbenzimidazole

DTT	dithiothreitol
E	early
E. coli	*Escherichia coli*
EBV	Epstein-Barr virus
EJC	exon junction complex
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acetic acid
eIF	eukaryotic initiation factor
FMRP-1	fragile X retardation protein 1
g	gram or glycoprotein
G	guanine
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
GMP	guanosine monophosphate
GTP	guanosine-5'-triphosphate
h	hour
HCMV	human cytomegalovirus
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HHV-1 to 8	human herpes virus type 1 to 8
HIV-1	human immunodeficiency virus type 1
hnRNP	heterogeneous nuclear ribonuclear protein
HPV	human papillomavirus
HS	heparin sulphate
HSV-1/2	herpes simplex virus type 1/ type2
HVS	herpes virus saimiri
ICP	infected cell protein
IE	immediate early
IFN	interferon
IRES	internal ribosome entry site
kbp	kilo-base pair
kDa	kilo-Dalton
KH	hnRNP K homology

KNS	hnRNP K nuclear shuttling signal
KSHV	Kaposi's sarcoma-associated herpesvirus
l	litre
L-1	leaky late
L-2	true late
LAT	latency associated transcripts
LMB	leptomycin B
lox	15-lipoxygenase
LPF	late processing factor
M	molar
m	milli
Mab	monoclonal antibody
MI	mock infected
min	minutes
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
N	amino (-terminal end of protein)
NES	nuclear export signal
NLS	nuclear localisation signal
NRE	Negative regulatory protein
nm	nanometres
NP40	nonidet--P40
NPC	nuclear pore complex
NuLS	nucleolar localisation signal
OD	optical density
ORF	open reading frame
ori	origin of replication
PABP	poly (A) binding protein
PAGE	polyacrylamide gel electrophoresis
PAP	poly (A) polymerase
PBS	phosphate buffered saline
pfu	plaque forming units

pre-mRNA	precursor mRNA
PREP	Pre-DNA replication particle
REF	RNA export factor
RGG	arginine rich RNA binding motif
RNAP II	RNA polymerase II
RRE	HIV-1 Rev-response element
SAP145	Splicing-associated protein 145
SDS	sodium dodecyl sulphate
snRNA	small nuclear RNA
snRNP	small nuclear ribonuclear protein
SV40	simian virus 40
T	thymine
TBP	TATA-binding protein
TE	Tris-EDTA
TEMED	N',N',N',N'-tertamethylethylenediamine
TF	transcription factor
tRNA	transfer RNA
U	uracil or units
u	micro
U2AF	U2 associated factor
U_L	unique long
U_S	unique short
UsnRNPs	small nuclear ribonuclear proteins bound with uridine-rich RNAs
UTR	untranslated region
UV	ultra-violet
V	volts
vhs	virion associated host shut off function
VP	virion polypeptide
VZV	varicella-zoster virus
w/v	weight/volume
Y2-H	yeast two hybrid
Y3-H	yeast three hybrid

Amino acid symbols

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

Chapter 1: Introduction

1.1 Herpesviridae family

Herpesviruses are large double stranded DNA viruses that share common morphological features. They constitute a large and diverse family of viruses that have been isolated from hosts ranging from fish, amphibians, reptiles, birds and mammals (including pigs, cattle and humans) (Roizman, 1993; Roizman, 2001). They vary considerably in their size, structure, pathology and biology and are highly host specific.

1.1.1 Distinctive structural characteristics

The characteristic morphology of the *Herpesviridae* family of viruses is shown in Figure 1.1. The virions consist of an electron dense core containing a double stranded linear DNA genome (124-235kbp), enclosed in an icosahedral capsid (approximately 100nm diameter) comprised of 162 capsomeres, of which 150 are hexamers (hexons), and 12 are pentamers (pentons) of the major capsid protein UL19. There are also 320 triplexes, each consisting of two molecules of UL18 and one of UL38, in the nucleocapsid (Schrag *et al.*, 1989; Wildy *et al.*, 1960; Zhou *et al.*, 1994) (Figure 1.2).

The tegument is an amorphous layer surrounding the capsid (Roizman, 1974), surrounded by a lipid bilayer containing virally encoded glycoprotein spikes approximately 8nm long (Spear and Roizman, 1972). The tegument layer consists of approximately 20 viral proteins and is of varying thickness, thereby affecting the size of the virion, resulting in diameters ranging from 150-200nm. Tegument proteins are mostly proteins required early in the course of infection including the transcription transactivators VP16 and a protein that induces host mRNA degradation UL41 (also known as the virion-induced host shutoff protein, vhs) (Mettenleiter, 2002). Furthermore, host cell proteins may be contained in the tegument compartment however very little is known of this. For example, cellular filaments have been observed in the

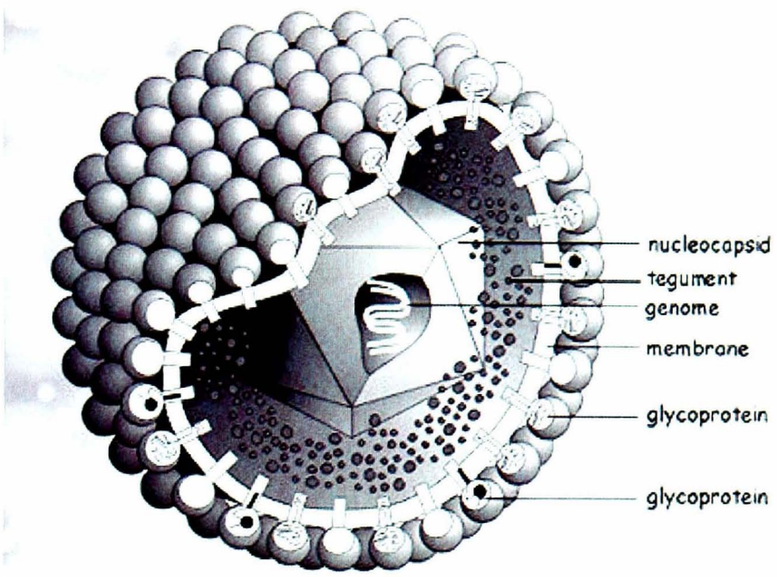


Figure 1.1: Schematic representation of the HSV-1 virion. Reproduced from Marko Reschke *et al.*, 1997.

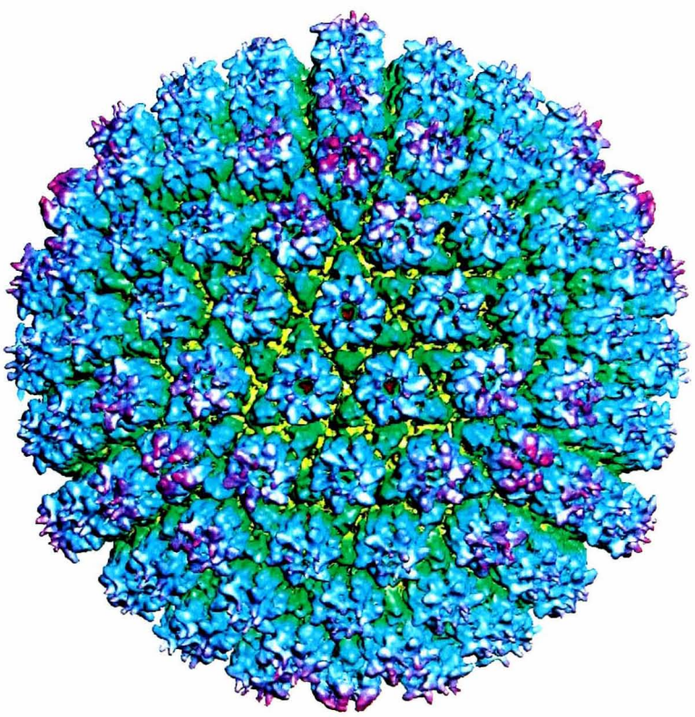


Figure 1.2: Reconstruction of HSV-1 B capsid. Produced from Zhou *et al.*, 1995.

tegument and the width of these structures suggests they are actin filaments, however, this has not been confirmed (Grunewald et al., 2003).

1.1.2 Biological properties

All herpesviruses have similar biological properties including production of enzymes involved in nucleic acid metabolism, and DNA synthesis. They replicate in the nucleus of the host cell, and budding occurs at the cell membrane where production of virions often leads to cell death. A characteristic of these viruses is their ability to enter a latent state after primary infection, thus establishing a quiescent infection throughout the lifetime of the host. The virus may then reactivate in response to stress, such as UV light or the host becoming immunocompromised. Biological variation between members occurs at the level of range of host species, speed of multiplication in the host, host cell specificity and clinical manifestations. There are eight herpesviruses known to infect man that have been identified and they are described in Table 1.1 (Flint., 2003).

1.1.3 Classification

Classification of herpesviruses is based on nucleic acid sequence, genomic organisation, cell tropism/host range, pathogenicity, duration of reproductive cycle and characterisation of latent infection (Roizman, 1982). These DNA viruses are separated into three subfamilies, called *Alphaherpesvirinae* (α), *Betaherpesvirinae* (β) and *Gammaherpesvirinae* (γ).

1.1.3.1 Alphaherpesvirinae

Alphaherpesvirinae have two genera identified so far, Simplexvirus (e.g. HSV-1) and Varicellovirus (e.g. HHV-3 also known as varicella-zoster virus (VZV)). They have variable host range *in vivo* and *in vitro* and a relatively short reproductive life cycle. Infection causes cell destruction that is quickly spread to other susceptible cells. These viruses also have the ability to establish latency in sensory ganglia. HSV-1 is the most

Herpesvirus	Class	G+C %	DNA size (kbp)	Associated illness
Herpes simplex virus type-1 (HSV-1)	α	68.3	152	Causes 80-95% of oral lesions (cold sores) and 30-50% of genital lesions plus other occasional symptoms.
Herpes simplex virus type-2 (HSV-2)	α	69	152	Causes 5-20% oral lesions (cold sores) and 50-70% genital lesions plus other occasional symptoms
Varicella zoster virus (VZV)	α	46	125	Primary infection: chicken pox (rash) accompanied by fever. Reactivation: shingles.
Epstein Barr virus (EBV)	γ	60	172	Primary infection in children is often asymptomatic. Infection in older children and adults is associated with infectious mononucleosis (glandular fever). Associated with Burkitt's lymphoma and nasopharyngeal carcinoma.
Human cytomegalovirus (HCMV)	β	57	229	Primary infection: enlargement and fusion of macrophages. Often asymptomatic though can be fatal in newborns.
Human herpesvirus 6 (HHV-6)	β	42	162	Infant rash, exanthum subitum
Human herpesvirus 7 (HHV-7)	β	45	145	Febrile illness
Kaposi's sarcoma associated herpesvirus (KSHV)	γ	53	140.5	Associated with Karposi's sarcoma, a vasculated nodular skin lesion.

Table 1.1: Human herpes viruses. Eight human herpesviruses, classification, G+C% content, genomic size and disease associated with them.

extensively studied of the herpesviruses and the prototype virus of the subfamily. The most common sites of HSV-1 infection are around the lips and mouth. The virus generally remains latent in sensory neurones and causes cold sores upon reactivation. HSV-2 is closely related to HSV-1 and is generally associated with genital infections.

VZV primary infections are classically recognised by the appearance of small pus filled vesicles on the skin that are associated with itching (chicken pox) and usually occurs during childhood. If reactivated in adulthood the virus can cause a complaint called shingles.

1.1.3.2 Betaherpesvirinae

Betaherpesvirinae have genera identified as human cytomegalovirus (e.g. HHV-5 also known as HCMV) and Roseolovirus (e.g. HHV-6), which are species specific. They have relatively long reproductive life cycles, replicate slowly in culture and cause enlargement of infected cells, i.e. cytomegalia, *in vivo* and *in vitro*. Latency generally occurs in lymphocyte precursors. HCMV is the most extensively studied of the *betaherpesvirinae* where infection is usually asymptomatic, however, it can cause serious foetal damage if acquired during pregnancy, organ transplant rejection, and retinitis in immunocompromised people.

Two betaherpesviruses, HHV-6 and HHV-7, are members of the Roseolovirus genus and have very similar genomes and gene products. HHV-6 can cause exanthema subitum, an early childhood disease that is characterised by high fever and rash. This disease can also occur in immunocompromised people.

1.1.3.3 Gammaherpesvirinae

Gammaherpesvirinae have two genera, Lymphocryptovirus and Rhadinovirus. They are a classic lymphoproliferative group where classification is based on their infecting characteristics i.e. they replicate and persist in lymphocytes and may cause lytic infection in epithelial or fibroblastic cells. They frequently, but not exclusively, infect

the same family or order to which the natural host belongs. The genera lymphocryptovirus (e.g. HHV-4) are specific for either B or T lymphocytes where lytic or latent infection is frequently found. The reproduction cycle and the extent of the cytopathology of the *gammaherpesvirinae* are highly variable. Epstein-Barr virus (EBV) can cause infectious mononucleosis in adolescents and young adults. Burkitt's lymphoma, nasopharyngeal carcinoma and Hodgkins disease have been associated with EBV.

Kaposi's sarcoma-associated herpesvirus (HHV-8) is a member of the rhadinovirus subfamily. This virus is the primary causative factor in all types of Kaposi sarcomas, that is, a multiple-pigmented sarcoma of the skin. KSHV has also been linked with other diseases such as primary effusion lymphoma and certain cases of multicentric Castleman's disease.

1.1.4 Herpesvirus DNAs

The majority of herpesvirus DNAs extracted from virions have linear, double stranded genomes 124-235kbp in length and contain 70-200 open reading frames (ORFs). Base composition varies from 31% G+C to 75% G+C (Table 1.1). The sequence arrangements vary in the copy number of terminal and internal repeat sequences and to date, seven genome structures have been identified in herpesviruses (Roizman, 1992) .

1.2 HSV-1 molecular biology

1.2.1 Genomic arrangement

The genome of HSV-1 is 152kbp in length (McGeoch *et al.*, 1988; McGeoch *et al.*, 1986a; McGeoch *et al.*, 1985; McGeoch *et al.*, 1986b) and encodes over 80 polypeptides. The genome is composed of long and short unique segments flanked by terminal repeat regions. The unique long (U_L) region is 107.9kbp long and encodes at least 59 genes. The unique short (U_S) region is 13kbp and encodes approximately 13

genes. The unique sequences are flanked by inverted repeats, R_L (9kbp) and R_S (6.5kbp) (McGeoch *et al.*, 1988). The R_L and R_S sequences are not related apart from the *a* sequences (400bp) 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 present at the L-S junction as an inverted repeat and again can be present as more than one copy (Wagner and Summers, 1978). During infection the L and S components invert relative to each other so that the progeny viral DNA consists of equimolar amounts of four isomers. They differ from each other solely in the relative orientation of the two unique components. These isomers are termed I_L (inversions of L), I_S (inversion of S) and I_{SL} (inversion of both S and L) (Figure 1.3) (Delius and Clements, 1976; Hayward *et al.*, 1975; Roizman, 1979).

1.2.2 Lytic cycle and particles

Figure 1.4 summarises the lytic life cycle of HSV-1 which takes approximately 18-20h in fully permissive cells (Roizman, 1996). Infected cells produce three types of HSV-1 particles; the virion, the L-particle and the PREP particle. The virion contains HSV-1 DNA and is thus capable of infecting other cells. The L-particle lacks the nucleocapsid and viral DNA and is therefore non-infectious (Szilagyi and Cunningham, 1991). Pre-DNA replication, or PREP particles (Dargan *et al.*, 1995), are synthesised when DNA replication is blocked; they are similar to L particles but lack the true late proteins or only possess them in minute quantities. The lytic cycle consists of virus attachment, entry, gene transcription, DNA replication, capsid assembly, virion maturation and egress, resulting in the death of the host cell.

1.2.3 Attachment and adsorption

The attachment of the HSV-1 virion to the host cell membrane requires the simultaneous interaction of the virion envelope glycoproteins with the cell surface receptors (Fuller and Lee, 1992). HSV-1 virions usually bind to heparin sulphate (HS) (Shukla and Spear, 2001) in most cell monolayers. Fusion of the virion membrane with

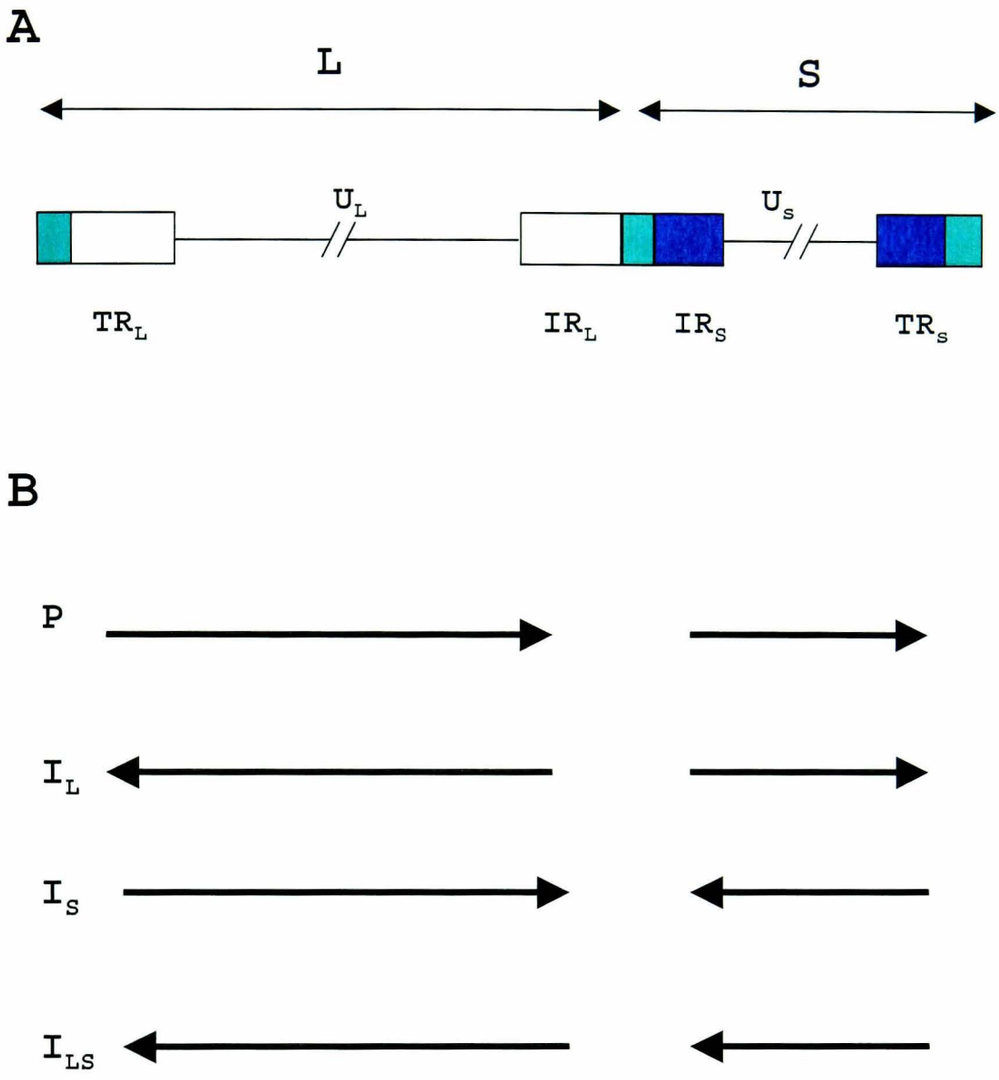


Figure 1.3: (A) Schematic diagram of the HSV-1 genome. The unique regions, U_L and U_S, denoted by single lines are flanked by the R_L and R_S repeat sequences, respectively (white and blue rectangles). The *a* sequence is represented by a green box and is present at each end of the genome in an inverted orientation, separating IR_L and IR_S (not to scale). **(B)** The four genomic isomers generated by the inversion of U_L and U_S regions are shown: P= prototype, I_L = inverted U_L, I_S = inverted U_S, I_{LS} = inverted U_L and U_S.

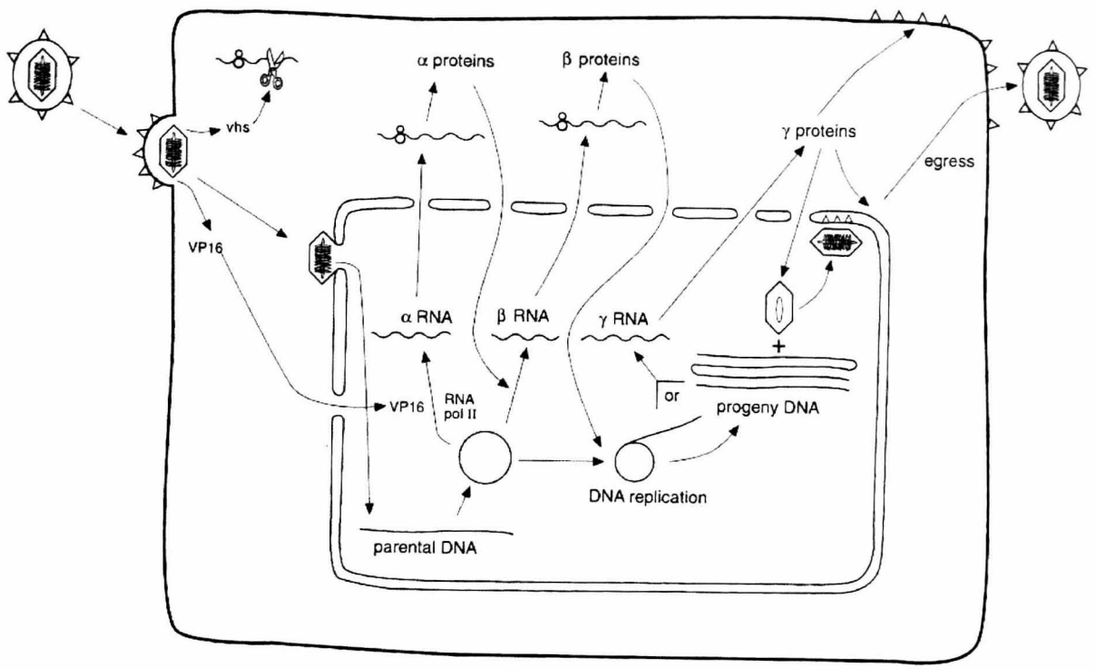


Figure 1.4: A schematic diagram of the lytic cycle. Adsorption is mediated by interactions between the host cell membrane and the virion glycoproteins leading to the release of capsid and tegument proteins into the cytoplasm. The vhs protein acts to cause degradation of mRNAs. The capsid is transported to the NPC and the viral DNA is released into the nucleus. Transcription, replication of viral DNA and assembly of nucleocapsid takes place in the nucleus. Viral gene expression is co-ordinately regulated in a temporal fashion starting with Immediate Early (α), Early (β) then Late (γ) gene expression resulting in the expression of around 80 gene products. Viral DNA is replicated initially through a theta replication mechanism, followed by the rolling circle mechanism and the virion DNA is packaged into the capsid in the nucleus. The virion is thought to mature and egress through the secretory pathway and is released into the surrounding medium. (From Howley, Fields Virology, 2001).

the cellular membrane is mediated by a number of viral glycoproteins including gB, gH (Spear, 1993), however, gD is absolutely required for virus entry. This requires the binding of gD to one of a few cell surface receptors including a herpesvirus entry mediator, a member of the tumor necrosis factor receptor family; nectin-1 or nectin-2, cell adhesion molecules; and specific modifications in HS (Spear *et al.*, 2006).

1.2.4 Regulation of gene expression

Viral gene expression occurs in a co-ordinately regulated and sequentially ordered fashion. Herpesvirus genes can be classified into at least three regulatory classes by the kinetics of their expression and their requirement for DNA replication, i.e. immediate early (IE), early (E) and late (L). IE gene expression occurs immediately following infection and expression peaks at between 3 and 4 h.p.i. Early genes are expressed next where the rates of polypeptide synthesis are highest from 5 to 7 h.p.i. Viral DNA synthesis begins soon after expression of early proteins and stimulates the expression of late genes that are made at increasing rates until at least 12 h.p.i (Zhang and Wagner, 1987).

1.2.4.1 Immediate early gene transcription

The α *trans*-inducing factor (α-TIF or VP16), a virion structural protein, is responsible for transactivating IE genes in the infected cell. Coordinated induction of IE genes requires the consensus DNA sequence TAATGARAT located upstream of all five IE gene promoters. The cellular Oct1 DNA-binding protein binds to this *cis* acting site and recruits α-TIF and other cellular proteins, including the RNAP II holoenzyme. They assemble into a transcriptional pre-initiation complex (Costanzo *et al.*, 1977; O'Hare and Goding, 1988). Four of the five IE gene products, ICP27, ICP4, ICP0 and ICP22 are involved in the regulation of early and late virus genes (Everett, 1987). Regulation of IE transcription is by various *cis* acting elements including TATA and CAAT elements, binding sites for the transcription factor Sp1, and occasionally binding sites for ICP4.

1.2.4.2 Early gene expression

E genes are generally involved in nucleotide metabolism and DNA replication and depend on the prior synthesis of the IE genes ICP0 and ICP4. Promoter regions of E genes containing a TATA box, a transcription cap site (McKnight and Tjian, 1986) and binding sites for cellular factors such as Sp1 and a CAAT binding protein, are found upstream of the promoter (Jones *et al.*, 1985). No upstream sequences specific for viral genes have been detected, though IE proteins (e.g. ICP4) may be involved in the stabilization of cellular transcription factor binding or aiding their recruitment.

1.2.4.3 Late gene expression

L genes can be subdivided into two classes, the leaky late or γ-1 (L-1) genes or the true late or γ-2 (L-2) genes. Genes from the L-1 class can be expressed at low levels prior to DNA replication whereas L-2 gene expression can only be activated during and after viral DNA replication has occurred (UL22, UL44, UL47 and US11 are examples of L-2 genes) (Honess and Watson, 1977).

Full expression of L1 genes requires viral DNA replication and some IE and E gene products (Honess and Roizman, 1974; Honess and Roizman, 1975). The onset and mechanism of L-2 transcription only at times of viral DNA synthesis is not fully understood, however this is thought to be due to a number of factors.

L-2 promoters are weak, compared to IE and E promoters (Guzowski *et al.*, 1994), however this weakness alone is not fully responsible for the strict regulation of L-2 gene expression. *In vivo* transcription assays determined that genome replication was a major factor in the activation of L-2 genes, however this is not due to restrictions on regions of the viral genome as E and L transcripts were rando mly distributed throughout the genome. Nor is it due to the global change in transcription factors as promoters controlling both E and L transcripts are active in uninfected cells. It was therefore postulated that promoters of each kinetic class require *cis*-acting sequences for their regulation of transcription (Mavromara-Nazos and Roizman, 1987).

Generally, sequences required for the efficient expression of all late genes include the TATA box and extend into the 5' non-coding regions. (Homa *et al.*, 1988; Johnson and Everett, 1986). Unlike IE and E gene promoters, L-2 promoters do not have *cis*-acting elements upstream of the TATA box regulating transcription. Instead, late gene promoters have an initiator element and a downstream *cis*-acting element called the downstream activation signal (DAS). (Homa *et al.*, 1988; Wagner *et al.*, 1998). It is thought that L gene expression is regulated by several factors; for example, ICP4 binds to the DAS and may interfere with transcription of the L gene before the onset of viral DNA synthesis. Also, the DNA-binding protein ICP8 can down regulate transcription of L genes before the onset of viral DNA replication (Rajcani *et al.*, 2004; Weir, 2001).

1.2.5 HSV-1 DNA replication

HSV-1 encodes seven genes required for DNA replication and several accessory enzymes required for the replication of the viral genome: UL5, UL8, UL9, UL29, UL30, UL42 and UL52 (Table 1.2) (Boehmer and Lehman, 1997). It is not known if additional host factors are required; however, two functions essential for cellular DNA replication, DNA topoisomarase and DNA ligase, are not encoded by HSV-1.

Viral DNA can undergo circularization within 1h of infection and is thought to serve as a template for DNA replication (Strang and Stow, 2005). The circularized DNA then enters a rolling circle mode of DNA replication generating branched concatameric DNA that is then cleaved and packaged as unit-length molecules. However, the presence of the origins of replication and the UL9 protein, that has helicase activity, suggests a theta mode of replication and led to the proposed bipartite model of replication where theta replication precedes the rolling circle mode of replication (Figure 1.5) (Lehman and Boehmer, 1999).

1.2.6 Latency and reactivation

The ability to establish a life long latent infection within neurones of the peripheral nervous system is the hallmark of HSV-1. The virus is able to exist in a non-replicative

Gene	Properties and Functions
UL5	Subunit of the helicase-primase complex in association with UL8 and UL52.
UL8	Subunit of the helicase-primase complex in association with UL5 and UL52.
UL9	Origin binding protein, binds as a homodimer to the HSV-1 ori sequences, also has helicase and ATPase activities.
UL29	Single-stranded DNA binding protein (also known as ICP8). Stimulates the helicase and ATPase activities of UL9. Recruits UL30 and UL42 proteins.
UL30	DNA polymerase with 3'-5' exonuclease and RNaseH activities. Forms a heterodimer with UL42.
UL42	Double-stranded DNA binding protein, forms a heterodimer with UL30 increasing the processivity of the UL30 DNA polymerase.
UL52	Subunit of the helicase-primase complex in association with UL8 and UL5.

Table 1.2: Summary of HSV-1 DNA replication proteins and their functions (adapted from Boehmer and Lehman, 1997).

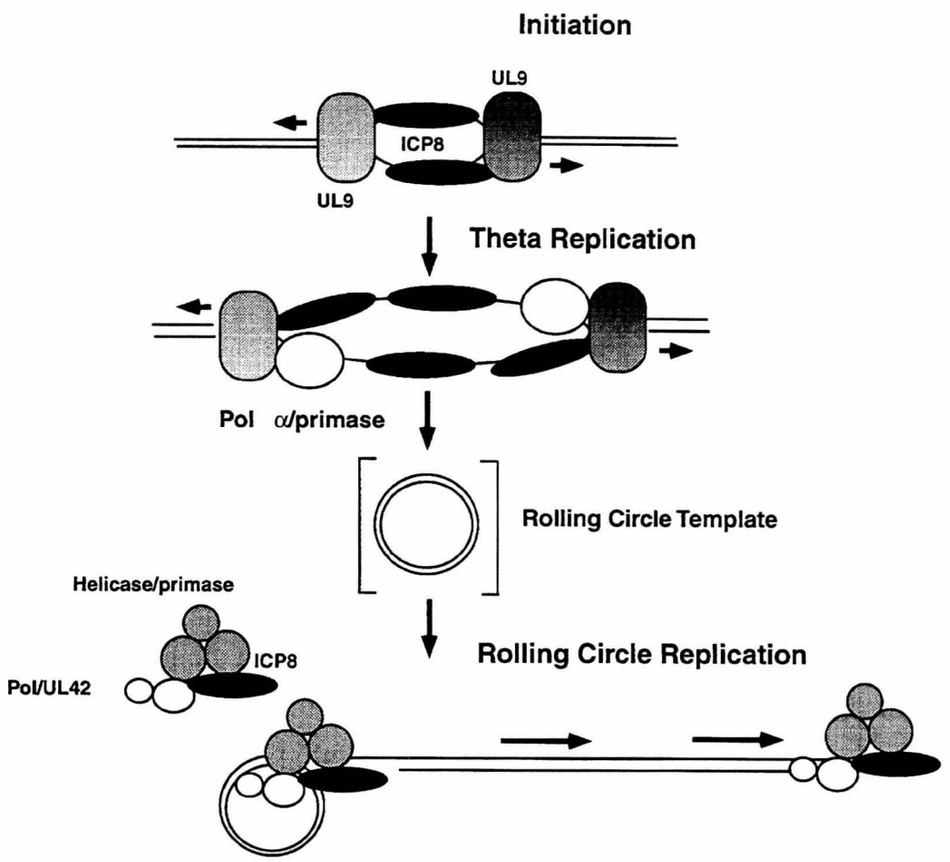


Figure 1.5: Model for the bipartite replication of the HSV-1 genome in which an initial transient phase of theta replication is followed by a rolling circle mode, the predominant mode of HSV-1 DNA replication (taken from Lehman, et al. 1999).

state, which is often followed by reactivation and asymptomatic disease (Rock, 1993). Lytic viral replication can be dissociated from establishment of latent infection as the HSV-1 mutant virus lacking the VP16 protein (the transactivators of IE genes) can establish a latent infection even though it fails to replicate during the acute phase of infection (Ecob-Prince *et al.*, 1993a; Ecob-Prince *et al.*, 1993b; Steiner *et al.*, 1990). Following infection, the virus is transported via retrograde axonal transport (Kristensson *et al.*, 1986) to the cell bodies of sensory neurones within the dorsal root ganglia to establish latent infection (Figure 1.6). During latent infection the only detecTable viral gene expression is that of the major class of transcripts called the latency associated transcripts (LATs). Although LATs are not essential for establishment or maintenance of latency it appears that both the LAT region of the genome (Block *et al.*, 1993; Deshmane *et al.*, 1993; Javier *et al.*, 1988; Leib *et al.*, 1989) and a functional ICP0 protein (Chen *et al.*, 1991; Harris *et al.*, 1989) are important for reactivation of latent virus. The molecular basis of reactivation is poorly understood but viral protein ICP0 as well as cellular proteins Oct1 and Brn3 have been implicated (Lillycrop *et al.*, 1995; Rock, 1993). These cellular proteins are mammalian transcription factors and are known to regulate gene expression, particularly in cells of the nervous system by binding to the octamer motif ATGCAAAT, which is found in the promoters of several cellular genes (e.g. neuronal nitric oxide synthase gene). Two members of the Brn 3 family, for example, can activate IE promoters (Latchman, 1999).

Infectious particles are carried back to peripheral tissues by axonal transport (Cook and Stevens, 1973). Reactivation takes 3-5 days and is triggered by external stimuli such as sunlight and stress (Jones, 2003). The ability of HSV-1 to reactivate from latency results in recurrent disease and virus transmission.

1.2.7 Host-shutoff

HSV-1 infection leads to the redirection of cellular factors and metabolism to the task of producing infectious virions. Upon infection, HSV-1 down-regulates host cell DNA synthesis, translation and splicing, in favour of the production of its own macromolecules. Numerous cellular components and pathways are modified during HSV-1 infection.

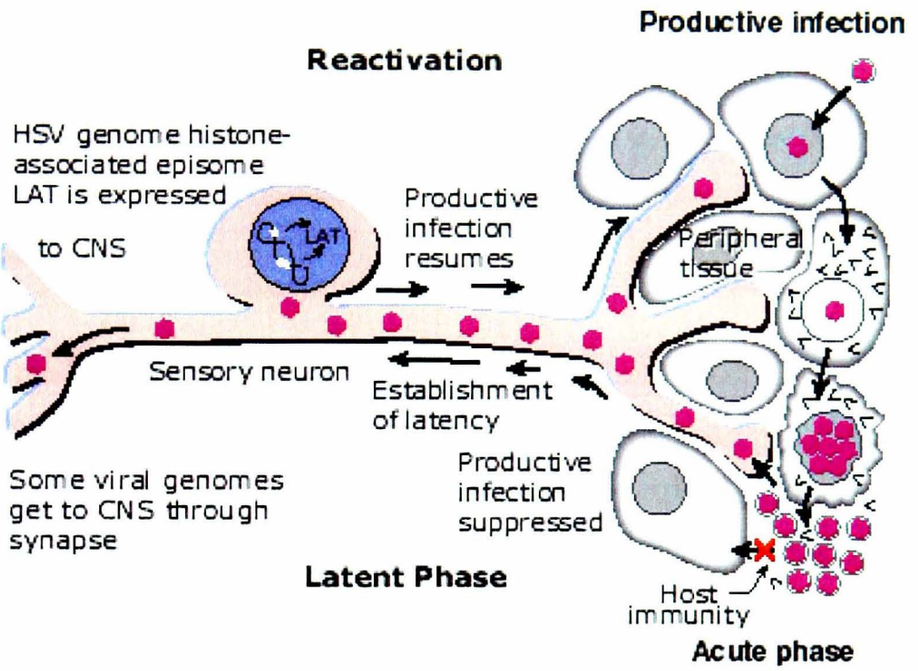


Figure 1.6: Establishment, maintenance and reactivation from latency. After replication at the peripheral tissue, HSV-1 enters the sensory neuron where it is transported to the ganglia that innervate the site of infection. Here it resides in a latent state. The virus begins to replicate during reactivation and is transported back to the peripheral tissues where it replicates to produce infectious virions.

The processof modulating host gene expression is initiated by the virion host shut-off (*vhs)* protein, which is delivered in the tegument. *Vhs* causes the destabilisation and degradation of mRNA (Kwong et al., 1988). The IE gene product ICP27 causes the redistribution of splicing factors (Bryant et al., 2001; Hardwicke and Sandri-Goldin, 1994; Phelan et al., 1996). Most cellular genes contain introns, compared with only five genes for HSV-1. The activity of ICP27, therefore, results in the nuclear retention of cellular genes. The role of ICP27 in pathways contributing to host shutoff is discussed at length in section 1.4.

Another HSV-1 protein with a role in host shutoff is the true late ICP34. Protein kinase R (PKR) is a cellular protein that can be activated by low concentrations of dsRNA and its antiviral action results in the global shutoff of translation. This is brought about by the ability of the activated PKR to phosphorylate the eukaryotic initiation factor 2 (eIF2) α subunit. Phosphorylated eIF2α forms a tight complex with the eIF2 guanosine-exchange factor, eIF2β, and inhibits the exchange of GDP for GTP in the eIF2 complex (Ramaiah et al., 1992; Rowlands et al., 1988). The recycling of the eIF2 complex to form the eIF2-GTP-Met-initiator tRNA ternary complex is therefore inhibited and the translation of mRNA is subject to global shutoff and growth arrest/cell death occurs (see section 1.3.5 for mammalian translation initiation). However, this global shutoff at the translation step is only observed during HSV-1 infection when ICP34.5 is absent or mutated. PKR, however, was activated in cells infected with wt or ICP34.5 virus mutants (Chou et al., 1995). The control of host cell protein synthesis during wt infection was later shown to be due to the interaction of ICP34.5 with protein phosphatase 1 to dephosphorylate eIF2 thus allowing protein synthesis to proceed (He et al., 1997).

1.3 Mammalian pre-mRNA processing

The HSV-1 IE protein ICP27 is known to play a role in the regulation of gene expression by influencing the cellular machinery involved in pre-mRNA processing. Cellular pre-mRNA processing will firstly be described and, later, the role of ICP27 with regard to these processes.

When a pre-mRNA has been produced by transcription, changes occur before the mRNA is ready for transport from the nucleus to the translation machinery. Post-transcriptional events such as capping, polyadenylation, splicing, nuclear export and translation initiation of transcripts all contribute to the final expression levels of the gene.

1.3.1 5' Capping

The 5' cap structure is essential and is required for efficient pre-mRNA splicing, export, stability and translation initiation, is a highly regulated process that occurs in the nucleus. The 5' terminal nucleotide of the primary transcript is capped by a 7-methyl guanosine linked by a 5'-5' triphosphate bridge (Figure 1.7) (Shatkin, 1976). Three enzymatic activities are required for cap formation: the 5' triphosphate end of the nascent transcript is hydrolysed to a diphosphate end by RNA triphosphatase; the diphosphatase end is capped with GMP by RNA guanylyltransferase; and the 5' guanine base is methylated by RNA methyltransferase at the guanine N7 position (Furuichi and Shatkin, 2000; Shuman, 2001). There have been no reports on the regulation of 5' capping by ICP27.

1.3.2 Polyadenylation

The formation of a poly (A) tail at the 3' end of pre-mRNA is also an essential step in the maturation of eukaryotic mRNA that contributes to the regulation of mRNA export, stability and translation (Edmonds, 2002; Proudfoot and O'Sullivan, 2002). Two

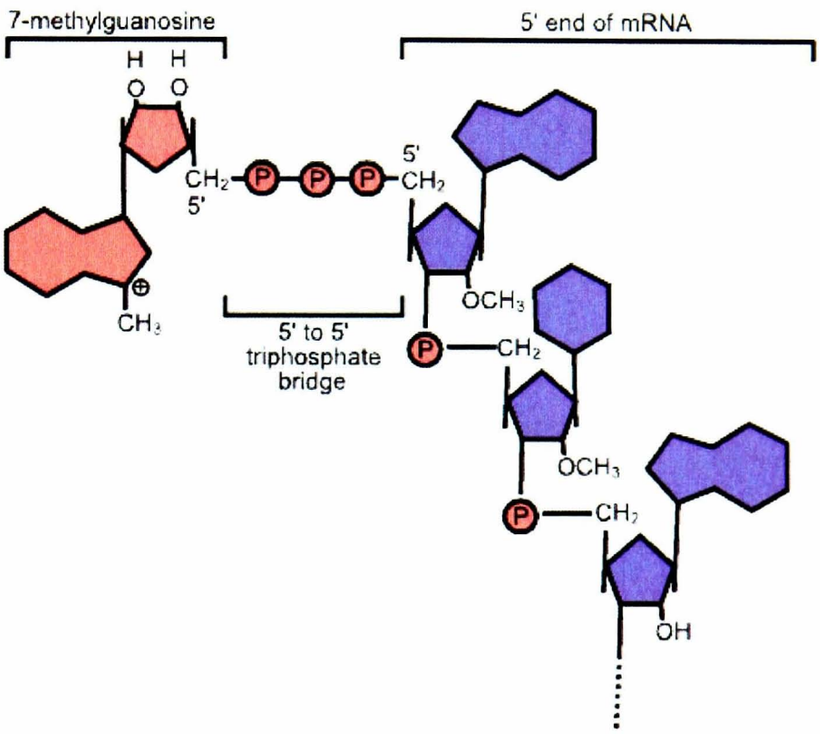


Figure 1.7: The 5' cap is found on the 5' end of an RNA molecule and consists of a guanosine nucleotide connected to the RNA via 5'-5' triphosphate linkage (taken from www.Wilkipedia.org).

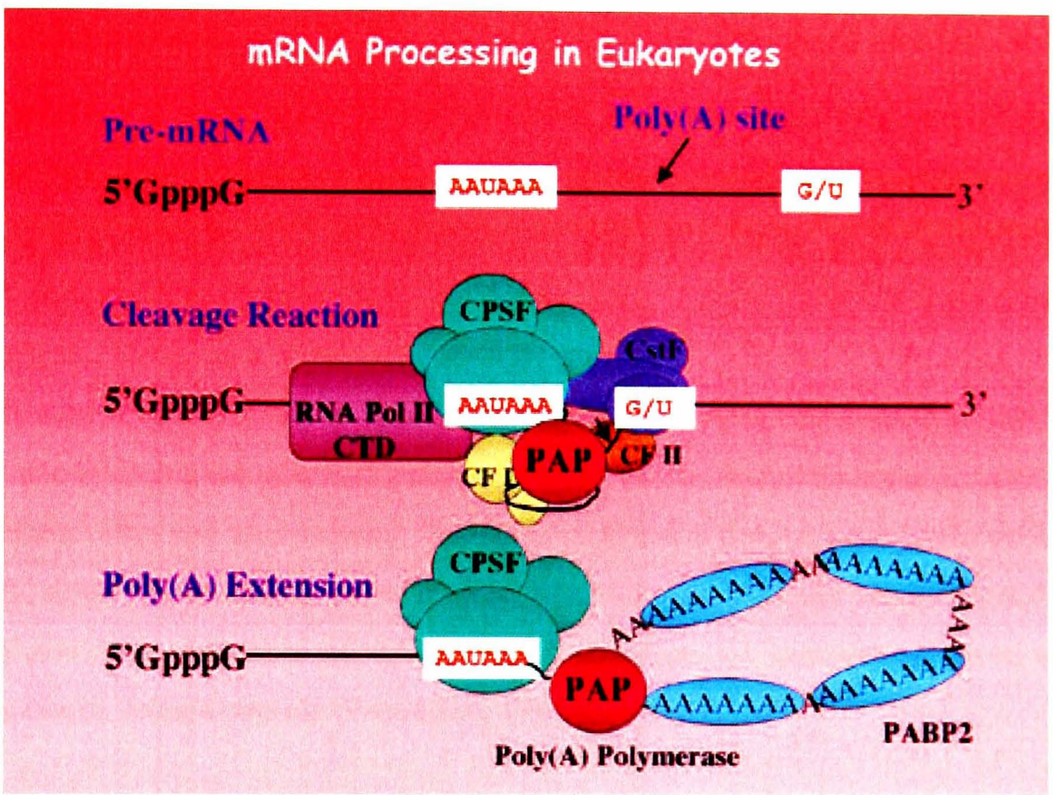


Figure 1.8: The polyadenylation machinery is composed of several multimeric proteins. Five factors are involved in the cleavage step: Cleavage and Polyadenylation Specificity Factor (CPSF), Cleavage stimulation Factor (CstF), Cleavage Factors I and II, and Poly(A) Polymerase (PAP), while three factors play a part in the polyadenylation step: CPSF, PAP, and poly(A) binding protein 2 (PABP2) (taken from www.uvm.edu/microbiology).

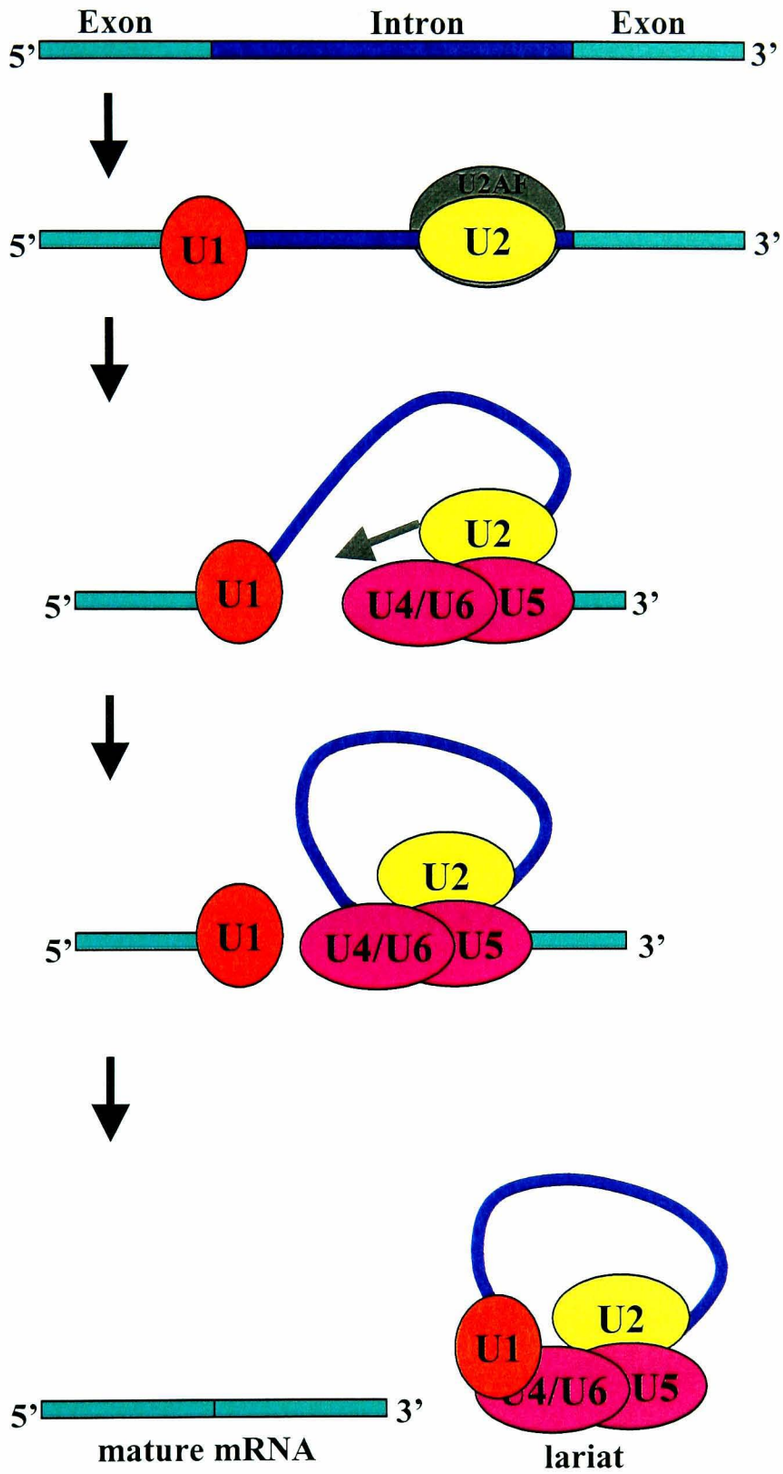
sequence elements that specify pre-mRNA cleavage and poly (A) addition have been identified. The highly conserved AAUAAA sequence is located about 10-30 nt upstream of the cleavage site, and the GU- or U-rich elements are located about 30 nt downstream of the cleavage site (Colgan and Manley, 1997; McLauchlan *et al.*, 1985).

mRNA 3' end formation occurs in a coupled two step reaction; firstly, the pre mRNA is cleaved and then poly (A) addition occurs. Two multi-protein complexes are involved in polyadenylation: cleavage and polyadenylation specificity factor (CPSF) binds the AAUAAA motif, while the cleavage stimulation factor (CstF) binds the downstream GU-rich region (Barabino and Keller, 1999) (Figure 1.8). CPSF and CstF, along with cleavage factors (CFI and CFII) and poly (A) polymerase (PAP), cleave the pre-mRNA, and CPSF and PAP add the poly (A) tail of between 20-200 (A) nucleotides. Alternative polyadenylation sites can also be used. That is, in addition to the AAUAAA and the GU rich motifs, some RNAs contain other genetic elements that influence the efficiency of polyadenylation, e.g. genomes of viruses and the human C2 complement gene and mouse calcitonin, related peptide (Proudfoot, 1996).

1.3.3 Splicing

Splicing is the process by which introns (non-coding sequences) are removed from pre-mRNA and exons (coding-sequences) are joined. The basic mechanism of splicing occurs in two steps. Firstly, 5' splice site cleavage and ligation of the intron's 5' end to the so-called branch point site (BPS) occurs. Secondly, 3' splice site cleavage occurs resulting in the excision of the intron and ligation of the 5' and 3' exons. The lariat is the result of a 2'-5' phosphodiester bond between the G at the 5' splice site and 3' end of the intron (Figure 1.9).

The splicing reaction is catalysed by the spliceosome, a macromolecular complex consisting of numerous proteins including small nuclear ribonuclear proteins bound with uridine-rich RNAs (UsnRNPs) (Will and Luhrmann, 2001). The major spliceosomal UsnRNPs U1, U2, U4, U5 and U6 are responsible for splicing the vast majority of pre-mRNA introns (called U2-type introns) (Figure 1.8). Assembly of the spliceosome begins with the association of the U1 snRNP with the 5' splice site while



An intron (non-coding sequences) flanked by two exons (coding sequences).

U1 snRNPs and U2AF splicing factor bind to 5' splice site and branch site respectively. U2 snRNP is recruited by U2AF.

Branchpoint A nucleotide in the intron sequence attacks the 5' splice site and cleaves it

The cut 5' end of the intron sequence becomes covalently linked to this A nucleotide

The end of the exons are joined cleaving the RNA molecule at the 3' splice site. The intron sequence is released as a lariat

Figure 1.9: The RNA splicing mechanism

the splicing factor U2AF binds to the pyrimidine tract between the BPS and the 3' splice site. A stepwise assembly of the spliceosome then occurs around the splice sites. U2 snRNP is recruited by U2AF and binds to a region encompassing the BPS. A pre-existing U4/U6/U5 snRNP (Konarska and Sharp, 1987; Konarska and Sharp, 1988) joins the complex bringing U1 and U2 snRNPs together (Bindereif and Green, 1987) ,and immediately prior to splicing a conformational change occurs destabilising the association of U4 snRNP, and the spliceosome is activated for catalysis (Staley and Guthrie, 1998; Turner *et al.*, 2004).

Like polyadenylation, the use of alternative splice sites is extremely common. A group of proteins most studied and implicated in splice site selection are the SR proteins. The SR proteins have a serine/arginine rich domain responsible for protein:protein interactions between SR proteins. This domain is thought to aid SR proteins in the recruitment and stabilisation of snRNPs with pre-mRNA at multiple and distinct sites during the splicing cycle. SR proteins can also interact with RNA *via* their amino terminal RNP domain (Manley and Tacke, 1996). ASF/SF2 is a typical SR protein involved in splicing. ASF/SF2 interacts with other proteins, namely SC35 and the small unit of the U2AF, forming a link between 3' and 5' splice sites (Huang and Steitz, 2005; Wu and Maniatis, 1993).

1.3.4 Transport of RNA

Export of mRNA occurs from the nucleus to the cytoplasm via the nuclear pore complex (NPC). NPCs are evolutionarily conserved and are composed of around 30 nucleoporins (nups). FG-nups are essential for translocation as they interact directly with transport receptors that are all members of the karyopherin-β family, e.g. importins or exportins, via their FG sequence repeats (Bednenko, *et al.*, 2003: Stewart, *et al.*, 2001).

Cellular RNA cargos (except mRNA) that contain signal import/export motifs are recognised by the transport receptors and are responsible for most of the exchanges between the nucleus and the cytoplasm (reviewed in Gorlich, *et al.*, 1999). These

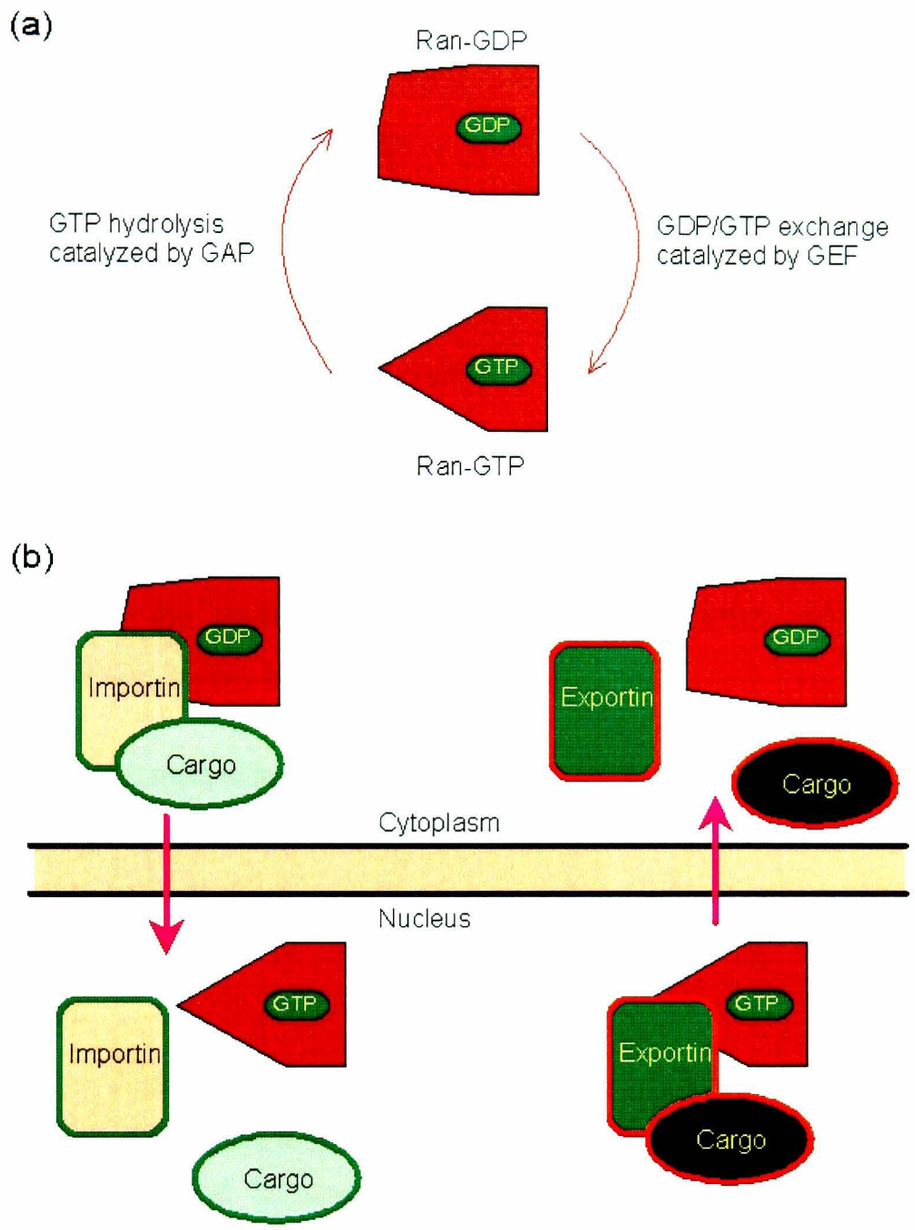


Figure 1.10: Nuclear transport *via* Ran, importin and exportin pathways. **(a)** The two states of Ran: GTP-bound and GDP-bound. **(b)** In the nucleus Ran will be mainly bound by GTP whereas in the cytoplasm Ran will be mainly bound by GDP. This asymmetric distribution gives the nucleoporin receptor (with cargo) directionality (taken from www.web-books.com).

receptors bind to the RNA cargo and the FG repeat on the nup in the NPC and the N-terminus of the receptor interacts with the receptor cofactor, Ran, in either its GTP or GDP-bound state (Moore, *et al.*, 1993). Transition from the GTP-bound to the GDP-bound state is catalyzed by a GTPase-activating protein (GAP), which induces hydrolysis of the bound GTP. The reverse transition is catalyzed by guanine nucleotide exchange factor (GEF) that induces exchange between the bound GDP and the cellular GTP. Ran-GEF is located predominantly in the nucleus while Ran-GAP is located almost exclusively in the cytoplasm. Therefore, in the nucleus Ran will be mainly in the GTP-bound state due to the action of Ran-GEF while cytoplasmic Ran will be mainly loaded with GDP. This asymmetric distribution has led to idea that Ran-GTP enhances binding between an exportin and its cargo but stimulates release of importin's cargo; Ran-GDP has the opposite effect (Figure 1.10) (Gorlich and Kutay, 1999; Izaurralde *et al.*, 1997b; Mattaj and Englmeier, 1998).

The specific sequence motifs in proteins for import are called the nuclear localisation signal (NLS) and the export motifs are known as the nuclear export signals (NESs). Some proteins that have an NLS rely on an interaction with importins although other proteins that lack an NLS can be carried 'piggy-back' through the NCP with a NLS-containing protein.

Another member of the karypherin-β family of nucleoporin receptors is chromosome region maintenance 1 (CRM 1). The HIV-1 Rev protein exports viral RNA using the CRM 1 pathway *via* the Rev NES and this was later shown to be the same route for the proteins involved in the export of U snRNAs (with the cap-binding complex) and 5S RNA (with TFIIIA) (Fischer *et al.*, 1995; Guddat *et al.*, 1990; Izaurralde *et al.*, 1995; Mattaj and Englmeier, 1998).

The splicing process and the presence of the 5' cap and the 3' poly (A) tail can influence the transport of transcripts from the nucleus to the cytoplasm. Pre-mRNA molecules bearing splice sites are mostly retained in the nucleus and appear to be due to the early stages of splicing where mutations of the 5' splice site or the branchpoint resulted in the escape of the unspliced RNA into the cytoplasm (Saguez *et al.*, 2005). The process of splicing appears to indirectly increase the rate of export where efficient export of some transcripts is dependent on splicing (Collis *et al.*, 1990), however expression of

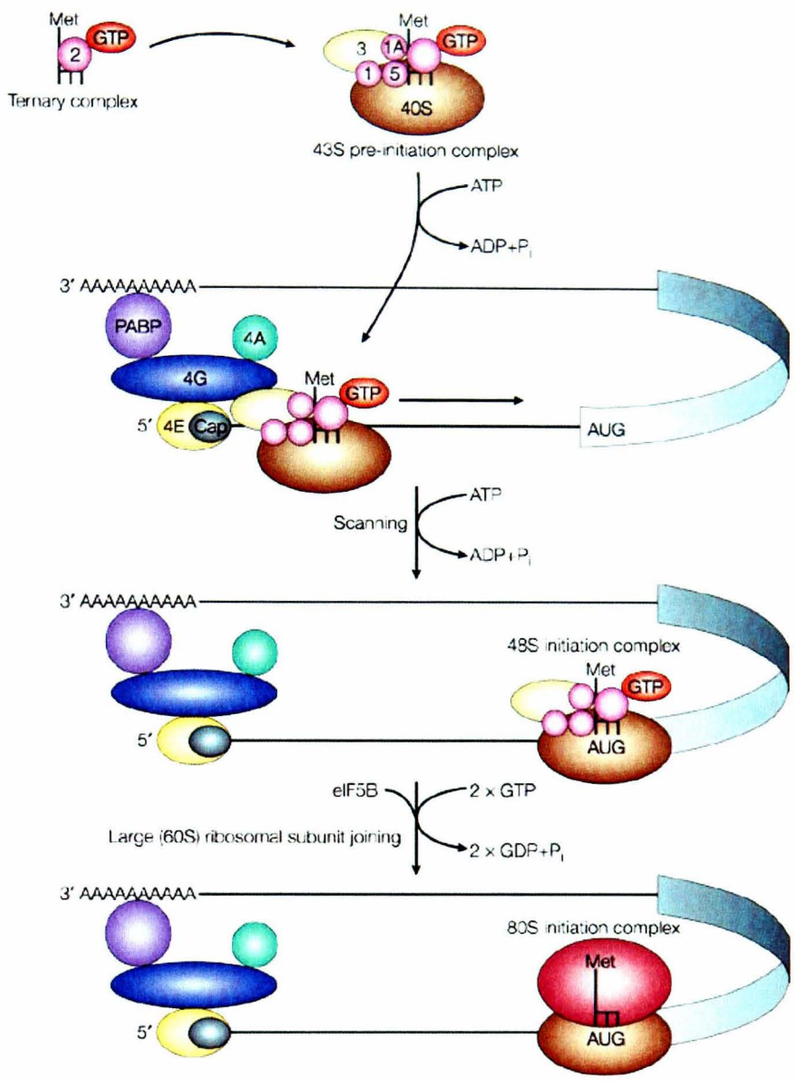
luciferase transcripts with and without an intron did not influence the export of that transcript (Nott *et al.*, 2003) . For some transcripts the presence of the 5' cap structure can enhance the rate of their export, as a transcript with a non-m^7G cap is exported more slowly than an mRNA synthesised with a m^7 G cap (Hamm and Mattaj, 1990). It is thought that proteins involved in capping are involved in export. The 3' poly (A) tail and a stem loop structure in the 3'UTR can also stimulate the export of mRNA but again is not essential (Eckner and Birnstiel, 1992; Saguez *et al.*, 2005).

The fact that the splicing process and the presence of the 5' and 3' secondary structures on the transcript are not absolutely required for export led to the idea that trans-acting protein factors, including hnRNPs, were involved. Some hnRNP proteins, including hnRNP A1 and hnRNP K, are shuttling proteins that are also associated with mRNA. This suggests that these proteins are associated with the mRNA from its emergence from the transcription machinery, though the nucleoplasm and finally during translocation *via* the NPC (Pinol-Roma and Dreyfuss, 1992). The discovery of a NES within hnRNP A1 (Siomi and Dreyfuss, 1995) supported the model that the hnRNP proteins mediate the nuclear export of mRNAs *via* their NES. TAP is another nuclear export factor that, in a complex with p15, binds to the FG-nups and cross-links to poly(A)+ RNA (Zenkluses, *et al.*, 2001), however TAP binds only weakly to RNA and requires an adaptor protein from the REF family that binds directly to RNA and TAP (Strasser, *et al.*, 2000; Stutz, *et al.*, 2000). The RNA/REF/TAP/p15 complex is exported as a whole to the cytoplasm through the direct interaction of TAP and the FG-nups (Conti, *et al.*, 2001).

1.3.5 Mammalian translation initiation

Translation initiation involves the interaction of a wide array of translation factors that work in conjunction with the ribosome and tRNA. Translation initiation involves the binding of the 40S ribosomal subunit to the 5' end of the mRNA and scanning it in a 5'-3' direction until the initiation codon is found. The 60S ribosomal subunit joins the 40S to form the catalytically competent 80S ribosome, however this process also involves many other factors. The small ribosomal subunit (40S) together with eukaryotic initiation factors (eIFs) including eIF3 and a ternary complex forms the 43S pre-

A



B

Name	Function
eIF-2	Binds initiator met-tRNA and GTP
eIF-3	Promotes met-RNA and mRNA binding
eIF-4A	ATPase, helicase, binds RNA
eIF-4E	Cap-binding subunit, part of eIF-4F complex
eIF-4G	Binds eIF-4A, eIF-4E and eIF-3, act as a scaffolding protein.
eIF-5	Promotes GTPase with eIF-2 and ejection of eIFs.

Figure 1.11: **(A)** Cap-mediated translation initiation. A ternary complex is formed between eIF-2, GTP and the methionine loaded initiator tRNA and then binds to the 40S ribosomal subunit plus initiation factors (including eIF-3) to form the 43S pre-initiation complex. The eIF-3 of the 43S complex binds to mRNA via eIF-4G, a scaffolding protein that binds eIF-4E, the RNA cap-binding subunit of the eIF-4F complex. The eIF-4G binds PABP and consequently circularising the RNA. The eIF-4A unwinds the secondary structure and scans the mRNA in a 5'-3' direction allowing the 43S ribosomal subunit to migrate to the initiator AUG, thus forming the 48S initiation complex. EIF-5B hydrolyses the GTP in the ternary complex resulting in the release of initiation factors and the 60S ribosomal subunit joins to form the elongation competent 80S ribosome (taken from Gebauer, 2004). **(B)** A table describing the characteristics of translation initiation factors.

initiation complex. The ternary complex contains the methionine charged tRNA (that will recognise the AUG codon) and initiation factor eIF2 coupled to GTP. The 43S complex bridges the mRNA by the interaction of eIF3 with the eIF4 complex. The eIF4 complex is composed of three initiation factors: eIF4E, which directly binds to the m7GpppN cap structure; eIF4A, a RNA helicase thought to unwind secondary structures in the 5'UTR; and eIF4G, a scaffold protein thought to interact with eIF3 (thus aiding the binding of the 43S initiation complex), eIF4E and eIF4A. Furthermore, the initiation factor eIF4G also interacts with poly (A) binding protein (PABP) and the simultaneous binding of all these factors brings about the circularisation of the mRNA, bringing the 5' and the 3' ends of the mRNA in close proximity to each other (Figure 1.11). Alterations in the 5' cap or the 3' poly (A) tail do influence the assembly of the initiation complex ultimately leading to translational control of that mRNA, or a global change in translation could occur when initiation factors are altered (Gray and Wickens, 1998; Mignone et al, 2002).

Efficiency of the initiation of translation is influenced at the 5' and 3'UTR in various ways including modifying the cap structure, the presence of secondary structures, RNA-protein interactions, upstream (u)ORFs, (u)AUGs, internal ribosome entry site (IRES) elements, RNA-RNA interactions, cytoplasmic polyadenylation elements, and changes in poly (A) tail length (Gray and Wickens, 1998; Mignone et al, 2002; Jackson and Kaminski, 1995).

1.4 The HSV-1 ICP27 protein

ICP27 is a 512 amino acid multifunctional phosphoprotein that is essential for virus replication, as HSV-1 ICP27-null viruses are defective for lytic infection (Sacks *et al.*, 1985; Smith *et al.*, 1992). HSV-1 mutant viruses that have mutations or deletions in the ICP27 gene display a range of phenotypes including a decrease or lack of L-2 gene products, a deficiency in viral DNA synthesis due to a decrease in E gene expression, and a defect in host-cell shut-off. ICP27 regulates viral gene expression through a variety of mechanisms to ensure a lytic infection occurs.

1.4.1 Transcriptional control by ICP27

Transient transfection assays have shown that ICP27 alone can transactivate a HSV-1 gB promoter fused to a CAT reporter gene (Rice and Knipe, 1988). However, reports on transactivation of viral genes have most commonly been observed in conjunction with other viral proteins. ICP27 can enhance or repress transcription from viral IE, E and L promoters activated by the IE proteins ICP0 and ICP4 (Everett, 1986; Rice and Knipe, 1988; Sekulovich *et al.*, 1988; Su and Knipe, 1989). In these cases, ICP27 works in combination with ICP0 and ICP4. ICP27 has been reported to interact with ICP4 (Panagiotidis *et al.*, 1997) and to co-localise with ICP4 in replication compartments in infected cells (de Bruyn Kops *et al.*, 1998). During wt HSV-1 infection, it was found that ICP27 acts to down-regulate the expression of certain IE and E genes and to up-regulate L-1 genes. During a ICP27-null HSV-1 infection some IE and E gene expression was increased and L-1 gene expression is decreased. Furthermore, there was a dramatic reduction in viral DNA synthesis thus leading to the failure of L-2 gene expression (Rice *et al.*, 1989). The effect on viral DNA synthesis during the IC27-null virus infection was most likely due to the abnormal sequence of viral gene expression rather than a direct effect on DNA synthesis. It is thought that ICP27, therefore, has an effect on one or more viral proteins involved in DNA synthesis, or perhaps the over expression of the early proteins has an inhibitory effect on DNA synthesis. Mutational analysis of the ICP27 gene identified that the transactivator and repressor functions were separable and that the C-terminal region is required for both functions (Hardwicke *et al.*, 1989; Rice *et al.*, 1989) (Figure 1.12). Furthermore ICP27, and at certain times after infection ICP8 (the single stranded DNA-binding protein required for viral DNA synthesis), has been reported to associate with RNA polymerase II complexes, and all HSV-1 genes are transcribed by RNA polymerase II (Zhou and Knipe, 2002). Thus, ICP27 was suggested to play a role in transcription initiation or elongation on the HSV-1 genome, however a mechanism for this function has not been elucidated.

1.4.2 ICP27 inhibits splicing of pre-mRNA

The HSV-1 genome contains only four genes that require splicing; the rest are intronless. There is therefore little requirement for the cellular RNA splicing machinery,

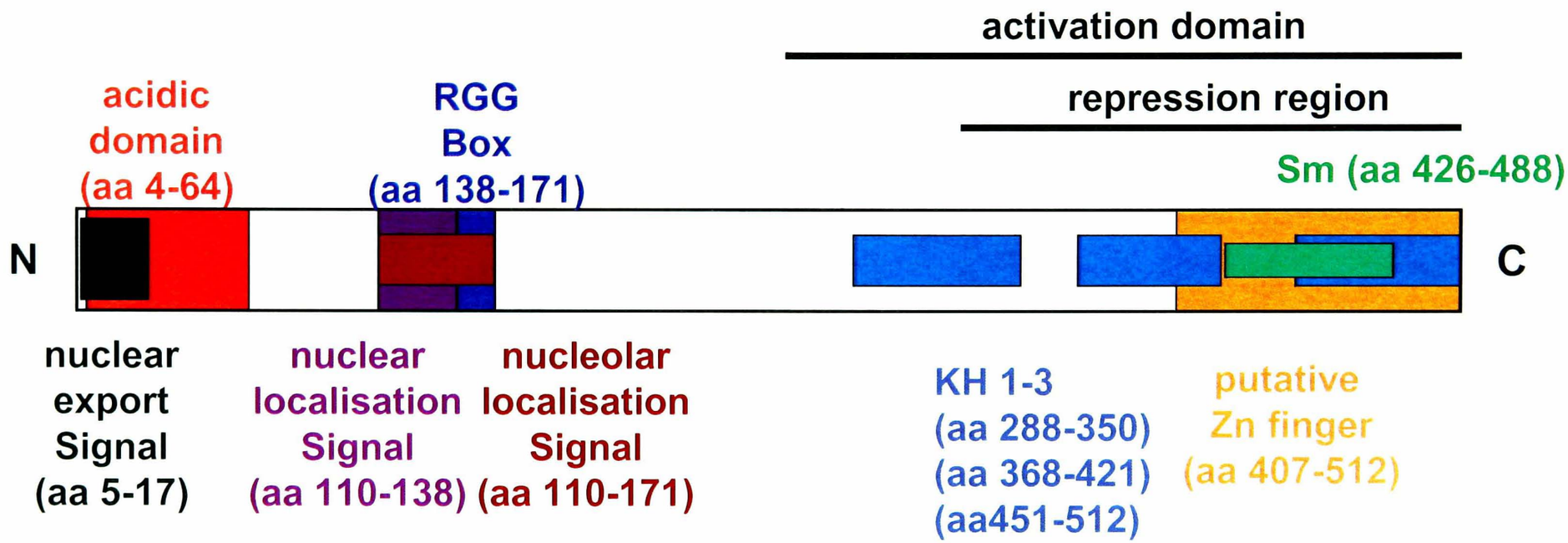


Figure 1.12: Cartoon of ICP27 highlighting its functional domains.

a fact that possibly contributes to host-cell shut-off (Bryant *et al.*, 2001). During early times of infection, ICP27 is responsible for the redistribution of splicing snRNPs in the nucleus. In uninfected cells, splicing proteins (e.g. SC35) exhibit a widespread distribution in the nucleus and as infection proceeds they relocate to a highly punctate pattern and co-localise with ICP27. ICP27 was shown to be essential for this effect during infection and, furthermore, ICP27 produced by transient transfection was sufficient for splicing factor redistribution (Phelan *et al.*, 1993). Further studies showed that ICP27 interacts with splicing factors including the SR proteins (involved in the assembly of the spliceosome), p32 (an inhibitor of the SF2/ASF SR protein), and the spliceosome-associated protein 145 (an essential factor of the splicing complex) (Bryant *et al.*, 2000; Bryant *et al.*, 2001; Sciabica *et al.*, 2003). Studies using an HSV-1 ICP27 null mutant reported that viral mRNAs that contain introns were spliced and located in the cytoplasm of the infected cell whereas during wt HSV-1 infection these transcripts were usually found to accumulate unspliced in the nucleus (Sandri-Goldin, 1994; Sandri-Goldin and Hibbard, 1996). Thus ICP27 was implicated as having an inhibitory effect on splicing although a direct role and mechanism have not been delineated.

1.4.3 ICP27 modulates poly (A) site usage

Studies using precursor RNA containing 3' processing sequences from different HSV-1 genes have shown that a viral factor named the late processing factor (LPF) can stimulate RNA 3' processing specifically at a late HSV poly (A) site of the UL38 gene. This effect was shown to be dependent upon the expression of ICP27 alone (McLauchlan *et al.*, 1989). 3' processing of two HSV-1 late genes, UL38 and UL44, was increased by an ICP27-induced activity whereas no change was observed for a number of IE and E transcripts and it was observed during *in vitro* polyadenylation studies that the late genes had predominantly weak poly (A) sites as compared to the relatively strong poly (A) site of the IE and E genes investigated (McGregor *et al.*, 1996a). UV cross linking experiments showed that the binding of the polyadenylation factor CstF to poly (A) sites of viral RNAs from all temporal classes is enhanced during infection and requires the expression of ICP27 (McGregor *et al.*, 1996a). These observations suggest the ICP27 property of enhancing the binding of CstF does not affect strong sites but increases the efficiency of 3' end formation at inherently weak L

poly (A) sites. This mechanism may contribute to ICP27's regulatory role in the switch between E and L gene expression.

1.4.4 ICP27 is an RNA binding protein

The work by Brown *et al*, 1995, led to the analysis of the ICP27 amino acid sequence revealing the presence of an RNA recognition domain. This was achieved using an in-vitro RNA binding assay, i.e. ^{32}P-labelled ribopropes were analysed for binding to bacterially expressed recombinant ICP27 with a GST tag using a Northwestern blotting technique. A cluster of arginines interspersed with glycines at the N-terminus of the ICP27 protein was seen in other RNA-binding proteins and termed the RGG box (Figure 1.12). UV cross-linking experiments using bacterially expressed protein showed ICP27 could bind directly to several RNAs (Ingram *et al.*, 1996), however the RNAs used in the Ingram investigation included fragments of an exon, a poly (A) site, a non-HSV-1 RNA and an ORF of three HSV-1 genes, leading to the supposition that this interaction was not specific for HSV-1 RNAs. ICP27 transcribed and translated in a eukaryotic expression system bound to poly (G) RNA homopolymers, weakly to poly (U) and did not bind to poly (A) or (C). However, ICP27 also bound to negative controls, an SV40 RNA, previously shown not to bind full length ICP27 (Brown *et al.*, 1995) and the IFN-β antisense RNA probe. Nevertheless, the fact that ICP27 binds efficiently to poly (G) RNA suggests the possibility that G-rich sequences play a role in RNA recognition by ICP27 (Mears and Rice, 1996a) which could result in preferential binding of HSV-1 RNA as this is also GC-rich.

In a study investigating ICP27 RNA binding *in vivo*, some HSV-1 transcripts that bind ICP27 were identified during UV cross-linking experiments, isolating seven HSV-1 intronless RNAs. Interestingly, ICP27 did not bind two intron-containing RNAs in these studies (Sandri-Goldin, 1998a). Investigations to identify further HSV-1 RNAs that bind ICP27 were carried out using a yeast 3 hybrid (Y3-H) assay (Sokolowski *et al.*, 2003). Thirty-one HSV-1 sense transcripts were identified to selectively bind ICP27; these ranged in length from 35-225 nucleotides. The identified transcripts mapped to regions of HSV-1 genes from 28 ORFs. Even though these isolated RNAs represented transcripts from all kinetic classes the corresponding gene, i.e. the gene from which the

RNA was isolated, encoded predominantly essential viral proteins with roles in viral DNA replication and virion maturation (Table 1.3). Furthermore, the ICP27-RNA interaction was abolished or severely reduced when ICP27 lacking an RGG box was used, highlighting the importance of the RGG box of ICP27 in the binding of these HSV-1 RNAs. In fact, previous work by Mears and Rice (1996a) determined that not only did ICP27 bind directly to RNA but also that ICP27 lacking an RGG box did not bind to RNA.

1.4.5 ICP27 stabilises RNA

The stability of an mRNA and therefore its lifetime can be crucial in the regulation of gene expression. Many mRNAs containing an AU-rich element (ARE), are known to be targeted by ARE-binding proteins (ARE-BP) for degradation, however, other ARE-BPs have been shown to mediate RNA stabilisation via these sequence motifs (Barreau *et al.*, 2005; Zhang *et al.*, 2002). AUF1 is a ARE-BP (also known as hnRNP D) and has been associated with both an increase in RNA instability and stability, depending on cell type (Loflin *et al.*, 1999). The Hu family of ARE-BPs (e.g. HuR) have been shown to be associated with RNA stability (Figueroa *et al.*, 2003)

Eukaryotically expressed ICP27 has been shown to bind to sequences at the 3' ends of RNA. ICP27 alone could increase the half-life and stimulate the steady state accumulation of reporter genes containing AU-rich untranslated regions, however, it is not known yet whether the binding involves these AU-rich motifs (Brown *et al.*, 1995).

1.4.6 Nucleocytoplasmic shuttling properties of ICP27

ICP27 binds to RNA and assists in exporting it from the nucleus to the cytoplasm. In situ hybridization assays revealed that during HSV-1 infection intron-containing transcripts were retained in the nucleus. Interestingly, immunofluorescence studies located these proteins, i.e. ICP0 and ICP4, to the punctate regions in the nucleus where the splicing factors had relocated (previously discussed in section 1.4.2). ICP27 was originally implicated in RNA export when, during an HSV-1 ICP27-null infection,

Isolate no	Transcript	Functional Role
69, 16, 17, 247, 352, 75	ICP4	Broad-range transcriptional trans-activator, E
324/16.2	UL4/5	Part of replication complex, NE/E
309	UL29	ssDNA binding protein, E
82, 5, 231	UL30	DNA pol subunit, E
13.2	UL39/40	Ribonucleotide reductase, NE
3	UL42	DNA pol subunit, E
8, 300	UL49/49.5	Tegument protein, NE
4, 242	US3	Protein kinase, NE
212	UL6	DNA cleavage/ packaging, E
12	UL13	Protein kinase, NE
21, 23	UL15	DNA packaging, E
274	UL16/17	Tegument/DNA packaging, NE/E
185/85/19, 251	UL19/20	Major capsid protein/ viral exocytosis, E/NE
55	UL31/32	Nuclear phosphoprotein/ DNA packaging, E
281.2	UL36	Tegument protein, E
334	UL48	Tegument protein, IE trans-activator, E
258	ICP34.5	Neurovirulence, NE

Table 1.3: Transcripts found to bind ICP27 and their functional role in the HSV-1 life cycle (adapted from Sokolowski et al, 2003). NE denotes non-essential and E is essential in tissue culture.

intron-containing viral transcripts (ICP0 and UL15) were located in the cytoplasm (Phelan *et al.*, 1996). Heterokaryon assays visualised the ability of ICP27 to continuously shuttle from the nucleus to the cytoplasm, and further ICP27 mutational analysis revealed that ICP27 with the *d*1-2 (deletion of amino acids 12-63) deletion significantly reduced shuttling and ICP27 with the M15 (containing mutations of a PG at amino acids 465 and 466 to an LE) mutation completely abolished shuttling (Mears and Rice, 1998).

Sequence analysis and ICP27 mutational analysis using the ICP27 deletion mutants *d*1-2, *d*leu (deletion of amino acids 6-19) and *d*3-4 (deletion of amino acids 110-137) revealed that ICP27 contains a nuclear export signal (NES) and a nuclear localisation signal (NLS) within the N-terminal region (Figure 1.12) (Lengyel *et al.*, 2002; Mears *et al.*, 1995). The presence of these signals indicated a Crm-1-independent pathway was used for export and experiments using the Crm-1 inhibitor, leptomycin B (LMB), on HSV-1 infected Vero cells showed a reduced cytoplasmic accumulation of ICP27 (Soliman and Silverstein, 2000a). However, contradictory to this, it was observed in two independent investigations that the localisation of ICP27 was not altered by LMB (Chen *et al.*, 2002; Koffa *et al.*, 2001). ICP27 binds directly to REF (the RNA export factor previously discussed) possibly functioning as an adaptor protein, and utilises the export receptor, TAP, to export viral mRNAs (Chen *et al.*, 2002). The HSV-1 ICP27 mutant virus (*d*3-4) that has a deletion of the REF binding site (and the NLS) exhibited a 40-fold reduction in titre but remained replication competent (Mears *et al.*, 1995) suggesting that ICP27 may utilise more than one export pathway during the virus life cycle.

1.4.7 ICP27 translational control

ICP27's role in the regulation of gene expression has been investigated at the level of translation initiation. ICP27 is required, post-export, for the expression of various HSV-1 transcripts. For example, during an HSV-1 ICP27-null infection the short transcript of UL24 was located in the cytoplasm yet UL24 protein levels were decreased by at least 70-fold as compared to wt HSV-1 infection (Pearson *et al.*, 2004), leading to the idea

that ICP27 is not essential for the export of that transcript but is required for its efficient translation.

ICP27 interacts with translation initiation factors eIF4G and PABP, contributing to the proposal that ICP27 has a function in promoting or inhibiting translation via these interactions (Fontaine-Rodriguez *et al.*, 2004). Translation initiation factor eIF4G is a scaffolding protein that binds to the cap-binding complex (eIF4F), and interacts with PABP and eIF3 (see section 1.3.5). ICP27 stimulated polysomal association of HSV-1 VP16 mRNA leading to the control of the levels of VP16 protein expression, however, this was not shown to be due to the direct stimulation by ICP27 (Ellison *et al.*, 2005). Larralde *et al*, (2006) however, confirmed the association of ICP27 with polyribosomes during infection and demonstrated that this association was abolished when ICP27 carried the M15 mutation. Using a tethered function assay in *Xenopus* oocytes, ICP27 was investigated for its effects on translation independently of any action on transcription, polyadenylation and mRNA export. ICP27, in the absence of any other viral proteins, directly stimulated the translation of mRNA by 10-fold and, furthermore, ICP27 with the M15 mutation failed to stimulate translation. Thus, the association of ICP27 with polyribosomes and its ability to stimulate translation are linked. The mechanism of this activity is still unknown.

1.4.8 Functional domains of ICP27

1.4.8.1 Essential acidic domain

ICP27 contains a highly acidic amino terminal region (amino acids 4 to 64) that is essential to the virus (Figure 1.12) (Rice *et al.*, 1993). A number of transcription transactivators, e.g. HSV-1 VP16, have an acidic region that has been shown to be involved in stimulating transcription by interacting with components of the RNA Pol II transcription complex, e.g. transcription factor IIA (TFIIA) (Kobayashi *et al.*, 1995; Strum, 1991). The HSV-1 ICP27 mutant virus *d*1-2 has a deletion of amino acids 12-63. The *d*1-2 mutant was shown to be deficient for growth in Vero cells, exhibiting a reduced level of DNA replication and a moderate reduction in viral gene expression,

demonstrating a regulatory function of ICP27's N-terminal region (Rice *et al.*, 1993). However, the *d*1-2 deletion overlaps with the NES and it is therefore unclear what contribution this has to the phenotype of *d*1-2.

1.4.8.2 Nuclear export signal

The NES of ICP27 is located between amino acids 3-17 (Figure 1.12). Sequence analysis identified a resemblance to the leucine–rich NESs first identified in HIV-1 Rev and PKI (the heat-sTable inhibitor of cAMP-dependent protein kinase) (Fischer *et al.*, 1995; Wen *et al.*, 1995). ICP27 mutational analysis revealed that ICP27 is still located in the cytoplasm when the ICP27 NES is replaced with the NES of Rev or PKI, however, a deletion of ICP27 NES resulted in ICP27 nuclear retention (Sandri-Goldin, 1998a). The heterokaryon assay to determine whether ICP27 shuttles between the nucleus and the cytoplasm (discussed in section 1.4.6) revealed the *d*1-2 ICP27 protein had severely impaired import/export abilities, i.e. when the NES is disrupted the protein's ability to shuttle is reduced (Rice, *et al.*, 1998).

A failure or reduction in cytoplasmic accumulation of other shuttling proteins due to the disruption of the NES has been previously been shown e.g. hnRNP A1 (Fritz *et al.*, 1995; Izaurralde *et al.*, 1997a), HIV Rev protein (Fischer *et al.*, 1994), the yeast mRNA transport protein Gle1 (Murphy *et al.*, 1996) and the transcription factor IIIA (Fridell *et al.*, 1996).

Interestingly, in cells infected with the ICP27 mutant virus, *d*leu, that has a deletion of the full NES (amino acid 6-19), ICP27 is highly restricted to the nucleus. This mutant virus has, however, only a modest defect in viral gene expression but is deficient in virion production (Lengyel *et al.*, 2002) suggesting that the NES of ICP27 is not absolutely essential to the replication of the virus.

1.4.8.3 Nuclear and nucleolar localisation signals

ICP27 contains multiple NLSs, both strong and weak, that mediate its nuclear localisation. The strong bipartite NLS maps to amino acids 110-137 (Figure 1.12).

Plasmids expressing ICP27 with a deletion of the NLS, *d*3-4, expressed ICP27 located in the nucleus and the cytoplasm as compared to wild type ICP27 that was almost exclusively nuclear. However, nuclear localisation of the ICP27 *d*3-4 protein indicates that the other weak NLS sequences are in use. The recombinant HSV-1 *d*3-4 virus, that expresses ICP27 lacking an NLS, gave a 9-34 fold defect in growth in Vero cells indicating the importance of the strong NLS during virus infection (Mears *et al.*, 1995).

ICP27 also contains a nucleolar localisation signal (NuLS) mapping to amino acids 110-152. Although ICP27 does not preferentially localise to the nucleolus, ICP27 has been isolated in this compartment (Knipe *et al.*, 1987). In cells infected with a recombinant HSV-1 virus that expresses only amino acids 1-263 of the ICP27 protein (*n*263R), ICP27 is predominantly located in the nucleolus, again indicating the use of another NLS(s). Two features, in common with the NuLS of ICP27, have been identified in the NuLSs of the HIV-1 Rev and Tat; they are both rich in arginine residues and they contain a functional NLS (Cochrane *et al.*, 1990; Dang and Lee, 1989).

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

Residues 139-153 encode the RGG box (Figure 1.12); RNA binding domain of ICP27 and these features are discussed in section 1.4.4. The C-terminal half of ICP27, based on limited sequence similarity, contains three RNA binding motifs that have homology to a series of hnRNP K homology (KH) domains (Soliman and Silverstein, 2000b). These have been mapped to amino acids 288-350, 368-421 and 451-512 and termed domains KH1, KH2 and KH3, respectively. KH domains were first identified in hnRNP K and later in other cellular proteins including FMRP-1 and Nova-1. RNA binding of the FMR-1 protein from a severely retarded fragile X patient is abolished due to a single point mutation in the KH domain of the protein (Siomi *et al.*, 1994). The precise function of FMRP is unknown, however it has been demonstrated to play a role in the negative regulation of translation (Laggerbauer *et al.*, 2001; Li *et al.*, 2001). Nova-1 is another neuronal RNA-binding protein involved in RNA splicing of two receptor proteins. The KH domains of Nova-1 have been shown to be required for RNA binding where inhibition of this RNA-binding is postulated to play a role in paraneoplastic

opsoclonusmyoclonus, an autoimmune disorder of the nervous system (Buckanovich *et al.*, 1993; Laggerbauer *et al.*, 2001).

The ICP27 mutation F303N is located in the KH-1 domain and was shown to be lethal when recombined into the virus. Analysis of protein synthesis revealed a distinct defect in a subset of viral late genes. However, the late genes inhibited during F303N infection are not essential, suggesting the mutation may also affect the expression of other essential HSV-1 genes.

1.4.8.5 Potential zinc finger

Metal chelate affinity chromatography has shown that ICP27 binds zinc *in vitro* through the C-terminal amino acids 407-512 (Figure 1.12) (Vaughan *et al.*, 1992). Zinc finger domains are associated with protein-nucleic acid and protein-protein interactions. The region of ICP27 that spans the zinc finger has been known to be involved and varying functions including gene activation and repression (Hardwicke *et al.*, 1989; McMahan and Schaffer, 1990). ICP27's self-association has been shown to require amino acids from the zinc finger domain and point mutations created in this domain were lethal to the virus, suggesting that self recognition is important for function (Zhi *et al.*, 1999).

1.4.8.6 SM domain

ICP27's putative SM domain lies at the C-terminus of the protein at amino acids 426-488 (Figure 1.12). These sequence motifs are known to be utilised by SM proteins that are involved in forming the core spliceosome complex (Kambach *et al.*, 1999). The identification of the SM motif in ICP27 may contribute to determining the role ICP27 plays during the inhibition of splicing during wt HSV-1 infection ultimately leading to host cell shut off. The HSV-1 ICP27 mutant virus D448L has a mutation within the SM domain that results in a defect in host cell shut off during infection thus contributing to the above hypothesis (Soliman and Silverstein, 2000a).

1.5 ICP27 Partner proteins

ICP27 interacts with a range of cellular proteins involved in gene expression. Figure 1.13 summarises ICP27 partner proteins.

1.5.1 HnRNP K

Like ICP27, hnRNP K is a multifunctional protein that plays a role in transcription, splicing and translation. It was first identified as a component of the hnRNP particle and can be found as at least five alternatively spliced isoforms (Dejgaard *et al.*, 1994; Matunis *et al.*, 1992). The nucleic acid binding KH domains were first identified in hnRNP K, and this protein binds poly (C) RNA or DNA via its three KH domains (Siomi *et al.*, 1993a). Between the KH2 and KH3 domains lies a K-protein-interactive (KI) region that is responsible for many of the protein interactions (Makeyev and Liebhaber, 2002). The hnRNP K protein contains an NLS and a nuclear shuttling domain (KNS). The KNS mediated bi-directional transport between the nucleus and the cytoplasm can act independently of the NLS (Bomsztyk *et al.*, 1997).

HnRNP K can transactivate a number of genes when using chimeric constructs with a variety of different promoters (Lee *et al.*, 1996). HnRNP K was also shown to be a transcription factor as it bound to the CT element of the c-*myc* gene and activated transcription. Affinity chromatography demonstrated that hnRNP K bound specifically to components of the RNA polymerase transcription machinery and later an interaction was observed with the TATA-binding protein (TBP). Thus, hnRNP K interacts with *cis* acting elements and binds to transcription machinery to activate transcription (Michelotti *et al.*, 1996).

HnRNP K protein has been reported to interact with splicing factors 9G8 and SRp20 (Shnyreva *et al.*, 2000). This was identified when investigating the chicken β-tropomyosin gene where hnRNP K acts as a component of an intronic enhancer complex that activates alternative splicing.

HnRNP K involvement in translation was first hypothesised when it was found to bind to the translation elongation factor-1A (Bomsztyk *et al.*, 1997). Later it was shown that hnRNP K silences translation of a luciferase reporter mRNA that contains a differentiation control element (DICE), that is, a CU-rich element normally found in the 3'UTR of the 15-lipoxygenase (lox) gene (Ostareck *et al.*, 1997). Lox is an enzyme expressed during erythroid cell differentiation and it must be silenced in early erythroid cells in the bone marrow and the peripheral blood. The silencing occurs at the level of translation initiation as hnRNP K binds to the 3'UTR DICE element effectively blocking the recruitment of the 60S ribosomal subunit (Ostareck *et al.*, 2001). HnRNP K/DICE binding is mediated by the tyrosine kinase c-Src, as phosphorylation of hnRNP K by this kinase, due to extracellular signals, removes hnRNP K from the DICE element and translation initiation can occur (Ostareck-Lederer *et al.*, 2002). Alternatively, during an investigation of the c-*myc* IRES, hnRNP K protein together with hnRNPE1/2 was shown to stimulate translation (Evans *et al.*, 2003).

HnRNP K binds HSV-1 ICP27, as determined by a yeast-two (Y2-H) hybrid system and immunoprecipitation assays. Interestingly, when hnRNP K was co-precipitated with ICP27 by anti-ICP27 serum, hnRNP K was phosphorylated by CK2 (another ICP27 partner protein, see later) (Wadd *et al.*, 1999). Phosphorylation of hnRNP K resulted in a number of actions including a decrease of its binding to RNA, and release from the DICE thus leading to hypotheses of its role in binding to ICP27 during infection. That is, the similarities between ICP27 and hnRNP K suggest similar functions and therefore possible common cellular pathways. During infection, the ICP27-mediated phosphorylation of hnRNP K could prevent RNA binding thus promoting ICP27 RNA binding and ultimately inhibiting any competition by hnRNP K. Alternatively; perhaps hnRNP K is recruited, by ICP27, leading to the speculation that the host of functions accredited to ICP27 are due, at least in part, to the interaction with hnRNP K.

1.5.2 REF and TAP

TAP in association with p15 is the nuclear export receptor for metazoans, and the yeast homologue, Mex67p, also functions as an export receptor (Braun *et al.*, 2001; Gruter *et*

al., 1998). Both of these proteins have the ability to shuttle in and out of the nucleus, cross-link to poly $(A)^+$ RNA, and associate with nucleoporins (Fribourg *et al.*, 2001; Katahira *et al.*, 2002). TAP was first identified as the cellular factor that interacts with the constitutive transport element (CTE) present in RNAs from type D retroviruses and aiding in the export of those RNAs (Bear *et al.*, 1999). Over expression of TAP was shown to stimulate mRNA export and inactivation of TAP by RNA interference resulted in the nuclear accumulation of poly $(A)^+$ mRNA, indicating a role for TAP in mRNA export (Herold *et al.*, 2003; Tan *et al.*, 2000).

REF is a component of the exon junction complex (ECJ) shown to function in splicing, nuclear export and RNA localisation. REF is recruited to the spliceosome complex by interacting with UAP56, a DEAD box helicase involved in spliceosome assembly (Gatfield *et al.*, 2001). REFs can associate with intronless RNAs independent of splicing, with variable efficiency depending on the sequence and length of sequence, to enhance the export of unspliced mRNAs (Rodrigues *et al.*, 2001).

REF was found to bind to ICP27 during a Y2-H assay (Wadd, 2000) and later from HSV-1 infected cells (Chen *et al.*, 2002). During *Xenopus* oocyte microinjection assays, ICP27 was shown to bind directly to REF where the interaction dramatically stimulated export of certain viral RNAs. ICP27 that fails to bind REF such as the ICP27 *d*3-4 (discussed previously as having a deletion in the NLS), failed to stimulate viral mRNA export (Koffa *et al.*, 2001). ICP27 is present in a complex with TAP and REF during infection, indicating a role for both cellular proteins for viral RNA export. Viral mRNA export was indeed stimulated in oocytes by REF and TAP and it was proposed that ICP27 recruits REF to intronless viral RNAs that are then transported *via* the TAP-mediated export pathway (Koffa *et al.*, 2001).

1.5.3 Casein Kinase II (CK2)

CK2 phosphorylates serine/threonine residues that are N-terminal to acidic amino acids. The protein kinase consists of two catalytic subunits (α or α') and two regulatory β subunits, and can exist in three forms: $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$ or $\alpha'_2\beta_2$ (Allende and Allende, 1995). CK2 is a regulator of cell growth, cell division, signal transduction and apoptosis

(Dobrowolska *et al.*, 1999; Guerra *et al.*, 1997). CK2 has been implicated in the cell-cycle dependent phosphorylation of the C-terminal domain of RNA polymerase II, altering transcription efficiencies (Bregman *et al.*, 2000). It plays an important role in positive and negative transcriptional regulation by phosphorylating transcription factors including Sp1 (Pugh and Tjian, 1990), Ap1 (Lin *et al.*, 1992) and serum response factor (Marais *et al.*, 1992).

Using Y2-H and immunoprecipitation assays ICP27 has been shown to interact with CK2 β subunit. Two regions of ICP27 were shown to involved in the interaction, the C-terminus containing the zinc finger and a portion of ICP27 containing the arginine rich region (Wadd *et al.*, 1999). ICP27 was shown to be required for the activation of CK2 and also the redistribution of CK2 relocating from the nucleus to the cytoplasm. Furthermore, phosphorylation of ICP27 by CK2 was required for the cytoplasmic localisation of ICP27 (Koffa *et al.*, 2003).

1.5.4 Splicing-associated protein 145 (SAP145)

SAP145 is an essential component of the spliceosome. It can bind pre-mRNA and is implicated in the tethering of U2 snRNA to the BPS (Champion-Arnaud and Reed, 1994; Staknis and Reed, 1994).

ICP27 interacts with SAP145 and during infection ICP27 and SAP145 colocalise in the nucleus of Vero cells. During HSV-1 ICP27 mutant virus infection this interaction is severely reduced or abolished. In vitro splicing reactions revealed that, in the presence of ICP27, splicing was inhibited prior to the first catalytic step (Bryant *et al.*, 2001). This data strongly indicated that ICP27 recruited SAP145 to inhibit splicing.

1.5.5 P32

P32 was originally isolated from a complex with the splicing factor ASF/SF2 (Krainer *et al.*, 1990). ASF/SF2 is a member of a family of splicing factors known to have a role in enhancing splicing and regulating alternative splicing (Fu, 1995). P32 regulates RNA

splicing by inhibiting ASF/SF2 RNA binding and phosphorylation (Petersen-Mahrt *et al.*, 1999). Immunofluorescence studies observed p32 to be located in the mitochondria and also in the nucleus as granule and tubules (Matthews and Russell, 1998). Previously it was shown that this pattern of localisation was altered upon adenovirus infection where p32 redistributes to the nucleus to co-localise with the viral core protein (Matthews and Russell, 1998). Many other proteins that interact with p32 have been reported including the HSV-1 ORF P protein (Bruni and Roizman, 1996), EBV EBNA 1 protein (Chen *et al.*, 1998) and HIV proteins Rev and Tat (Luo *et al.*, 1994; Yu *et al.*, 1995).

A Y2-H assay and immunoprecipitation assays have revealed an interaction with p32 and HSV-1 ICP27. The p32 that co-immunoprecipitated with ICP27 is phosphorylated by CK2, which is also precipitated with ICP27. During an HSV-1 infection ICP27 co-localises with p32 and alters its distribution to form a speckled pattern in the nucleus (Bryant *et al.*, 2000). These data led to the speculation that ICP27 recruits p32 to aid in the inhibition of splicing.

1.5.6 Cellular RNA polymerase II and ICP8

The transcription of all viral genes is dependent on cellular RNA polymerase II; furthermore several viral proteins stimulate viral gene transcription, e.g. ICP4, ICP0 and ICP8. ICP8 is a single stranded DNA binding protein that negatively affects transcription from the parental viral genome (Godowski and Knipe, 1985; Godowski and Knipe, 1986). ICP27, ICP8 and RNA polymerase II has been reported to form a complex at early time of infection, as determined by immunoprecipitation assays and western blot techniques, and formation of the complex was dependent on viral DNA synthesis (Zhou and Knipe, 2002). These data suggest a role for ICP27 in stimulating E and L gene expression and or a role in inhibiting cellular transcription. The association with ICP8 suggests a role in stimulating L gene expression, as the association with ICP27 was not detected until 5 h.p.i. (Zhou and Knipe, 2002).

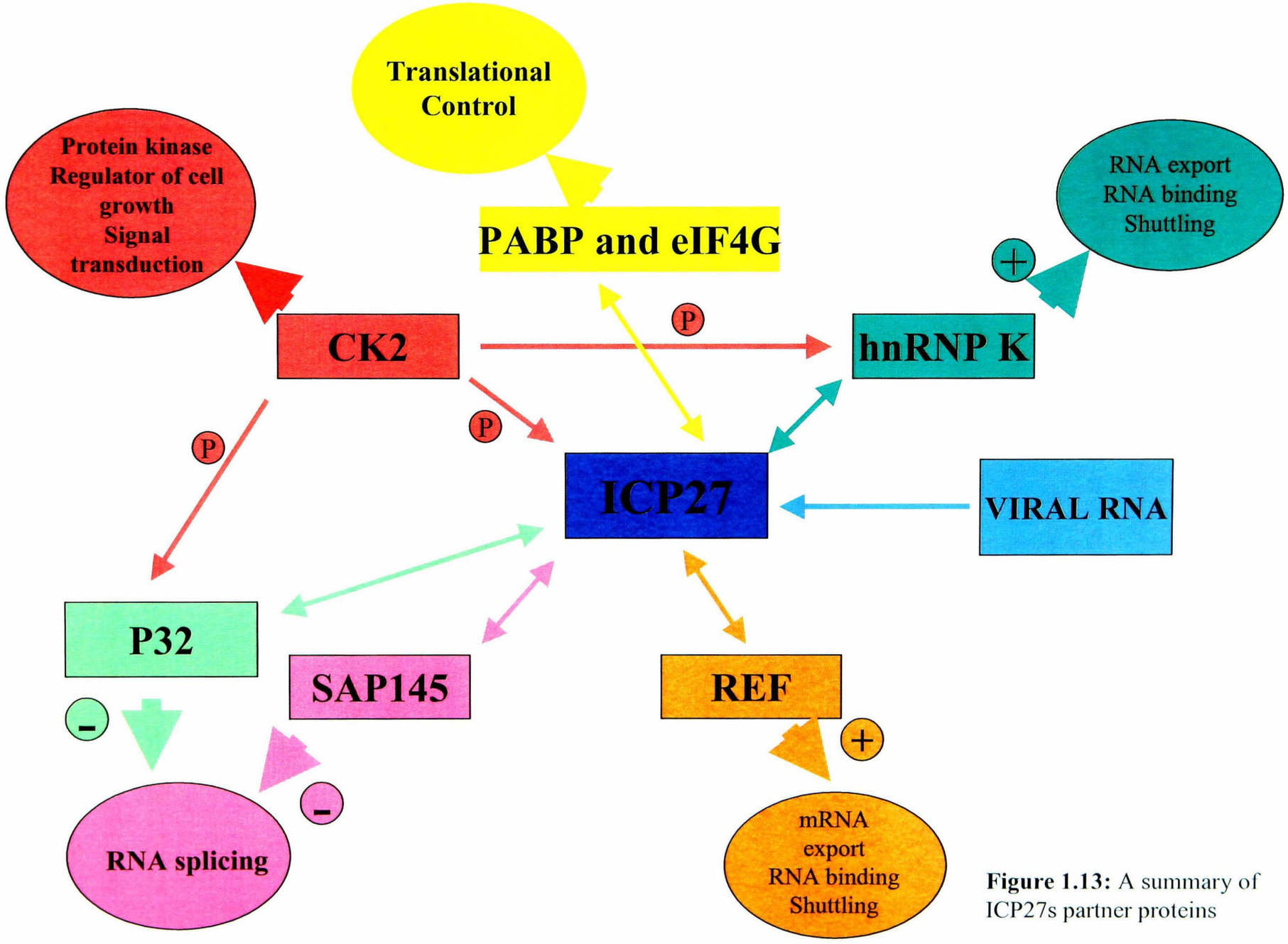


Figure 1.13: A summary of ICP27s partner proteins

1.6 ICP27 Homologues

ICP27 is a regulatory protein homologous to proteins identified in all classes of herpesviruses, implicating a regulatory function that is conserved throughout the *Herpeseviridae* family. However, although sequence similarities have been identified, the ICP27 homologues exhibit functional diversity. Comparing the structural and functional properties of these conserved genes with ICP27 aids in the understanding of each homologue and ultimately of their evolution during the emergence of the different herpesviruses.

The gammaherpesvirus homologues contain an N-terminal region from an additional exon that the alpha and betaherpesviruses do not have. The N-terminal region is not conserved throughout the herpesviruses, however the central region is conserved within the herpesvirus family.

1.6.1 BHV-1 BICP27

BICP27 is an alphaherpesvirus ICP27 homologue of bovine herpesvirus 1. The BICP27 protein is expressed early during infection, in contrast to ICP27 that is IE, and localises to the nucleus of the infected cell. This protein has a role in processing efficiencies of mRNAs that contain weak poly (A) sites (Singh *et al.*, 1996), like ICP27.

1.6.2 HCMV UL69

The betaherpesvirus HCMV gene UL69, is a positional homologue of the HSV-1 ICP27 and, unlike ICP27, is expressed at early to late times during infection and ultimately becomes a phosphorylated tegument protein of the virion (Winkler and Stamminger, 1996). A function of the HCMV protein, similar to that of ICP27, is its ability to transactivate particular viral promoters (Winkler *et al.*, 1994).

1.6.3 Herpesvirus saimiri (HVS) ORF57

ORF57 from the gammaherpesvirus HVS, is a multifunctional regulatory protein that shares limited homology with HSV-1 ICP27. ORF57 is involved in the activation and repression of early and late gene expression at the level of transcription. The repressor function was determined by the different spliced forms of ORF57 (Whitehouse *et al.*, 1998). Like ICP27, ORF57 protein structure contains a transactivation and repressor domain at the C terminus.

During HVS infection, ORF57, like ICP27, is responsible for the redistribution of the splicing factor SC35 and was found to relocate with this factor at distinct nuclear aggregates (Cooper *et al.*, 1999). Further similarities of ORF57 to ICP27 include viral RNA binding, shuttling between nucleus and cytoplasm, and the export of viral RNA (Goodwin and Whitehouse, 2001). Taken together, these data suggest that ORF57 regulates gene expression using similar pathways to HSV-1 ICP27.

1.6.4 HHV-8 ORF57

The gamma herpesvirus HHV-8 ORF57 is a spliced gene that is expressed very early in lytic cycle (Lukac *et al.*, 1999). Like HSV-1 ICP27, ORF57 localises to the nucleus, co-localises with SC35 and can shuttle between the nucleus and the cytoplasm (Bello *et al.*, 1999; Gupta *et al.*, 2000; Kirshner *et al.*, 2000).

A range of HSV-1 ICP27 identified partner proteins that are also found to associate with ORF57 include hnRNP K, CK2 and REF (Malik *et al.*, 2004a; Malik *et al.*, 2004b; Malik and Clements, 2004). ORF57 has a role in viral RNA export *via* the same pathway as HSV-1 ICP27, by forming a complex with the cellular export factors REF and TAP (Malik *et al.*, 2004b).

1.7 AIMS

The HSV-1 protein ICP27 has an essential role in viral gene expression at the level of transcription and post-transcription. It is an RNA-binding protein, however, little is known about the RNA ligands of this signature herpesvirus protein that has a counterpart in all sequenced herpesviruses that infect mammals and birds. HSV-1 RNAs that interacted specifically with ICP27 were identified using a genomic DNA library yeast three-hybrid screen and were found to belong to transcripts that encode predominantly essential proteins with roles in viral DNA replication and virion maturation: use of ICP27 mutant protein largely abolished the interactions, indicating their biological relevance (Sokolowski *et al.*, 2003). HSV-1 sense RNAs identified were 35 to 225 nucleotides in length and located to the 5'UTR and ORF's of HSV-1 IE, E and L genes. With the knowledge of the RNA sequences that bind ICP27 my project aimed to investigate any activational or inhibitory properties of these sequences in the presence of ICP27. To this end, plasmids were constructed with a selection of these sequences inserted into the 5'UTR of a reporter construct, and the functional properties of the sequences in the presence or absence of ICP27 were studied. This was achieved by measuring the levels of CAT activity after transfecting the plasmid into BHK cells and subsequently infecting with wt HSV-1, ICP27-null or ICP27 mutant viruses. CAT RNA levels were also measured during these experiments, using Q-RT-PCR, to identify the level at which ICP27 acts.

ICP27 is known to affect viral late gene expression. During an ICP27-null HSV-1 infection the expression of certain late genes are severely reduced or not detected. To analyse late gene expression when a function of ICP27 was altered, my initial experiments utilised a panel of ICP27 mutant viruses. After infecting BHK cells with these viruses, cell lysates were obtained and used to analyse expression levels of three true late genes and two leaky late genes using western blotting techniques. Furthermore, an immunofluorescence study was carried out to confirm or identify the localisation of ICP27 during each mutant virus infection. Unfortunately, as these experiments proceeded it became obvious that this was an extremely complicated system with regards to the affect ICP27 has on viral DNA replication, which in turn would influence late gene expression. True late gene expression is dependent on the onset of viral DNA

replication thus ICP27's effect on late gene expression and viral DNA replication must be separated. Furthermore, ICP27s effect on viral DNA replication when an ICP27 mutant virus is infected into different cell types is extremely variable making results difficult to interpret. Due to these difficulties, current literature regarding the affect on late gene expression is conflicting. It was decided, therefore, to discontinue this approach and with the new knowledge and identification of viral RNAs that bound to ICP27 it was decided that an investigation into the properties of these RNAs would be more informative.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Enzymes

All restriction enzymes were purchased from Roche. *Bam*HI, *Eco*RI, 10U/μl and calf intestinal phosphatase, 10U/μl

T4 DNA ligase, Invitrogen; 1U/μl

RNase A, Promega; 10U/μl

Acetyl coenzyme A; Sigma

DNase I, Promega RQ1 RNase-Free DNase; 1U/μl

Alkaline Phosphatase; Biolabs, 10U/μl.

PFU Turbo DNA Polymerase; Stratagene, 2.5U/μl

2.1.2 Radiochemicals

Radiochemicals were purchased from PerkinElmer Life Sciences Inc.

[^{14}C]-Chloramphenicol (CAM); Specific activity of 60Ci/mmol (0.05 μCi/μl)

2.1.3 Plasmids/Vectors

pcmv10 A eukaryotic expression vector containing the immediate-early (IE) promoter of human cytomegalovirus (HCMV) and RNA processing signals of SV40 (Stow *et al.*, 1993).

pcmv63 The pcmv63 plasmid consists of the complete open reading frame of ICP27 downstream of an IE HCMV promoter. This fragment was ligated

into the *Eco*RI/*Bam*HI digested pcmv10 plasmid (Donated by N. Reuthmeir, Institute of Virology, Glasgow).

pcmv-βgal The plasmid pcmv-βgalactosidase (β-gal) contains the β-gal coding sequence downstream of a CMV promoter. Provided by Nigel Stow, MRC Virology Unit , Glasgow.

psvCAT This plasmid has a SV40 promoter upstream of a CAT ORF (Figure 4.1) (a kind gift from Dr R. Everett, MRC Virology Unit, Glasgow).

2.1.4 Antibodies

Monoclonal antibodies (MAb) used during this investigation and their sources are listed in Table 2.1

2.1.5 Viruses

HSV-1 wild type (wt) strain 17^+ was used in this study. HSV-1 ICP27 mutation/deletion viruses including phenotypes and references are listed in Table 2.2.

2.1.6 Bacterial strain

The *A. coli* strain DH5α was used for maintenance and propagation of plasmid DNA.

2.1.7 Bacterial culture media

Bacterial culture media were supplied by the Institute of Virology, Glasgow.

LB-Broth:	10g NaCl, 10g Bacto-tryptone, 5g yeast extract per litre.
LB-Agar:	LB-Broth with 1.5% (w/v) agar.
YT broth	5g NaCl, 16g Bactopeptone, 10g yeast extract per litre.

PRIMARY ANTIBODY	WESTERN BLOT DILUTION	SOURCE AND REFERENCE
ICP27 (1113)	1:3000 (1:500 for IF)	Goodwin Institute for Cancer Research, Florida. Ackerman, 1984.
ICP27 (1119)	1:3000	Goodwin Institute for Cancer Research, Florida. Ackerman, 1984.
ICP4 (10176)	1:5,000	R.Everett, 1993.
ICP4 (58s)	1:100	Showalter, 1981.
ICP0 (11060)	1:10,000	R.Everett, 1993.
VP22A (LP13)	1:2,000	Serotech, Oxford, UK.
VP19 (CB2068)	1:10	C.Boutel, Institute of Virology
UL9 (13924)	1:4,000	N.Stow, Institute of Virology
VP5	1:100	D.McClelland, J.Virol 199
UL44 (4916)	1:5,000	H.Marsden, Institute of Virology
US11	1:2,500	Roller and Roizman, 1992

Table 2.1: Monoclonal antibodies used during this investigation

HSV-1 ICP27 mutant/deletion virus	Mutation type	Functional domain(s) affected	Amino acid(s) affected	Growth in BHK-21 cells	Reference
27LacZ	Deletion	All	1-512	Defective[1]	Rice, 2000
d1-2	Deletion	Acidic	12-63	Deficient[2]	Rice, 1993
d3-4	Deletion	NLS	109-138	Deficient	Rice, 1995
d4-5	Deletion	RGG	139-153	Defective	Rice, 1996
M15	Point, PG to LE	Zn finger, KH3	465,466	Defective	Rice, 1994

Table 2.2: HSV-1 ICP27 mutant/deletion viruses used during this study. The following abbreviations were used: [1] HSV-1 mutant virus that cannot grow in BHK-21 cells, [2] HSV-1 mutant viruses that have reduced titres when grown on BHK-21 cells.

Where necessary, media and LB-agar were supplemented with antibiotic: 100μg/ ml kanamycin (for pEGFP transformed cells) or 100μg/ ml ampicillin (for psv or pcmv transformed cells).

2.1.8 Cells and tissue culture media

BHK-21 C13 cells

The fibroblast line derived from baby hamster kidney cells (Macpherson, 1962) was grown in Glasgow Modified Eagles Medium (GMEM) supplemented with 10% new born calf serum, 10mM L-glutamine and 10% (v/v) tryptose-phosphate broth (the medium already contained sodium bicarbonate), 100 units/ ml penicillin and 100μg/ ml streptomycin.

M49 cells

M49 was a BHK derived ICP27-complementing cell line (Lilley et al., 2001). M49 cells were cultured in DMEM supplemented with 10% FCS and 5%TPB. Continual selection was achieved by maintaining cells in 800μg of G418/ ml and 750μg of zeocin/ ml. Provided by R.S.Coffin, Department of Molecular Pathology, University College, London.

2.1.9 Synthetic oligonucleotides

Oligonucleotides were synthesised by Sigma-Genosys.
The sequences, names and uses of the oligonucleotides are described in Table 2.3

2.1.10 Solutions

5x Agarose gel loading buffer	1x TAE, 1% SDS (w/v), 50% (v/v) glycerol, 1 mg/ ml bromophenol blue.
Blocking buffer:	PBS-Tween with 5% MARVEL skimmed milk

A: PCR primers for vector inserts

Insert	5'primer	3'primer
ICP27 mutant gene, from pAlter plasmids (d1-2, d3-4, d4-5 and M15), cloned into pEGFP-C1.	CCGGAATTCCATGGCGACTGACATTGATATGC	CGCGGATCCAAACAGGGAGGTGCA
NRE	ACGGATCCGCTAAACGCAAAA	ACGGATCCACATACAAACATA

B: Annealing primers for full vector inserts with BamHI overhangs

Insert	5'primer	3'primer
#212	GATCAAAAAAGGCCTCGGCCCTCCCTGGAACGGCTGGTCGGT CCCCGGGTTGCTGAAGGTGCGGCGGGT	GATCACCCGCCGCACCTTCAGCAACCCGGGGACCGACCAGC GTTCCAGGGAGGGCCGAGGCCTTTTTT
#247	GATCACCCGCGGTGTCGCGGCAGCACGCCTACCTGGCGTGCG AGCTGCTGCCCGCCGTGCAGTGCGCCGTGCGCTGT	GATCACAGCGCACGGCGCACTGCACGGCGGGCAGCAGCTCG ACGCCAGGTAGGCGTGCTGCCGCGACACCGCGGGT
#300	GATCAACTCCGCCCCCGCGAGTAGCGACGGCCGTGTGCCAGT CGCCATCGTT	GATCAACGATGGCGACTGGCACACGGCCGTCGCTACTCGCG GGGCGGAGTT
#82	GATCACCCCGAGTTCGCGACCGGGTACAACATCATCAACTTC GACTGGCCCTTCTTGCTGGCCAAGT	GATCACTTGGCCAGCAAGAAGGGCCAGTCGAAGTTGATGAT TTGTACCCGGTCGCGAACTCGGGGT
#334	GATCACCCCCCCGCTGTACGCAACGGGGCGCCTGAGCCAGGT	GATCACCTGGCTCAGGCGCCCCGTTGCGTACAGCGGGGGGGT
#212,1-30	GATCAAAAAAGGCCTCGGCCCTCCCTGGAACGGCTT	GATCAAGCCGTTCAGGGAGGGCCGAGGCCTTTTTT
#212,15-35	GATCACCTCCCTGGAACGGCTGGTCGT	GATCACGACCAGCCGTTCCAGGGAGGT
#212,31-63	GATCAGGTCGGTCCCCGGGTTGCTGAAGGTGCGGCGGGT	GATCACCCGCCGCACCTTCAGCAACCCGGGGACCGACCT
#212,15-24	GATCACCTCCCTGGAT	GATCATCCAGGGAGGT
#212	GATCAACGGCTGGTCGT	GATCACGACCAGCCGTT
6Gs	GATCAGGGGGGT	GATCACCCCCCT
6As	GATCAAAAAAAT	GATCATTTTTTT

C: Primers for Quantitative RT-PCR

Gene amplified	5'	3'
CAT	AATCACTGGATATACCACCGTTGA	TGAACGGTCTGGTTATAGGTACATTG

Table 2.3: (A) Primers used in the PCR of fragments for cloning. (B) The primers that were annealed and cloned into the *Bam*HI site of psvCAT vector. (C) Primers used in quantitative RT-PCR.

	powder.
Diethyl pyrocarbonate (DEPC) treated dH_20	300µl DEPC added to 500 ml dH_2O, Incubated overnight at room temperature and baked at 80°C for 20 mins to inactivate the DEPC.
6x DNA loading buffer	0.25% bromophenol blue, 0.25% xylene cyanol, 40% (w/v) sucrose made up in dH_2O.
HEPES extract buffer	50mM HEPES, 50mM NaCl, 0.1% (v/v) NP40, complete protease inhibitor Tablet (Roche), pH 7.5.
PBS	170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.2
PBS-Tween	PBS plus 0.05% (v/v) Tween 20.
7x Protease Inhibitor Cocktail	One Tablet of protein inhibitor (without EDTA) (Roche) was dissolved in 1.5 ml distilled water.
Protein Loading Buffer:	100mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 200 mM β-mercaptoethanol, 20% (v/v) glycerol, 0.2% (w/v) bromophenol blue.
Protein gel running buffer	50mM Tris base, 50mM glycine, 0.1% (w/v) SDS.
RNA loading buffer	15% (w/v) ficoll, 10mM sodium phosphate (pH 7.0), 0.25% (w/v) bromophenol blue, 1mM EDTA

STET solution	1% (v/v) Triton-X100, 40mM EDTA, 50mM Tris-HCl, 8% (w/v) sucrose, pH 8.
10x Tris-Borate-EDTA (TBE):	890mM Tris, 890mM boric acid, 10mM EDTA.
TAE buffer	40mM Tris-acetate 1mM EDTA
TE buffer	10mM Tris-HCl, 1mM EDTA, pH 8.
TEN buffer	50mM Tris pH 7.2, 1mM EDTA, 0.15mM NaCl.
Towbin's Blotting buffer	25mM Tris base, 192mM glycine, 20% (v/v) methanol.
Trypsin	0.25% (v/w) trypsin, in Tris-saline, containing phenol red, adjusted to pH 7.5 with $NaHCO_3$.
Versene	0.6mM EDTA in PBS, 0.002% phenol red.
Water (dH_20)	Sterile dH_20 obtained from a "Milli-Ro 60 plus" deioniser (Millipore, USA) and sterilised by autoclaving.

2.1.11 Chemicals and reagents

All chemicals and reagents were purchased from BDH Chemicals UK or from Sigma Chemicals Co. unless otherwise stated in this section or in the methods.

30% Acrylamide solution, acrylamide:bis-acrylamide, 37.5:1
40% Acrylamide solution, acrylamide/bis solution 19:1 (Bio-Rad)
3MM paper (Whatmann)
Ampicillin (Penbritin)
Ampliscribe T7 Transcription Kit (Cambio)
Bradford's reagent (Bio-Rad)
Diethyl Pyrocarbonate (DEPC)
ECL Western Blotting Detection Reagents (Amersham)
Ethidium bromide
Ethyl acetate (Fisher Bioreagents)
High Pure PCR Purification kit (Roche)
Lipofectamine 2000 (Invitrogen)
Mini Quick Spin RNA Columns (Roche)
Nitrocellulose Membrane (Hybond-ECL, Amersham)
Nonidet P-40 (NP40)
Nuclear Extract Kit (Active Motif)
Omniscript RT Kit (QIAGEN)
Penicillin/Streptomycin (Gibco)
Precision Broad Range Prestained Protein Marker (BioRad)
QIAfilter Midi Prep Kit (QIAGEN)
QIAquick Gel Extraction Kit (QIAGEN)
Rapid DNA LigationKit (Roche)
RNeasy Mini Kit (QIAGEN)
SYBR Green PCR Mastermix (Applied Biosystems)
TEMED (N,N,N',N' Tetramethylethelenediamine)
TLC plastic sheets (Merk)
TRIzol reagent (Invitrogen)

2.2 Methods

2.2.1 DNA manipulation

2.2.1.1 Polymerase chain reaction (PCR) amplification of DNA

PCR reactions were carried out in a Hybaid PCR Express machine in thin walled 0.5 ml tubes. Typical PCR reactions were performed in a final concentration of 50µl containing 1U Pfu turbo (DNA polymerase), 1x Pfu PCR buffer (supplied with Pfu turbo), 0.5mM dNTPs and 10-50pmol/µl primer. The DNA templates were used at a final concentration of 0.2ng/µl (plasmid F11) or 2ng/µl (pAlter plasmid). Prior to PCR cycles, reactions were heated at 94°C for 3 min to allow complete denaturation of the nucleic acids. PCR reactions were carried out under the following conditions: i) strand-separation at 94°C for 30 sec, ii) primer annealing at 54°C or 65°C for 30 secs (temperature varied depending on the primer), iii) strand elongation at 72°C for 30 sec for 30 cycles, iv) a final elongation step at 72°C for 5 min.

2.2.1.2 Oligonucleotide annealing

All sequences to be inserted into the psvCAT (except the control sequence) were ordered as full-length oligonucleotides (see Table 2.3 B) and annealed as follows:

5' oligonucleotide	7µl
3' oligonucleotide	7µl
Buffer B (Roche)	5µl
dH_2O	31µl

Each reagent was pipetted into 1.5 ml reaction vials, placed in a beaker of boiling water and allowed to cool. The annealed primers were purified, as below, to allow a change of buffer during ligation.

2.2.1.3 Purification of DNA after annealing or digesting

Annealed or digested DNA was cleaned before further manipulation by using High Pure PCR Product Purification Kit (Roche) in which DNA binds to glass-fibres in spin-columns in the presence of guanidine isothiocyanate. The protocol was performed according to the manufacturer's instructions. Briefly, 500µl of binding buffer was added to the PCR reaction and vortexed briefly. This mixture was applied to a spin column and centrifuged at 15,700 xg for 1 min in a bench top centrifuge at room temperature. The flow through was discarded and 500µl of wash buffer was applied to the upper chamber of the column, which was centrifuged again for 1 min. Again the flow through was discarded, 200µl of wash buffer was added and the column centrifuged for 1 min once more. The column was centrifuged for 1 min to remove residual buffer, the column attached to a fresh 1.5 ml centrifuge tube and 50µl of elution buffer added to the centre of the column. The column was left for 1 min and then centrifuged for 1 min at 15,700 xg, leaving the eluted DNA. The DNA was analysed by agarose gel electrophoresis prior to its use, to determine the amount and quality of DNA.

2.2.1.4 Agarose gel electrophoresis of DNA

Gel electrophoresis of DNA samples was carried out using 1% solution of agarose (w/v) in TBE containing, 0.4µg/ ml ethidium bromide, on a horizontal slab gel apparatus.
1x TBE was used as a running buffer. Samples were loaded in 5x DNA loading buffer. Between 75 and 150 V was applied to the apparatus for 30-50 min. Gels were analysed on a ultra-violet transilluminator and images recorded.

2.2.1.5 Purification of DNA from agarose gels

PCR DNA fragments or digested plasmids were electrophoretically separated on an agarose gel as described above. The gel was visualised on a long-wave UV transilluminator and the band corresponding to the DNA fragment of interest was excised from the gel with a scalpel. Care was taken to avoid contamination with other bands in the lane. The DNA was purified using the QIAquick Gel Extraction Kit. Briefly, 300µl buffer QC was added to the gel slice and incubated at 50°C for 10 min, vortexing every 2-3 min. 100µl isopropanol was added and the sample was placed into QIAquick column and centrifuged at 15,700 xg for 1 min. 750µl wash buffer PE was added to wash the column and centrifuged for 1 min. The QIAquick column was placed into a fresh tube and the DNA was eluted from the column by adding 50µl elution buffer. Samples were analysed on an agarose gel.

2.2.1.6 Restriction enzyme digestion of DNA

Small-scale restriction enzyme digestion of DNA was used to digest plasmids, PCR fragments or identify recombinant plasmids. Digests were generally carried out in 20µl reaction volumes using 10U of restriction enzyme per µg of DNA and 1x enzyme specific buffer. Reactions were incubated at 37°C for 3 h before further treatment or analysis.

2.2.1.7 Alkaline phosphatase treatment of plasmid DNA

Removal of 5' phosphates from vector fragments with complementary ends, produced by digestion with a single restriction enzyme, was performed by incubating the DNA with 1U of alkaline phosphatase in the provided buffer, for 30 min at 37°C. The alkaline phosphatase was heat-inactivated at 65°C and the treated DNA was later re-purified using the purification method above.

2.2.1.8 Ligation of DNA using T4 DNA ligase

Samples of the purified fragments were run on an agarose gel to determine the concentrations of each to be used in the ligation reactions, and molar ratios of vector to insert DNA between 1:1 and 1:5 were used. The standard reaction carried out was as below:

vector DNA	1µl	(100ng)
insert DNA	19µl	
T4 DNA ligase	3µl	(1U/µl)
10 x ligase buffer (Sigma)	3µl	
dH_2O	4µl	

Reactions were left for 16 h at room temperature and transformed into bacterial cells immediately as described below.

2.2.1.9 Ligation using Rapid DNA Ligation Kit

Purified annealed oligonucleotides were ligated into the psvCAT using this method. The plasmid and oligonucleotide DNAs were diluted in DNA dilution buffer (as supplied) to a total volume of 10µl. The molar ratio of vector to insert used was 1:5. 10µl ligation buffer and 1µl T4 DNA ligase was added to the reaction mixture, left at room temperature for 15 min and transformed immediately, as described below.

2.2.1.10 Making competent E.coli

A single colony of DH5α bacteria was inoculated into 10 ml of L-broth and grown at 37°C for 16 h. 2 ml of the overnight culture was used to inoculate 200 ml L-broth and incubated at 37°C with shaking until an OD_{600} of 0.4 was reached. The culture was centrifuged at 2100 xg for 10 min at 4°C, the pellet resuspended in 20 ml ice-cold 0.1M $CaCl_2$ and incubated on ice for 30 min. Cells were centrifuged at 2100 xg and the resulting pellet resuspended in 10 ml ice-cold 0.1M $CaCl_2$ and incubated in ice for a further 15 min. The suspension was centrifuged once more and the pellet was

resuspended in 4 ml ice-cold 0.1M $CaCl_2$ with the addition of 15% glycerol, dispensed, snap frozen and stored at -70°C.

2.2.1.11 Transformation

Transformation of plasmids into competent cells used approximately 0.2μg of DNA, which was mixed with 75μl of competent bacteria and the mixture was chilled on ice for 30 min. The cells were then heat-shocked at 42°C for 2 min and immediately placed back on ice for 2 min. 200μl of YT broth was added and tubes were placed in a 37°C water bath for 1 h. 100μl of this suspension was spread onto an LB-agar plate containing 100μg/ ml ampicillin or, in the case of EGFP plasmids, 100μg/ ml kanamycin.

2.2.1.12 Miniprep and midiprep plasmid DNA preparations

(i) Quick analysis of plasmids during cloning.

Single colonies of transformed bacteria were picked and inoculated into 3 ml aliquots of L-broth containing the appropriate antibiotic. Cultures were grown for 6 h at 37°C in a shaking incubator. 1.5 ml of each culture was centrifuged at 15700 xg for 1 min. The bacterial pellets were resuspended in 200 μl STET solution and 20μl of lysozyme (10 mg/ ml) was added before placing into a boiling waterbath for 40 sec. Cell debris was removed by centrifugation at 15700 xg for 10min. The supernatant was removed to a fresh tube, 0.9 volumes of isopropanol with 0.1 volumes of 3M sodium acetate were added to precipitate the DNA, and the tube was centrifuged at 15700 xg for 10 min. The DNA pellet was allowed to dry before resuspending in 20μl dH_2O. 1μl of RNase A was added and placed in a 37°C water bath for 1 h before digesting and analysing on an agarose gel.

(ii) Small scale plasmid preparation: Qiagen 'QIAprep spin' miniprep kit

This method was used to prepare high quality DNA for sequencing. The kit is based on an alkaline lysis method followed by selective adsorption of plasmid DNA onto a silica-gel column and subsequent elution from the silica gel by water.

The use of this kit was according to the manufacturer's instructions. 1.5 ml of bacterial culture was centrifuged for 1 min 15700 xg. The bacterial pellet was resuspended in 250µl of chilled Buffer P1. To lyse the cells, 250µl Buffer P2 was added and the tube inverted 6 times. 350µl Buffer N3 was added to neutralise the solution, and the tube once more inverted 6 times. After centrifugation for 10 min at 15700 xg the supernatant was transferred to a QIAprep column and centrifuged for 1 min at 15700 xg. 0.75 ml of wash buffer Buffer PE was added to the column, which was again centrifuged at 15700 xg for 1 min. The column was centrifuged for 1 min again, to remove any residual buffer, transferred to a clean 1.5 ml tube and 50µl dH_2O added to the centre of the silica gel. After 1 min, to allow the water to filter throughout the silica gel, the column and tube were centrifuged for 1 min at 15700 xg, after which the DNA had eluted in the water.

(iii) Large scale preparation of DNA

Midi prep DNA preparation was carried out on all EGFP plasmids.
Qiagen midi preps were used to prepare large-scale preparations of plasmid DNA for transformation or expression in mammalian cells.

0.2 ml of an overnight 10 ml culture from a single colony of the relevant bacterial strain was inoculated into 50 ml L-Broth (plus antibiotic) and incubated at 37°C for 12-16 h (overnight) with shaking. The culture was centrifuged at 6000 xg for 15 min at 4°C and the bacterial pellet was resuspended in 4 ml of buffer P1. The suspension was lysed with 4 ml buffer P2 for 5 min and 4 ml of chilled buffer P3 was added to neutralise the suspension, which was immediately poured into a QIAfilter cartridge and allowed to settle for 10 min. The lysed bacterial suspension was pushed through the QIAfilter

cartridge to clear any precipitated bacterial debris by a syringe method. Passing the filtrate through an equilibrated QIAGEN-tip 100 purified the DNA. This was then washed twice with buffer QC. The DNA was eluted by adding 5 ml buffer QF and allowing the eluant to drip through by gravity flow. The DNA was precipitated by adding 3.5 ml isopropanol and immediately centrifuged at 15000 xg for 30 min. The DNA pellet was washed with 70% ethanol and centrifuged at 15000 xg for a further 10 min. The pellet was air dried and re-dissolved in 50µl dH_20. The DNA content was measured with a spectrophotometer and the concentration calculated was roughly confirmed by running the sample on an agarose gel in comparison with known standards.

(iv) Large scale preparation of high quality DNA.

Caesium chloride banded plasmid DNA was used for analysis of CAT expression.

500 ml of overnight bacterial culture was pelleted by centrifugation at 9200 xg for 10 min at 4°C. The pellet was resuspended in 10 ml solution 1 (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA). The cells were lysed by adding 10 ml of freshly prepared solution 2 (0.2M NaOH, 1% SDS), mixed by inversion and incubated at room temperature for 10 min. The sample was neutralised by adding 15 ml ice-cold solution 3 (3M potassium acetate, 11.5% acetic acid), inverted 5 times and incubated on ice for 10 min to precipitate the protein complexes. Cell debris was pelleted by centrifugation at 9200 xg for 15 min at room temperature. The supernatant was filtered through muslin and the plasmid DNA was precipitated from the filtrate by adding 26 ml isopropanol, incubating at room temperature for 10 min and pelleted by centrifuging at 9200 xg for 15 min at room temperature. The DNA pellet was allowed to air dry and was resuspended in 5.5 ml TE buffer with 5.5g CsCl and 400µl ethidium bromide (10 mg/ ml). This solution was centrifuged at 12100 xg for 10 min at room temperature to remove any undissolved material. The supernatant was transferred to an ultracentrifuge tube and centrifuged at 197400 xg for 16 h at room temperature. The plasmid DNA band was removed by side-puncturing the tube using a syringe and needle and transferred to a 15 ml tube. Ethidium bromide was removed from the plasmid DNA by repeated extraction with TE-saturated butanol. The DNA was precipitated with chilled

ethanol, incubated on ice for 30 min and pelleted by centrifuging at 12100 xg for 10 min. The DNA pellet was washed with 70% ethanol and finally resuspended in 500µl TE buffer. The DNA content was measured with a spectrophotometer and the concentration calculated was roughly confirmed by running the sample on an agarose gel in comparison with known standards.

2.2.1.13 Sequencing of DNA fragments

DNA sequencing was performed by the University of Glasgow MBSU using an ABI 377 DNA sequencer. The procedure used was the Sanger method based on chain termination. DNA was denatured to give single stranded DNA by heating to 96°C. A site-specific primer was annealed to one of the DNA strands and the sequencing reaction continued in the presence of nucleotides (dNTPs) and dideoxynucleotideds (ddNTPs) labelled with a fluorescent dye. The ddNTPs are identical to the NTPs except they contain a hydrogen group on the 3' carbon instead of a hydroxyl group. This modification prevents the addition of further nucleotides and thus the DNA chain is terminated with a fluorescent ddNTP. As the reaction starts at the same nucleotide, the continually synthesised DNA chain will terminate at all possible positions, creating DNA stretches of different lengths. The DNA is denatured again and the contents of each tube are run on a polyacrylamide gel to separate each band. As each dye has a different wavelength, a laser can read the gel to determine the identity of each band (G, A, T or C). The results are given as a chromatograph where each coloured peak represents a nucleotide in that location of the sequence i.e. blue is C, green is A, black is G and red is T.

2.2.2 RNA extraction and manipulation procedures

2.2.2.1 Extraction of total RNA from BHK cells

Extraction of RNA from transfected and/or infected cells was carried out using TRIzol reagent, a mono-phasic solution of phenol and guanidine isothiocyanate. Whole cell extracts were disrupted by the solution, following which the addition of chloroform separated the lysate into protein, DNA and RNA phases. Use of this reagent was according to the manufacturer's instructions.

Initially, 1 ml of TRIzol reagent was added to a 35mm cell culture dish, transferred to a 1.5 ml sample tube and vortexed to give an even suspension. This was incubated at room temperature for 5 min and 0.2 ml chloroform was added, the tubes vigorously shaken for 20 sec and then incubated at room temperature for 5 min. The suspension was centrifuged at 15700 xg for 15 min, at 4°C, which allowed a clear separation into a lower organic phase (proteins and cell debris), an interphase (DNA) and an upper aqueous phase of approximately 600 µl containing the RNA. 500µl of the aqueous phase was transferred to a new 1.5 ml tube. 500µl of isopropanol was added and the tubes mixed vigorously for 20 sec. The solution was incubated for 10 min at room temperature and centrifuged at 15700 xg for 10 min at 4°C. The pellet was washed with 1 ml 70% ethanol in DEPC-treated dH_20. Pellets were air-dried and resuspended in 50µl of DEPC-treated dH_20 and stored at -20°C.

2.2.2.2 Isolation of cytoplasmic RNA

All solutions were supplied from the Nuclear Extract kit (Active Motif). A 35mm dish of confluent cells was washed with 2.5 ml of the supplied PBS/Phosphatase Inhibitor solution and the cells harvested by scraping in 1.5 ml PBS/Phosphatase Inhibitor solution. Cells were centrifuged for 5 min at 4000 xg at 4°C and the cell pellet resuspended in 0.25 ml 1x hypotonic buffer solution and incubated for 15 min on ice. 12.5µl of the detergent solution was added, the suspension vortexed for 10 sec and

centrifuged for 30 sec at 15700 xg at 4°C. The supernatant contained the cytoplasmic fraction and RNA was extracted using the TRIzol method above. Extracted RNA was analysed on an agarose gel and quantified and standardised using a NanoDrop spectrophotometer.

2.2.2.3 DNase treatment of RNA

19µl TRIzol extracted RNA was incubated with 2µl 10x DNAse buffer and 1µl DNase for 30 min at 37°C. The solution was extracted twice by phenol/chloroform extraction, once with chloroform and then the RNA was ethanol precipitated.

2.2.2.4 Phenol/chloroform extraction and ethanol precipitation of RNA

Precipitated RNA was resuspended in 200µl water and 200µl phenol:chloroform:isoamyl alcohol (25:24:1) was added. The mixture was vortexed for 30 sec and centrifuged at 15700 xg for 5 min. The upper phase was carefully removed and transferred to a clean microfuge tube. The RNA was precipitated with the addition of 0.5 volumes of 7.5M ammonium acetate and 2.5 volumes of ice-cold ethanol and incubated at -20°C for 1 h. The RNA was pelleted by centrifuging at 15700 xg for 10 min, washed once with 70% ethanol and resuspended in dH_20.

2.2.2.5 Generation of cDNA from chloramphenicol acetyltransferase (CAT) RNA by RT-PCR

Using TRIzol extracted, DNAse treated RNA, generation of cDNA was performed using the Omniscript Reverse Transcription kit (QIAGEN). 3µl (120ng) standardised RNA was added to 9.7µl dH_2O and 20µl mineral oil was placed on top. To denature any secondary structures, the RNA was incubated at 65°C for 5 min and cooled to 4°C. A mastermix was prepared consisting of the following quantities per sample:

10x buffer RT	2.0µl
dNTP (5mM of each dNTP)	2.0µl
Primer (40 pmol/µl)	1.0µl
RNasin (40U/µl)	0.3µl
Omniscript	1.0µl
dH_2O	1.0µl

The mastermix was added to the denatured RNA, ensuring that pipetting was below the mineral oil. Each sample was placed on a BIOMETRA PCR machine and incubated at 37°C for 1 h, 93°C for 5 min and 4°C for up to 24 h.

2.2.2.6 Quantification of extracted RNA by Quantitative PCR (Q-PCR)

The resulting CAT cDNA from the above RT-PCR procedure was subjected to quantitative PCR (Q-PCR) using the SYBR Green I mastermix (Applied Biosystems) which contains SYBR Green I dye, Amplitaq ® Gold, DNA polymerase, dNTPs and PCR buffer. SYBR Green I is a fluorescent dye that binds to the minor groove of double-stranded DNA upon which fluorescence increases greatly.

Highly purified salt-free primers for the target within the CAT gene were designed using the Primer Express package (Table 2.3 C). These primers were designed to produce a product of 104bp and optimised to an annealing temperature of 60°C. A mastermix (SYBR Green I mastermix and primers) was made up in bulk to allow for each RNA sample to be tested in triplicate i.e. 12µl SYBR Green I mastermix and 0.75µl of each primer (10pmol/µl) and 10µl of dH_2O. This mix was vortexed for 1 sec before 24µl of mastermix was dispensed into each well of a 96 well plate and 1µl of cDNA was added, ensuring that no air bubbles were present. The plate was placed on a TaqMan PCR machine linked to ABI Prism software.
The cycling program used was as follows:

initial stage	50°C	2 min
initial denaturation	95°C	10 min
then 40 cycles of:		
denaturation	95°C	15 sec
annealing/elongation	60°C	1 min

Analysis was carried out using the ABI Prism package, which calculates the relative amount of RNA in each sample on a scale of 0 to 40, with 40 indicating no detectable RNA and 0 being the maximum detectable RNA.

2.2.3 Tissue culture techniques

2.2.3.1 Maintenance of cell lines:

Mammalian cells were grown in 175 cm^2 flasks for 3-4 days until confluent. Cells were washed twice with 20 ml versene and the versene was discarded. 4 ml of a 1:4 solution of trypsin:versene was added to the cells, followed by incubation at 37°C for 2 min. When the cells started to detach from the flask, they were harvested by adding 10 ml of medium. This cell suspension was passaged by splitting in a 1:10 ratio into a new flask. Cells and were grown at 37°C in a humidified incubator supplied with 5% (v/v) CO_2.

2.2.3.2 Liposome-mediated transfection of BHK cells:

Transfections were carried out in 24 well plates when BHK cells were 80% confluent. The medium was changed to penicillin/streptomycin free medium 30 min before transfection. 50µl Opti-MEM was mixed with 1µl Lipofectamine 2000 and incubated for 10 min at room temperature. 300ng of plasmid DNA was added to the Lipofectamine/Opti-MEM mix and incubated for 15 min at room temperature. The DNA/ liposome solution was added gently to the cells and left for 16 h.

2.2.3.3 Infection of cells

80% confluent BHK monolayers were infected with wt HSV-1 or HSV-1 ICP27 mutant viruses at a multiplicity of 1 or 10, or no virus (mock). Adsorption was for 1 h at 37°C, after which the medium was changed and infection was allowed to proceed at 37°C in a humidified incubator supplied with 5% (v/v) CO_2.

2.2.4 Virus propagation techniques

2.2.4.1 Preparation of virus stocks

BHK21 C13 (for wt HSV-1 virus) and M49 cells (for HSV-1 ICP27 mutant viruses) were grown in roller bottles to a confluency of 80% and infected at an MOI of 1 plaque forming units (pfu) in 300 cells in 40 ml of medium at 31°C for 3-5 days until a cytopathic effect (CPE) was observed. Cells were detached by shaking the roller and harvested by centrifugation at 950 xg for 30 min at 4°C. To isolate the cell-release virus, the supernatant was removed and centrifuged at 22100 xg for 2 h. The pellet was resuspended in 1 ml/roller bottle of culture medium, sonicated briefly, dispensed, and frozen on dry ice and stored at -70°C. The cell-associated virus was harvested by resuspending the cell pellet in 5 ml medium and sonicated for 10 min in a sonibath. The cell debris was pelleted by centrifugation at 950 xg for 10 min and the supernatant removed. The pellet was again resuspended in 5 ml fresh medium, sonicated and pelleted. The supernatant was added to that gained in the first round, dispensed, frozen on dry ice and stored at -70°C. Serial dilutions (10^{-1}-10^{-8}) of the virus stock were titrated on BHK cells (for wt HSV-1 virus) or M49 cells (for HSV-1 ICP27 mutant viruses). Cells were grown on 60mm dishes to 90% confluency and infected for 1 h at 37°C. Cells were overlaid with BHK or M49 media containing 1% methylcellulose. After 2-3 days the medium was removed and the cells were overlayed with Giemsa stain for 2 h and gently washed with water to remove excess stain. The Giemasa stain stained the fibroblastic cells and allowed detection and counting of plaques (cleared areas due to cell cell death) under a microscope. The titre was calculated as pfu/ ml. Virus stocks were checked for bacterial contamination by streaking a loopfull of virus stock onto

blood agar plates, containing 10% (v/v) horse blood (blood agar) and incubating at 37°C for 7 days; if colonies appear the virus stock should be discarded.

2.2.5 Protein manipulation techniques

2.2.5.1 Preparation of cell lysates for western blot analysis

The culture medium was removed from monolayers of BHK cells grown in 35mm dishes, the cells were washed with PBS and the solution discarded. Cells were scraped into 1 ml fresh PBS and centrifuged at 4000 xg for 10 min. Cell pellets were resuspended in 200µl HEPES buffer, sonicated and centrifuged at 15700 xg at 4°C for 10 min. The supernatant was removed to a fresh tube and stored at -20°C.

2.2.5.2 Preparation of cell lysates for CAT assays

BHK cells grown in a 24 well dish were washed twice with PBS and the solution was discarded. 400µl TEN buffer was added to each well and left until cells detached from the well. Cells were collected into a 1.5 ml vial, centrifuged at 15700 xg for 10 min and the supernatant discarded. Pelleted cells were resuspended in 30µl 0.25M Tris.HCl pH 7.5 and lysed by three rounds of freeze/thawing. Centrifuging at 15700 xg for 10 min pelleted cell debris, and supernatants were removed to a fresh tube and stored at -20°C.

2.2.5.3 β-Galactosidase(β-gal) Enzyme Assay system

BHK cells were co-transfected with psvCAT constructs and β-gal plasmids. Cell extracts were assayed for β-gal activity. Samples obtained were treated using the β-gal Enzyme Assay System (Promega) on a 96 well plate format and activity was measured by absorbance of the samples at 405nm in a plate reader (Anthos htII plate reader).

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

35µl protein loading buffer was added to standardise cell lysates and boiled at 100°C for 2-3 min prior to separation by SDS-PAGE. Denatured proteins were resolved by electrophoresis through polyacrylamide minigels containing SDS. Gel mixes were as follows

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

After the resolving gel mixes were poured between the glass plates of the mini gel kits, dH_2O-saturated butanol was overlaid to produce a smooth interface. Once set, the butanol was removed and the gel rinsed with dH_2O prior to overlaying the stacking gel. Samples were loaded onto the gel and electrophoresed at 150V in 1x running buffer until the dye front reached the bottom of the gel.

2.2.5.5 Western blot analysis of denatured proteins

Denatured proteins resolved on a SDS-PAGE were transferred to nitrocellulose membranes by the method of Towbin *et al* (Towbin *et al.*, 1992) using a BioRad transblot cell. The western blot gel sandwich components, pre-soaked in Towbin's

buffer, were assembled as follows. The gel was placed onto an appropriate sized piece of Whatman 3MM paper. A piece of nitrocellulose membrane was placed onto the gel and air bubbles removed with a pipette. Another piece of Whatman paper was placed on top of the membrane and the sandwich was put between two sponges provided by BioRad and inserted into the blotting cassette. Transfer was carried out at 100mA for 1 h. Following transfer, the membrane was incubated in 50 ml blocking buffer overnight at 4°C. The membrane was washed twice for 10 min with PBS before the primary antibody was added (at the appropriate dilution in 1% blocking buffer). Incubation was continued for 1 h at room temperature. The nitrocellulose membrane was washed three times for 10 min in PBST before being left for 1 h in horseradish peroxidase (HRP) conjugated antibody (at appropriate dilution in 1% blocking buffer). The membrane was finally washed three times in PBST for 10 min each and proteins detected by ECL western blot detection reagents. The membrane was placed between two sheets of transparent plastic and exposed to Kodak X-Omat S film for varying lengths of time.

2.2.5.6 CAT assays

Chloramphenicol acatyltransferase (CAT) is a popular reporter gene and protein. CAT catalyses the transfer of the acetyl group from acetyl coenzyme A (acetyl CoA) to chloramphenicol resulting in the formation of mono- and di-acetylated chloramphenicol. The amount of acetylation is directly proportional to the amount of CAT enzyme present, thus the amount of acetylated chloamphenicol in different protein extracts can be measured. In this protocol, [^{14}C]-chloramphenicol (CAM) was used and the products were analysed by thin layer chromatography (TLC). This involves extraction with organic solvents, resuspension and spotting of the sample onto the origin of a TLC plate. The TLC plate was lowered into a chamber containing a solvent 2cm from the bottom and the sample was allowed to migrate up the TLC plate thus separating the different acetylated forms of [^{14}C]-CAM (Figure 2.1). The TLC plate was then dried and exposed to a phosphoscreen for analysis.The protocol used in this investigation was as follows:

2, 10 OR 20µl BHK cell lysate obtained for CAT assays was incubated with 1µl 50mM acetyl CoA, 1µl [^{14}C]-CAM and 18µl 0.25M Tris.HCl pH 7.5 at 37°C for 1 h. The reaction was stopped by adding 200µl ethyl acetate, mixed by vortexing and centrifuged

at 15700 xg for 5 min. The upper organic phase was retained and placed in a Speedivac to be lyophilised for 30 min. Each sample was resuspended in 25µl ethyl acetate and vigorously vortexed for 20 sec. Samples were spotted onto a TLC plate, which was placed into a shallow pool of solvent consisting of 95 ml chloroform, and 5 ml methanol allowing separation of acetylated and unacetylated forms of [^{14}C]-CAM as the solvent rose through the sample. When the solvent reached near to the top of the plate, the TLC plate was removed and dried in a fume hood. The plate was exposed to a previously erased phosphor screen for approximately 2 h and scanned to give an image of each sample on the TLC plate. Each band was analysed and quantified using Bio-Rads' Quantity One software. The amount of acetylated [^{14}C]-CAM was quantified in cmp, which was then divided by the total amount of [^{14}C]-CAM, in cpm, and multiplied by 100 to give % CAT activity. Each plasmid was transfected in BHK cells on at least three separate occasions and CAT assays carried out on the lysates obtained.

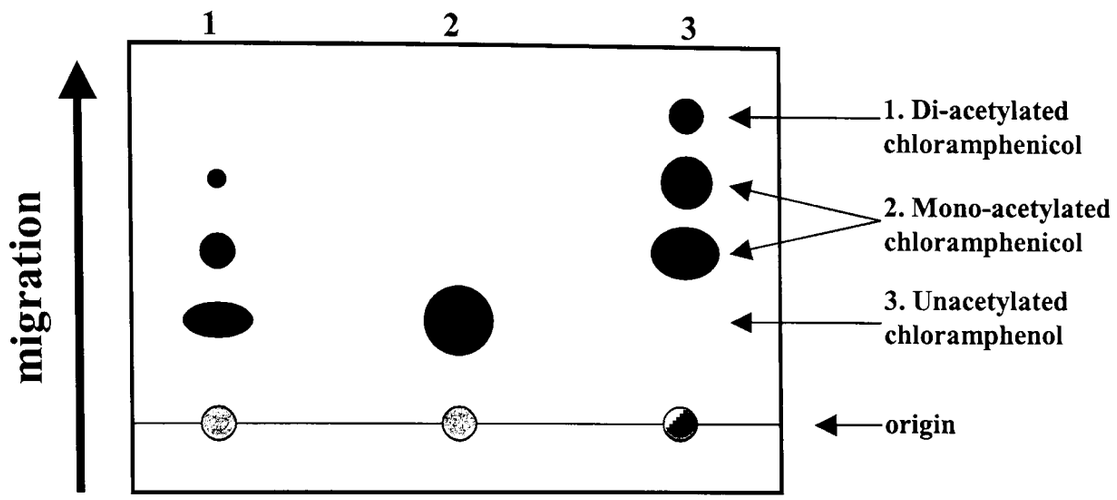


Figure 2.1: This diagram represents a phosphoscreen exposed to a TLC plate after TLC demonstrating the separation of acetylated and unacetylated forms of [^{14}C]-CAM. Lane 1: psvCAT construct 1; Lane 2: negative control; Lane 3: positive control.

CHAPTER 3: The use of a CAT expression vector to investigate the properties of ICP27-binding sequences in BHK cells.

3.1 Introduction

The RNA binding properties of ICP27 are crucial to virus viability (Lengyel *et al.*, 2002; Mears and Rice, 1996b; Sandri-Goldin, 1998a). Functional properties of ICP27 in connection with the RNA binding domains of ICP27 have been studied using various viruses containing deletions or mutations of the RGG box, or of the putative KH domain(s) of the ICP27 protein (Rice and Lam, 1994; Soliman and Silverstein, 2000b) both of which are RNA binding domains. Although these mutant forms of the ICP27 protein are produced during infection, the disruptions of these respective RNA binding domains have detrimental effects on virus survival producing no, or severely reduced numbers of, infectious progeny. Furthermore, the mutations greatly reduce the nucleocytoplasmic shuttling capabilities of ICP27 and ultimately inhibit the expression of some late genes, e.g. gC (Jean *et al.*, 2001). The shuttling capability is possibly an RNA export function of ICP27 that diminishes when ICP27 cannot bind RNA (Sandri-Goldin, 1998a; Soliman *et al.*, 1997; Soliman and Silverstein, 2000b).

Previously, a few HSV-1 RNAs were found to bind ICP27 by performing UV cross-linking experiments, covalently binding protein to RNA. Seven poly(A)$^+$ HSV-1 RNAs, those encoding ICP4, TK, ICP8 gD, gC, UL41 and ICP27, were found to bind ICP27. This result also confirmed the association of ICP27 with intronless RNAs, as two RNAs containing introns, ICP0 and UL15, did not bind to ICP27 (Sandri-Goldin, 1998a).

A more wide-ranging investigation to identify HSV-1 RNAs that bind ICP27 was carried out (Sokolowski *et al.*, 2003). This involved an extensive yeast 3-hybrid (Y3-H) study isolating from a genomic library HSV-1 RNAs that interact with ICP27 protein.

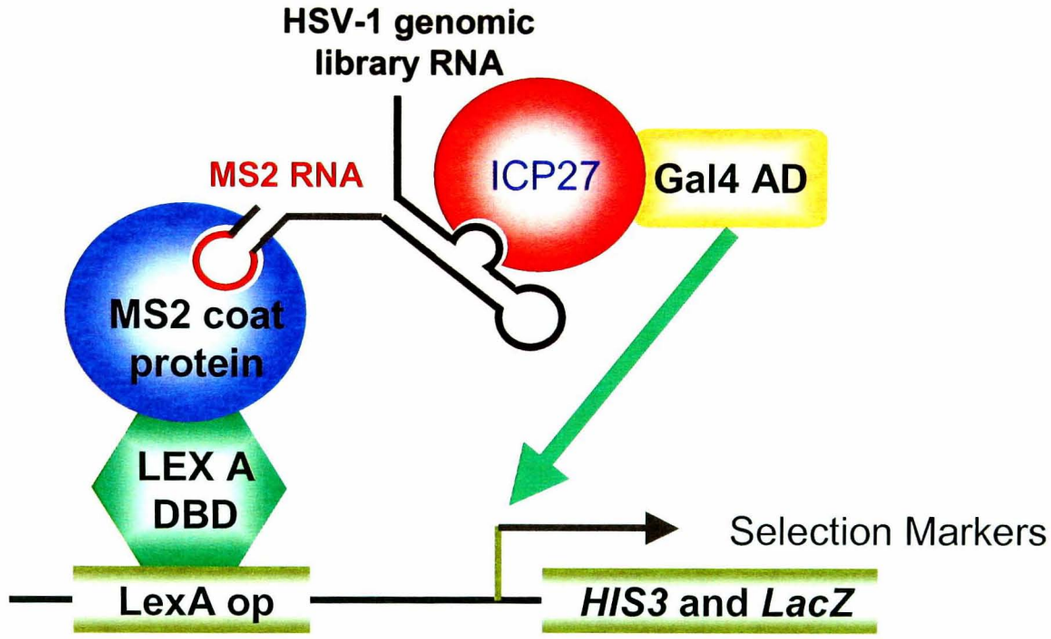


Figure 3.1: Schematic drawing of the complex formed during the yeast-3 hybrid system

The Y3-H system relies upon a physical RNA-protein interaction resulting in the assembly of two protein hybrids and a fusion RNA. This forms a complex capable of *trans*-activating promoters of *LacZ* and *HIS3* reporter genes. Briefly, a yeast strain that stably expresses the bacteriophage MS2 coat protein was transformed with a plasmid expressing an ICP27/GAL4-binding domain fusion protein. These cells were also transformed with an RNA expression vector containing a range of small HSV-1 inserts, from an HSV-1 genomic library, cloned upstream of an MS2 binding site. When ICP27-RNA binding occurs a complex is formed with their respective fusion proteins and the reporter genes on each plasmid are transactivated (Figure 3.1). Isolation of transformants containing this complex was achieved by using selective media. These isolates were subjected to further rounds of selection and eventually 68 single transformants showed activation of the *LacZ* gene with the ICP27 fusion protein and did not activate when the negative control protein (iron regulatory protein 1) was present. Sequence analysis of these selected HSV-1 library plasmids revealed that 31 of the 68 isolated transformants contained plasmid inserts encoding RNA corresponding to HSV-1 transcripts in the correct orientation (Table 3.1). The remaining 37 RNAs were found to have no biological significance as they were located in the reverse orientation as compared to the HSV-1 sequence or were found to contain more than one insert. β-galactosidase liquid assays were carried out on the 31 selected transfomants and the results confirmed that the ICP27-RNA interaction was specific (and was also a possible measure of binding affinity). Further analysis was undertaken using ICP27 protein with a deletion of the RGG-box (*d*4-5) or a mutation in the KH3 domain (M15). Both domains are thought to be involved in RNA binding (Mears and Rice, 1996b; Sandri-Goldin, 1998b; Soliman and Silverstein, 2000b). Separate assays carried out with these mutant ICP27 proteins either abolished or gave an extremely weak interaction with the 31 isolates. Thus, these results indicate not only a relationship between ICP27 functions and these binding domains but also that the function of ICP27 and its ability to bind RNA are connected.

Although these viral transcripts were recognised and their sequences analysed in the Y3-H investigation, little was known of their roles in gene expression or the significance of ICP27 binding during infection *in vivo*. Extension of the Y3-H results discussed above is essential to identify a possible functional relationship between ICP27 and the isolated ICP27-binding HSV-1 sequences. To enable the function of these

Gene ,kinetic class[a]		Isolate no.	Length (nt)	Genomic position[b]
ICP4	α	69	95	130251c
ICP4	α	16	50	128982c
ICP4	α	17	36	128003c
ICP4	α	247	71	128005c
ICP4	α	352	196	127879c
ICP4	α	75	148	127879c
UL5u	β	324/ 16.2	66	15333c
UL29	β	309	108	59581
UL30	β	82	61	64180
UL30	β	5	72	66092
UL30	β	231	100	66092
UL39	β	13.2	104	88443
UL42	β	3	62	93504
UL49	β	8	40	106100c
US3	β	4	72	135702
US3	β	242	87	135702
UL6u	γ	212	63	15022
UL13	γ	12	160	27101c
UL15i	γ	23	35	32178
UL15u	γ	21	209	28826
UL16	γ	274	41	30757c
UL19	γ	19/85/185	122	38856c
UL19	γ	251	159	38902c
UL32u	γ	55	225	69322c
UL36	γ	281.2	47	77686c
UL48	γ	334	36	104979c
UL49Au	γ	300	40	106100c
ICP34.5	γ	258	139	1306

Table 3.1: HSV-1 RNAs interacting specifically with the ICP27 protein

The following abbreviations were used: u, partially or completely in the untranslated region of the corresponding gene; i, located in intron;αimmediate-early; β, early; γ, late; c, complementary genomic strand; [a] HSV-1 transcribed genes in which the identified RNAs are encoded, with expression kinetics for the genes as described previously (55, 58). [b] Position of the 5′-end of each RNA isolate in the HSV-1 17^{+} genome. For ICP34.5 and ICP4, nucleotide numbers refer to one of the inverted repeats (adapted from Sokolowski et al, 2003).

sequences to be measured in a system that responds to endogenous regulatory proteins (i.e. host cell factors) or input regulatory proteins (e.g. ICP27), it was decided to study these HSV-1 RNA sequences in a mammalian cell system. A series of recombinant plasmids containing the reporter gene, CAT, was used to measure the impact of these sequences and ICP27 protein on CAT expression, under varying conditions.

3.2 Criteria for choosing Y3-H identified sequences for investigation

HSV-1 RNAs found to bind ICP27 were examined for sequence homology and secondary structures, however, the resulting identified sequences showed no obvious similarities. Due to the plethora of isolated RNAs, the choice of sequences for further analysis was based on various criteria to allow a full range of ICP27-binding sequences to be studied. Criteria were as follows:

1. Each sequence chosen should originally be from a different gene. Analysis should cover a range of genes to prevent results being confined to one gene. Experiments should be carried out on sequences from different genes to determine whether any change in gene expression when ICP27 is present is specific to a particular gene.
2. Sequences should be chosen that are found in genes belonging to different classes i.e. IE, E or L genes. This would provide a sequence from a gene from each of the temporal classes.
3. Isolated RNAs were found from different areas of the gene, for example, isolate #212 is located to the 5'UTR of the UL6 gene whereas isolated sequence #82 is located within the ORF of the UL30 gene. The ICP27 binding location within each gene may be an important factor.
4. Sequences chosen should direct a range of β-gal activities in the Y3-H assay. β-gal activity values may be indicators of the affinities of these RNAs for ICP27. RNA-protein binding affinities may significantly affect gene expression.

After applying the above criteria, 5 sequences were selected for further investigation (Table 3.2). A schematic diagram of the HSV-1 genome is shown in Figure 3.2, which indicates the position of all the transcripts identified by the Y3-H assay. The isolates

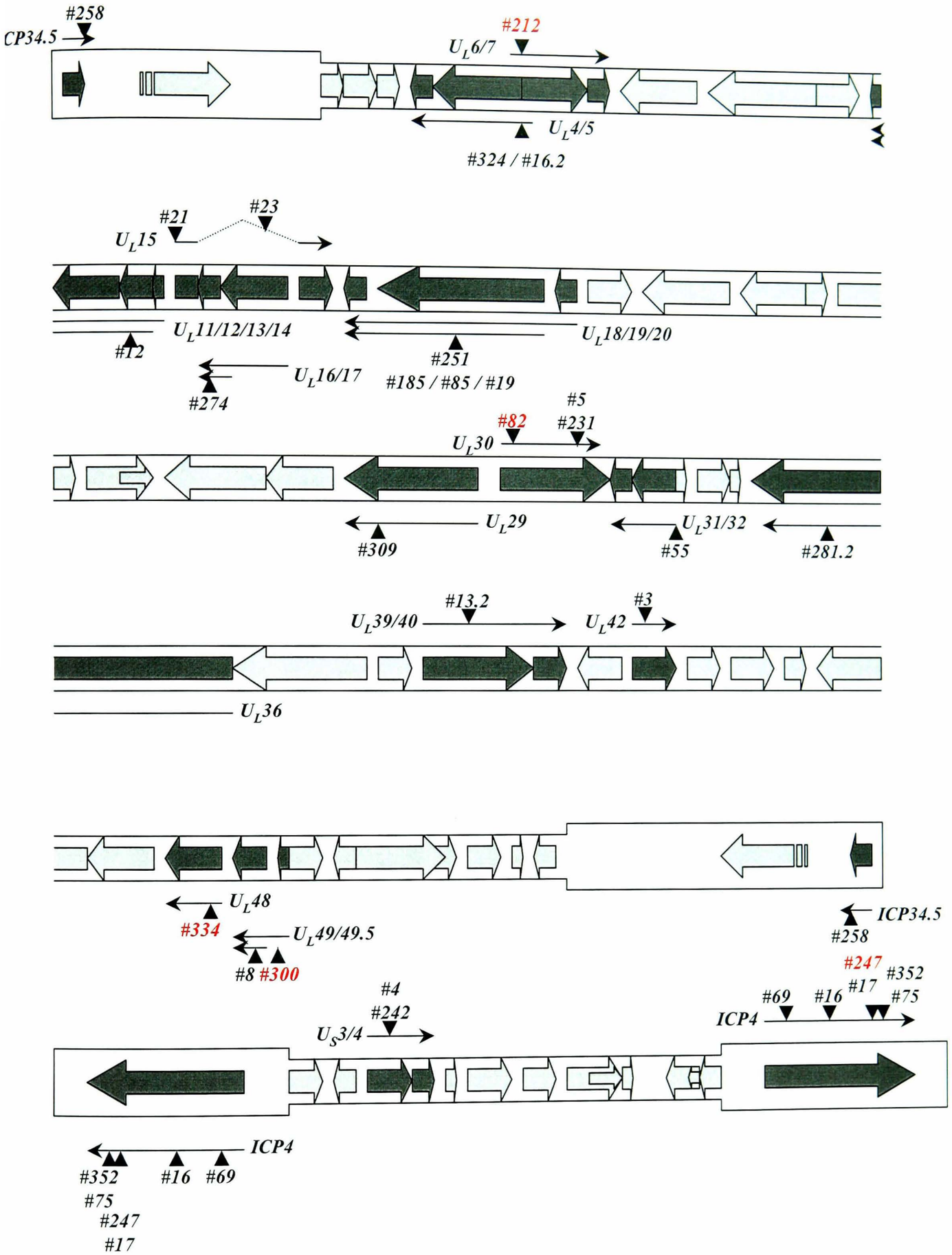


Figure 3.2: Schematic diagram of the HSV-1 genome highlights the transcripts identified using the Y3-H (indicated by #). Dark shaded thick arrow show ORFs that encode the 3' co-terminal transcripts. Indicated in red are the transcripts chosen for further investigation in this study, all of which do not overlap with any other gene.

ISOLATE NO.	MATCH TO:	NT POSITION	INSERT LENGTH	GENE POSITION	KINETIC GROUP: DESCRIPTION
212	46 nt upstream of UL6 start	15022-15084, sense	63	5'UTR	γ: a virion protein required for DNA cleavage/packaging
247	RS1 (Vmw175, ICP4)	127934-128005, antisense and 150228-150298	71	5'ORF	α: transcription transactivator.
82	UL30 (DNA polymerase)	66092-66163, sense	61	ORF	β: essential for DNA replication
300	75 nt upstream of UL49.5 ATG	107068-107113, antisense	46	5'UTR	γ_2: putative membrane associated protein
334	UL48 (VP16)	104944-104979, antisense	36	3'ORF	γ: induces alpha genes

Table 3.2 : ICP27-binding HSV-1 RNAs selected for investigation. ORF denotes open reading frame.

212F	AAAAAGGCCTCGGCCCTCCCTGGAACGGCTGGTCGGTCCCCGGGTTGCTG AAGGTGCGGCGGG
212R	CCCGCCGCACCTTCAGCAACCCGGGGACCGACCAGCCGTTCCAGGGAGGG CCGAGGCCTTTTT
247F	CCCGCGGTGTCGCGGCAGCACGCCTACCTGGCGTGCGAGCTGCTGCCCGC CGTGCAGTGCGCCGTGCGCTG
247R	CAGCGCACGGCGCACTGCACGGCGGGCAGCAGCTCGCACGCCAGGTAGGC GTGCTGCCGCGACACCGCGGG
82F	CCCCGAGTTCGCGACCGGGTACAACATCATCAACTTCGACTGGCCCTTCT TGCTGGCCAAG
82R	CTTGGCCAGCAAGAAGGGCCAGTCGAAGTTGATGATGTTGTACCCGGTCG CGAACTCGGGGA*TG*
300F	ACTCCGCCCCCGCGAGTAGCGACGGCCGTGTGCCAGTCGCCATCGT
300R	ACGATGGCGACTGGCACACGGCCGTCGCTACTCGCGGGGGCGGAGTA*TG*
334F	CCCCCCCGCTGTACGCAACGGGGCGCCTGAGCCAGG
334R	CCTGGCTCAGGCGCCCCGTTGCGTACAGCGGGGGGG

Table 3.3: A list of chosen sequences shown in both forward and reverse orientation. The red characters in sequence 300R and 82R show the start codon found only in this orientation. Refer to Table 3.2 for data on each sequence. The italicized ATG at the end of sequence 82R and 300R was not incorporated into the oligo, instead it represent the start codon of the CAT gene to identify whether the ATGs within 82R and 300R are in-frame.

coloured in red highlight the transcripts chosen for investigation in this study, and are found not to overlap with any other HSV-1 genes. Sandri-Goldin (1998a) previously identified an interaction with the HSV-1 RNA transcript ICP4, and ICP27 protein, otherwise, there has been no previously published data showing interactions of the selected HSV-1 transcripts with ICP27.

3.3 Introduction to CAT reporter assays

The CAT gene was originally used as a reporter to assay indirectly the transcriptional activity of mammalian promoters and enhancers in transiently transfected cells (Gorman *et al.*, 1982). The *Bam*HI site within the 5'UTR of the plasmid was used, as the majority of the isolated RNAs from the Y3-H investigation were located to the 5'UTR of the corresponding gene. The effect of sequences inserted into the *Bam*HI site at the 3' end of the 5'UTR was quantified by measuring the amount of CAT enzymatic activity. A CAT enzyme assay determines the amount of CAT protein being expressed from each construct as CAT converts [^{14}C]-labelled chloramphenicol ([^{14}C]-CAM) into mono- and diacetylated forms. In this assay, the labelled products were separated by thin layer chromatography, allowing the modified forms to be separated and measured as units of [^{14}C]-CAM converted per µl cell lysate. This assay was sensitive, reproducible and devoid of enzymatic activities of the host cell. In this section I report the effects the ICP27-binding sequences have on CAT activity in the presence or absence of ICP27 and also the effects on CAT activity when sequences are inserted in the reverse orientation.

3.4 Cloning isolated HSV-1 sequences into the CAT reporter vectors

In this study, a reporter plasmid (psvCAT) that contains a CAT gene under the control of an SV40 promoter, 95bp of 5' untranslated sequence and an HSV-2 IE4/5 polyA site (Gaffney *et al.*, 1985) was used (Figure 3.3). psvCAT is an intronless vector and was

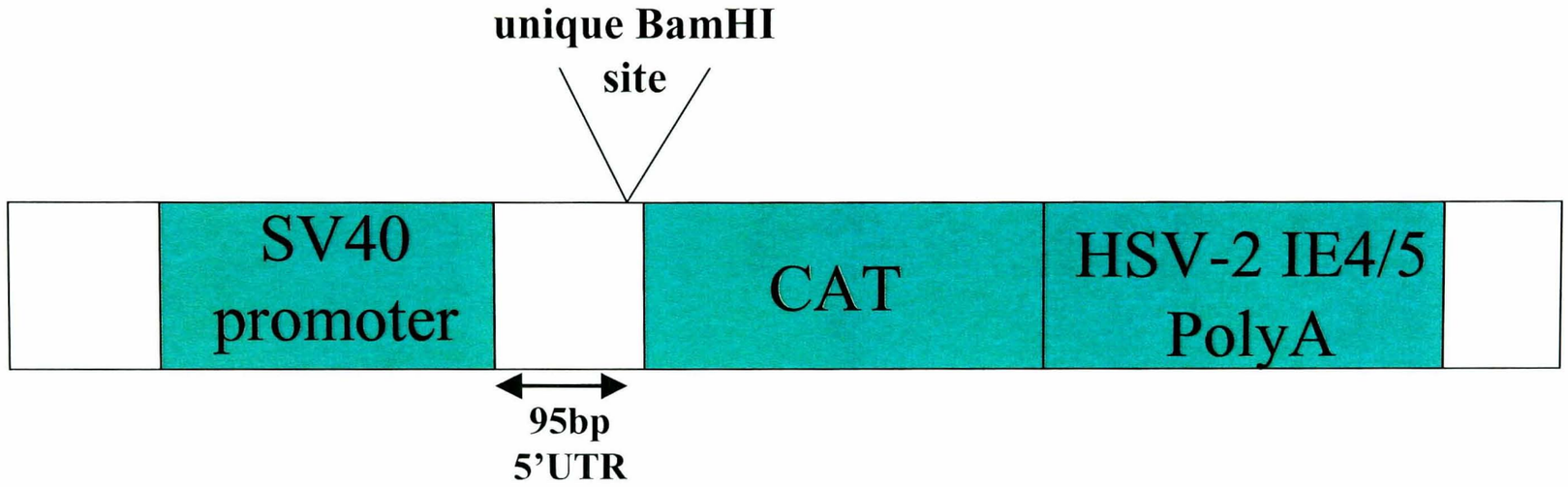


Figure 3.3 : psvCAT plasmid. This plasmid was originally derived from a pUC8 plasmid and reconstructed into the CAT reporter plasmid pLW1 by Gaffney et al 1985, which contained a HSV-2 IE4/5 promoter and polyA site. R.Everett altered this vector further and the IE4/5 promoter was replaced with an SV40 promoter. A single BamHI site was available for cloning into the 5'UTR of this vector.

chosen as ICP27 is known to affect the expression and binds preferentially to intronless viral transcripts (Phelan *et al.*, 1996; Sandri-Goldin, 1998a). The sequences to be inserted into the reporter plasmids were short (only 36 to 71 bp in length), therefore, oligonucleotides encompassing these sequences with introduced *Bam*HI sites at both 5 and 3' ends were synthesised. The oligonucleotides were annealed and ligated into the single *Bam*HI site within the 5'UTR of the plasmid as described. The *Bam*HI sequence on each annealed oligonucleotide contained a mutation from GATCC to GATCA on its 5' end and a T on the 3' end (see Table 2.3 for list of primers). This allowed the ligation mixture to be digested with the BamHI restriction enzyme, eliminating re-circularised plasmids and preserving plasmids containing inserts. Several clones from each ligation were sequenced to enable each HSV-1 sequence to be identified in both forward and reverse orientations (Table 3.3). Caesium chloride banded plasmid DNA prepared (using the large-scale preparation of DNA method) allowed the isolation of the closed circular DNA only.

3.5 Quantifying expression of CAT enzyme from a psvCAT construct

Each plasmid was transfected at 200, 300, 400 and 500ng per well, into BHK cells, using the liposome-mediated transfection method. Expression of the CAT enzyme was allowed to proceed for 16 h before cell lysates were harvested and the CAT assays performed. A transfection time of 16 h was chosen as future experiments aimed to include the co-transfection of a plasmid expressing ICP27, where expression of this plasmid for times longer than 16 h was known to be detrimental to the cells. Transfection of varying psvCAT plasmid DNA concentrations gave an increasing response of CAT activation and [^{14}C]-CAM conversion units were calculated using the volume analysis function in Bio-Rad's Quantity One software. The titration assay was carried out for each psv plasmid discussed in the following chapters. Despite the observation that CAM conversion units for each transfected CAT plasmid differed, the structure of the curve was similar for each plasmid, giving a steady increase in activity as the amount of DNA transfected was increased. I have therefore included one graph as an example of the profile obtained for the transfected plasmids (Figure 3.4). The CAT

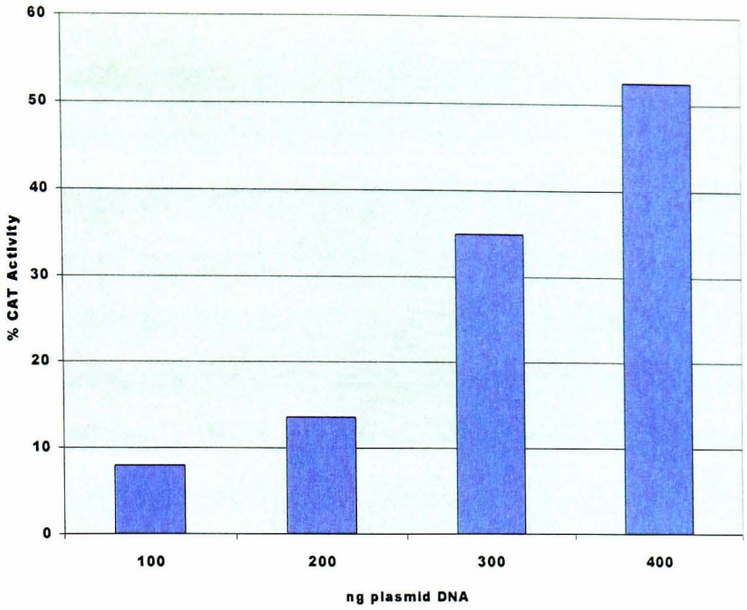


Figure 3.4 : Quantifying CATgene expression after transfection of a psvCAT construct. No error bars are present on this graph as this is a typical example of a CAT assay carried out after a plasmid transfection DNA titration.

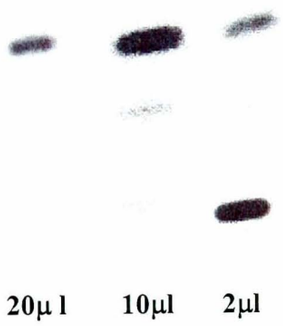


Figure3.5: TLC plate showing the products of CAT assays carried out with reducing concentrations of BHK cell lysates

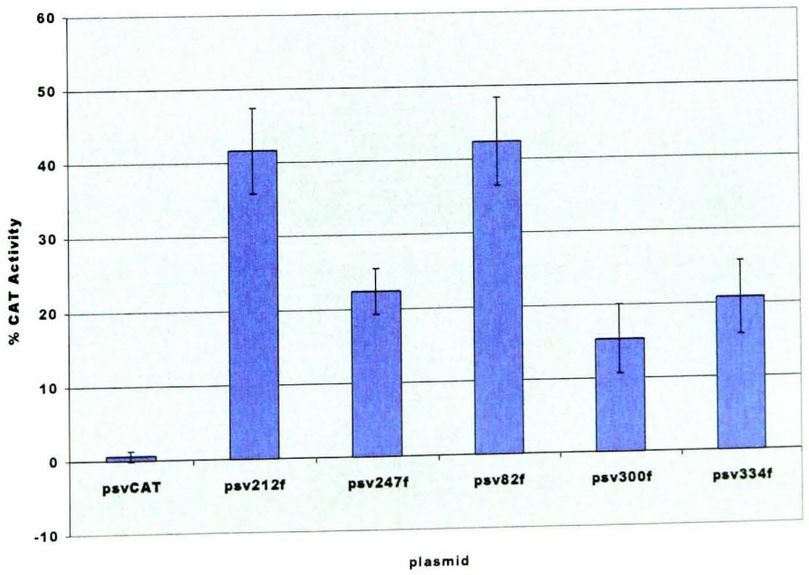


Figure 3.6: CAT activation when ICP27-binding sequences are present in the absence of any viral proteins (n=3).

production at 400ng DNA gave a high [^{14}C]-CAM conversion level therefore there was a risk of failing to detect a response to additional factors due to saturation of the assay. Conversion levels at 100 and 200ng DNA were low which may not have allowed the clear detection of a possible inhibitory affect when other factors were present during transfection. Transfections of all plasmids at 300ng DNA/well were chosen as a good response allowing the possible detection of an activation or inhibitory affect of CAT gene expression to be observed. The psvCAT plasmid without insert gave a consistently low level of CAT activity and was consequently used as a control for each transfection carried out. CAT assays were further carried out on 20, 10 and 2μl cell lysates after BHK cells were transfected with 300ng plasmid DNA. Figure 3.5 demonstrates a typical TLC plate showing the products of a CAT assay using these volumes. From these data 2μl cell lysates were used in each CAT assay unless otherwise stated. To ensure transfection efficiencies were similar between wells a β-gal plasmid was co-transfected with a psvCAT construct into BHK cells and CAT and β-gal assays were carried out. These results are presented in chapter 4 and demonstrate transfection efficiencies were similar between wells.

3.6 CAT gene expression increases when HSV-1 isolated sequences are present in the reporter plasmid. The effect does not require HSV-1 proteins and is not orientation dependent

After inserting the five chosen, isolated sequences into psvCAT all plasmids plus psvCAT control were transfected into BHK cells as described above. CAT assays were performed on cell lysates and volume analysis, using BioRad software, of each converted [^{14}C]-CAM band on each TLC plate was quantified and values plotted on an excel graph.

CAM conversion units were considerably higher for all plasmids containing inserts compared to the control vector with increases ranging from 40-80 fold, depending on the sequence inserted (Figure 3.6). This indicates that, after transfection of the plasmid

into the cell, the inserted sequences enhanced CAT gene production. Interestingly, this effect did not involve any HSV-1 proteins, suggesting that a cellular component(s) possibly in association with these small inserts, located 5' to the CAT gene, aids in synthesis of CAT.

Sequence analysis of the clones inserted in the forward orientation did not detect any obvious homologies, however, small RNA sequence or structure specificity may still be a factor in the 5'UTR's ability to increase CAT gene expression. To test for sequence orientation requirements, CAT assays were performed on extracts prepared from BHK cells transfected with psvCAT constructs containing the above sequences in reverse orientation. An increase in CAT synthesis was observed from all reverse constructs (Figure 3.7), except psv300R. The psv300R construct was found to have a start codon in its reverse inserted sequence and this gave no CAT activity after transfection. The results imply that properties of the viral sequences, other than orientation, were important, such as RNA structures or very small HSV-1 sequence motifs, for enhancement of CAT expression. Alternatively, the effect could be due to a non-specific RNA spacer with no sequence specificity at all. Furthermore, the presence of an intron within the 5'UTR of some CAT reporter plasmids can increase the expression of CAT. It was therefore postulated that by inserting these HSV-1 sequences into the 5'UTR of the psvCAT plasmid an intron might be created. Sequence analysis of isolates #212F, #212R, 247F, #247R, #82F and #82R including the 5'UTR sequence of the psvCAT plasmid (found 5' and 3' to the insert), was performed using software from two independent Internet sites that allowed splice site predictions i.e. NetGene2.com and Biogen.com. Results identified only a single 5' splice site in sequence #247, therefore by the insertion of sequences #212, #247 and #82 in both orientation, no conventional introns were likely to be formed.

Later, in Chapter 4, I discuss the length of the inserted sequence and the G or C base content of each insert. A control sequence that was inserted into the psvCAT plasmid to detect any specificity for HSV-1 sequences is also discussed later.

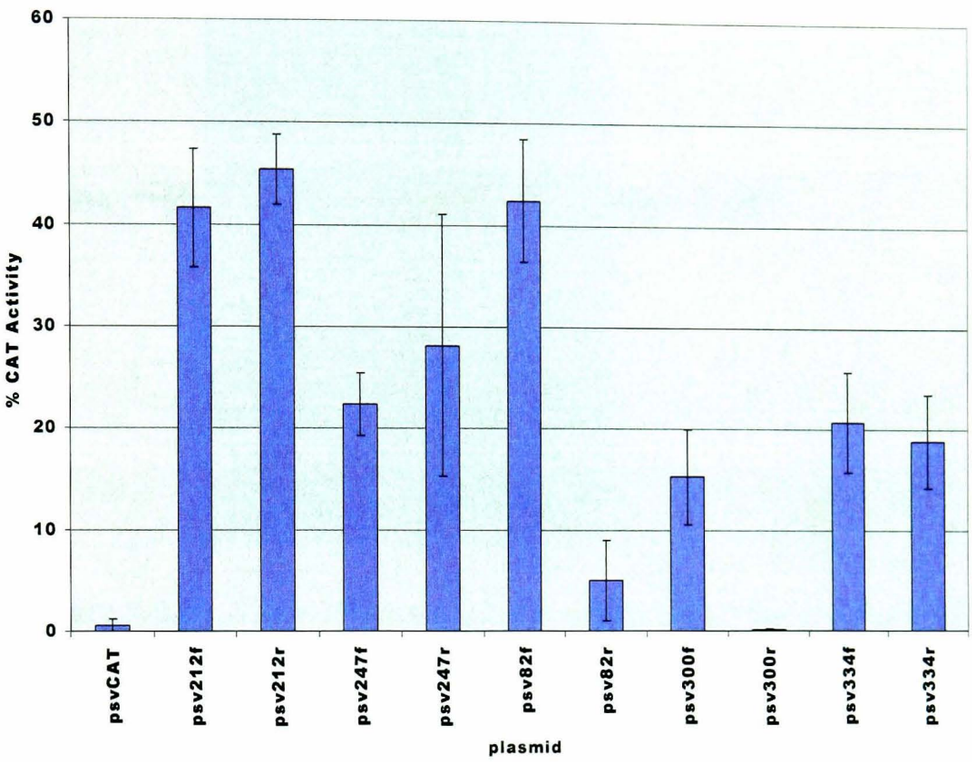


Figure 3.7: CAT activation is independent of orientation of sequence. Sequences inserted in both forward and reverse orientations, within the psvCAT plasmid, activate CAT gene expression in the absence of viral proteins (n=3).

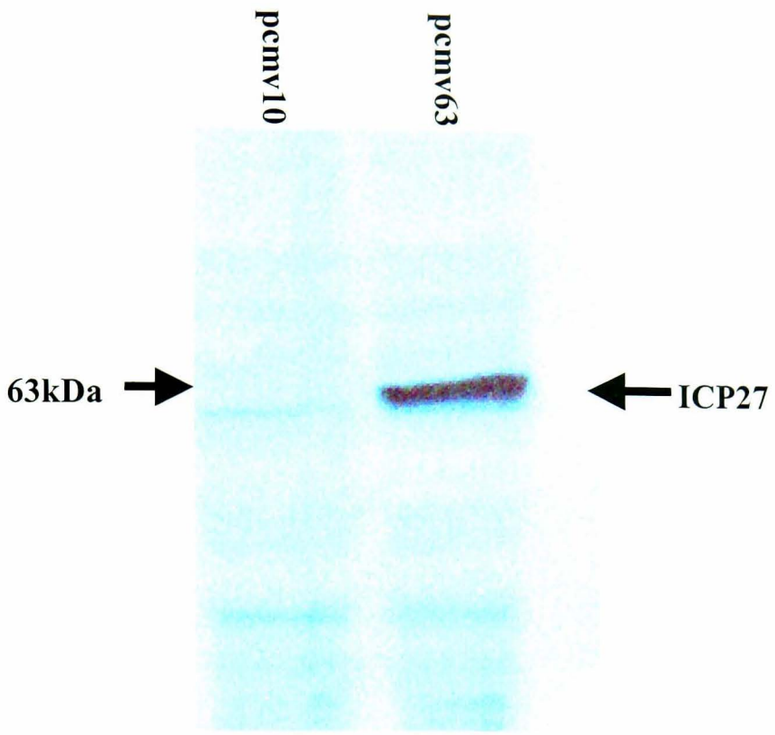


Figure 3.8A : Western blot analysis showing a prominent band at 63KDa identified as ICP27 was present when 50ng of plasmid pcmv63 was transfected into BHK cells for 16 h. The control plasmid, pcmv10, does not contain the ICP27 gene.

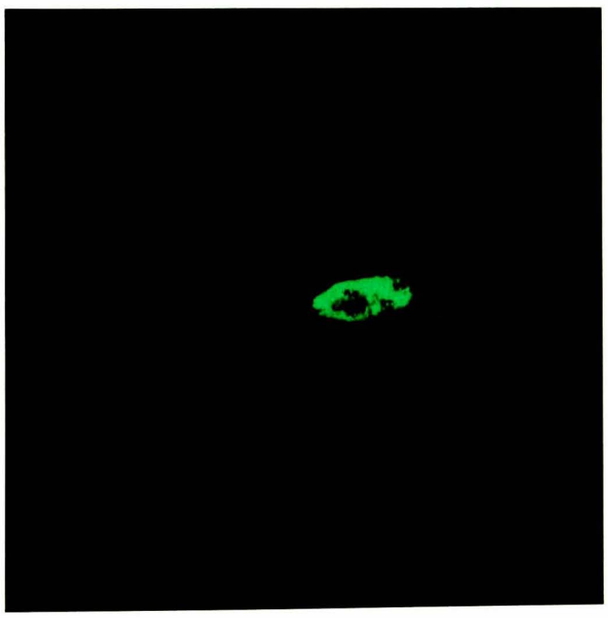


Figure 3.8B: Immunofluorescence data of a BHK cell transfected with pcmv63 and probed with an anti-ICP27 antibody (1113).

3.7 Transiently transfected ICP27 has no effect on HSV-1 sequence induced CAT activation

To investigate the effect of ICP27 on CAT production when inserts were present, a plasmid containing the ICP27 gene under the control of a HCMV promoter (pcmv63) was co-transfected with the psvCAT clones. The plasmid pcmv10, which did not contain the ICP27 gene insert, was used as a control to ensure any affect is not due to the amount of DNA transfected or due to the plasmid alone. To detect any effect of the pcmv plasmids on CAT activity, both pcmv10 and pcmv63 were co-transfected at DNA concentrations ranging from 50-500ng/well. This DNA titration revealed that with both pcmv10 and pcmv63, CAT activity levels were reduced at concentrations greater than 50ng/well due to a detrimental effect on the cells resulting in cell death. ICP27 was undetecTable by western blot using lysates from transfections of less than 50ng/well pcmv plasmid DNA in this assay. A value of 50ng/well pcmv plasmid DNA was therefore the chosen amount for transfection. This allowed the introduction of ICP27 into the cells without any obvious cytopathic effects. Western blots were carried out on lysates obtained from each transfection and probed with anti-ICP27 antibody (1113) to confirm that ICP27 was expressed (Figure 3.8A). The transfection of pcmv63 plasmid DNA into BHK cells followed by immunofluorescence using an anti-ICP27 antibody (1113) identified the location of ICP27 as predominantly nuclear with some staining in the cytoplasm, using confocal microscopy (Figure 3.8B). The transfection of pcmv63 plasmid DNA into BHK cells resulted in approximately 1:100 cells being transfected with that plasmid DNA. Transfection of the pcmv63 was previously shown not to affect the expression of another reporter gene by co-transfecting with pcmvβ-gal (Leiper, 2004). β-gal assays and western blotting assays (probing with anti-ICP27) were performed and results indicated that, during the expression of ICP27, β-gal expression levels remained similar to levels seen when no ICP27 was present (refer forward to chapter 4, Table 4.1).

Plasmids pcmv10 or pcmv63 (50ng/well) were co-transfected with psvCAT, psv212F, psv247F or psv82F for 16 h and CAT assays carried out on cell lysates. There was no significant difference in the amount of CAT produced when pcmv63 or pcmv10 was co-

transfected with the psvCAT constructs containing psv212, psv247 and psv82 (Figure 3.9). There was also little to no difference when pcmv63 or pcmv10 was co-transfected with the psvCAT control plasmid.

3.8 Analysis of RNA secondary structures using the Rfam database

It was of interest to examine the RNA structures of each HSV-1 sequence found to bind ICP27 during the Y3-H study as compared to a database of structures of known small RNAs.

Rfam is a database of structure-annotated multiple sequence alignments, covariance models and family annotations for a number of non-coding RNA families (http://www.sanger.ac.uk/Software/Rfam/index.sht ml). Rfam contains 503 such families. The alignments are hand curated and aligned using available data, and covariance models are built from these alignments using the INFERNAL software suite (http://infernal.wustl.edu/). The full alignments are created by searching the Rfamseq database using the covariance model, and then aligning the hits above a family specific threshold to the model. Important sequence similarities can be analysed on the bases of their biological importance as information regarding the known RNA within each database is cited. The aim here was to enter all HSV-1 sequences previously identified by the Y3-H assay to bind ICP27 protein, to identify structural similarities with any of the small RNA structures within the Rfam library and further to note any biological relevance or relation to the input sequence, if similarities were encountered.

All sequences of interest were 'trimmed' for any duplicate sequences per HSV-1 gene and entered into the INFERNAL program to align any possible homologues. After entering all 24 sequences, the INFERNAL program identified only 4 hits with 10 input sequences. These hits were identified as:

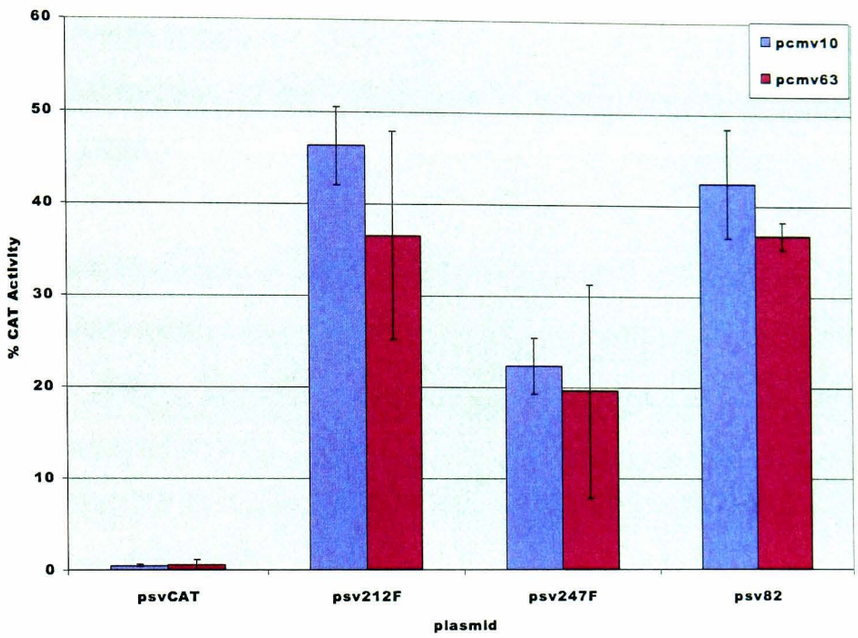


Figure 3.9: CAT activation levels following co-transfection of insert-containing CAT reporter plasmid DNA and plasmid containing either the ICP27 coding region (pcmv63) or no insert (pcmv10) (n=3).

1) **Histone 3'UTR stem loop**, known to be involved in nucleocytoplasmic transport of the mRNA, and in stability regulation and translation efficiency in the cytoplasm (Wang *et al.*, 1999). The **#212** sequence gave a hit to the histone 3' RNA in Rfam. **#212** is a 63nt sequence found within the 5'UTR of the HSV-1 UL6 gene; a protein known to be required for the formation of full virion capsids and for the processing and packaging of replicated viral DNA.

2) **Cardiovirus CIS-acting replication element (CRE),** a sequence coding for the VP2 protein of Theiler's virus containing this element required for replication of the genome (Lobert *et al.*, 1999). The **#82** sequence, contained within the HSV-1 UL30 gene encoding the catalytic subunit of the DNA polymerase; and the #13_2 sequence found within the HSV-1 UL39 gene, i.e. the large subunit of the ribonucleotide reductase, gave a hit with the CRE RNA in Rfam.

3) **Iron response element (IRE),** a sequence found in the 5'UTR of mRNAs involved in iron metabolism (Hentze and Kuhn, 1996). This IRE hit with six input sequences; **#247**, a sequence found within the HSV-1 RS1 gene encoding the immediate early transactivational protein ICP4; #16_2 sequence found within the 5'UTR of the UL4 gene, where the non-essential function of the encoded protein is not known; #352, a sequence found within the RS1 gene; #4, found within the UL34 gene; #55, found within the 5'UTR of the UL32 gene which encodes protein involved in viral DNA packaging; #12 from the UL13 gene encoding the VP57 protein involved in the post-translational processing.

4) **Hepatitis C virus stem loop IV**, found to be important in replication but not essential for colony formation by the HCV subgenomic replicon (exact function in the virus is not known) (Lee *et al.*, 2004). This aligned with one input sequences, #258, found within the 3'UTR of the HSV-1 RL1 gene, encoding a neurovirulence factor involved in promoting host-cell shut-off.

The secondary RNA structures and the score, in bits, of each sequence with a hit in Rfam was analysed and can be seen in figure 3.10 A, B and C. These structures were compared here with the secondary RNA structures obtained when analysing thermodynamic folding of each sequence entered into the Rfam program using the

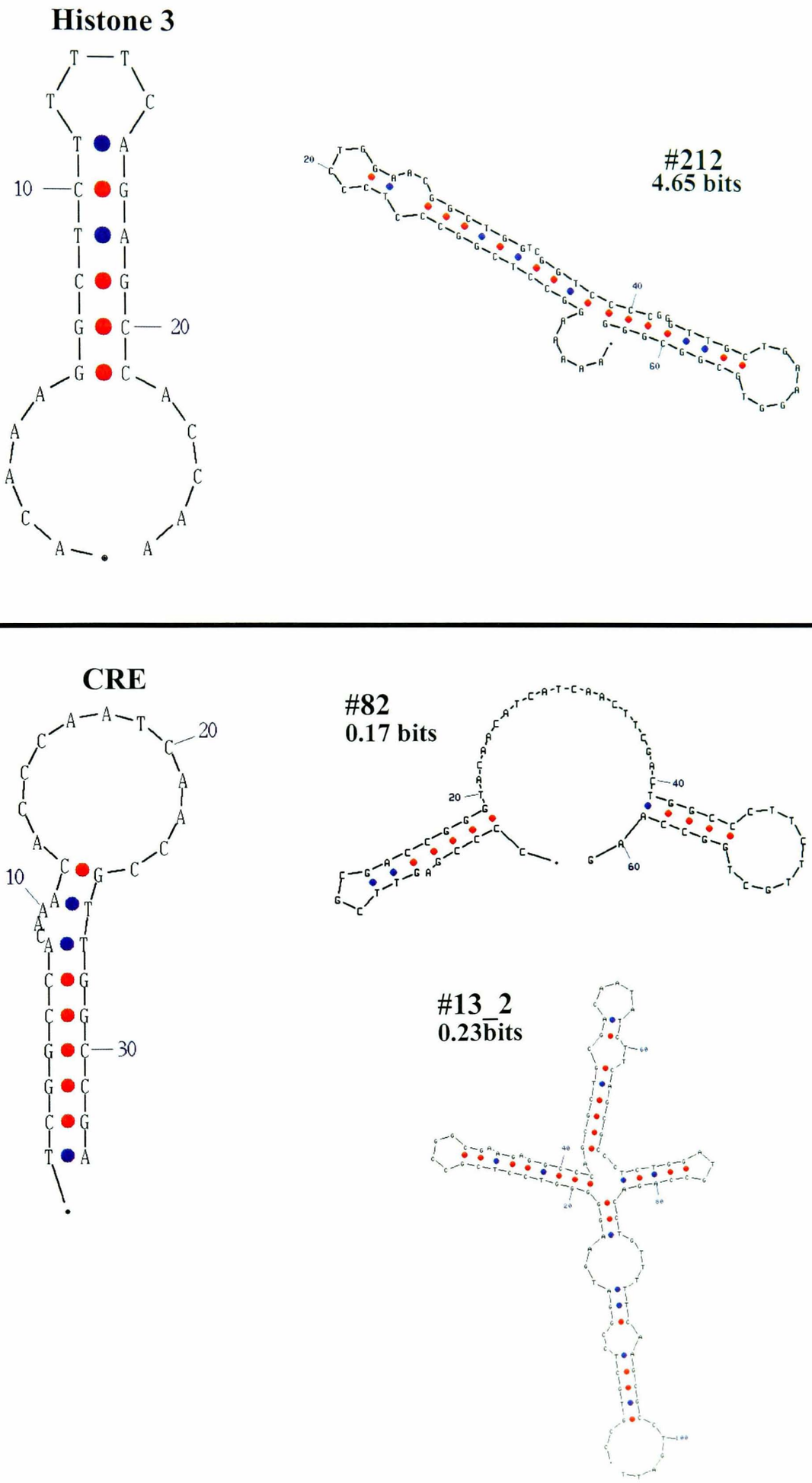


Figure 3.10 A : Comparison of structures between RNAs of known function with RNAs found to bind ICP27 in the Y3-H assay

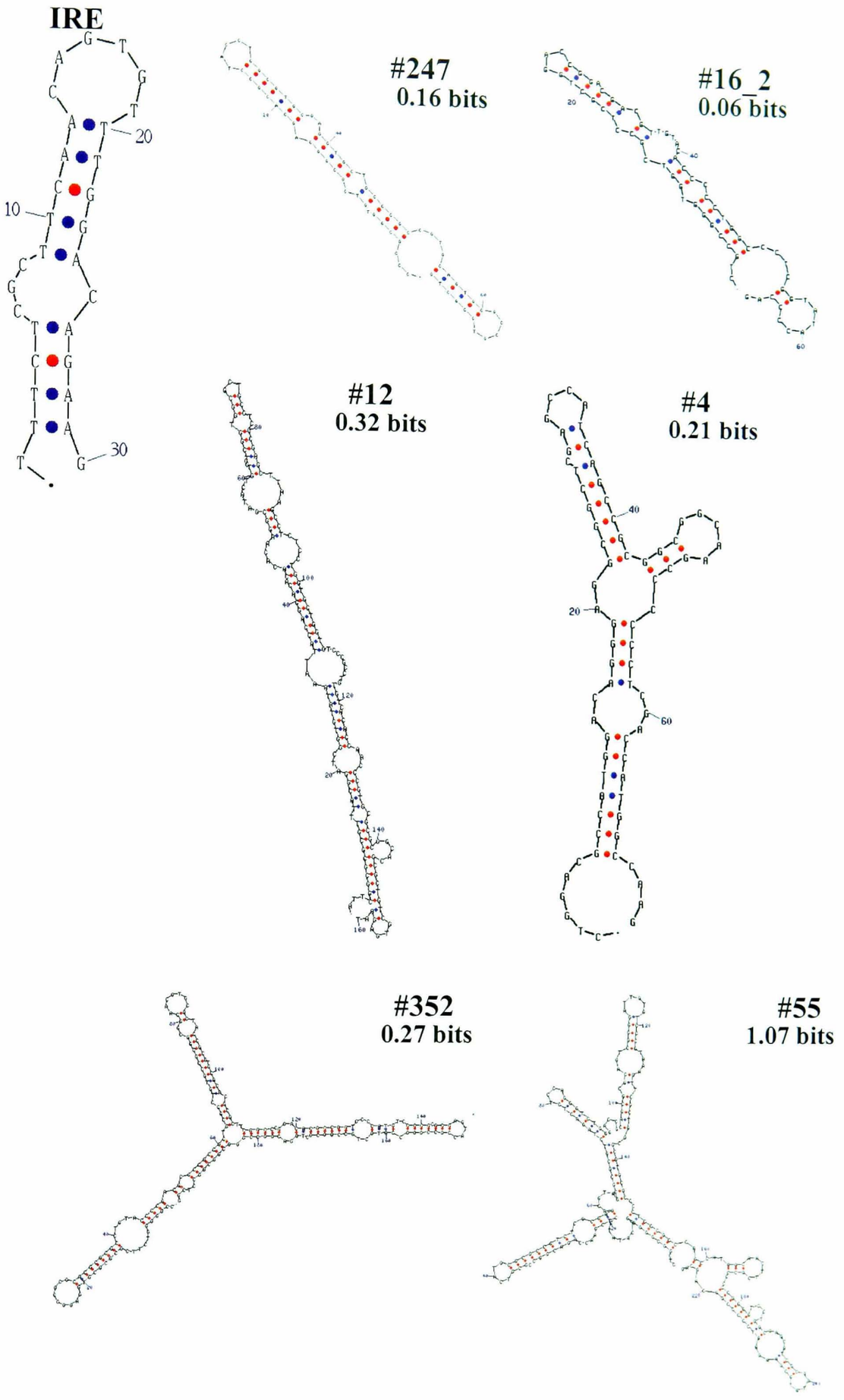


Figure 3.10B : Comparison of structures between RNAs of known function with RNAs found to bind ICP27 in the Y3-H assay

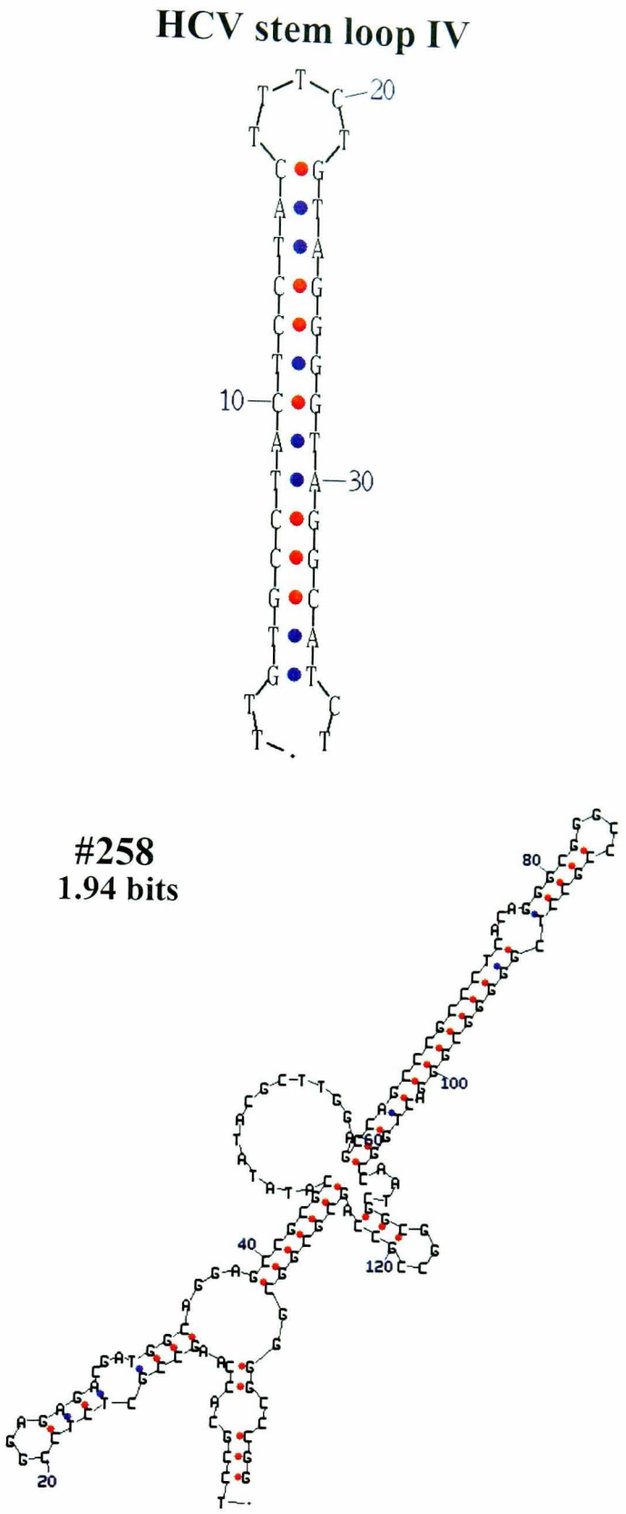


Figure 3.10C : Comparison of structures between RNAs of known function with RNAs found to bind ICP27 in the Y3-H assay

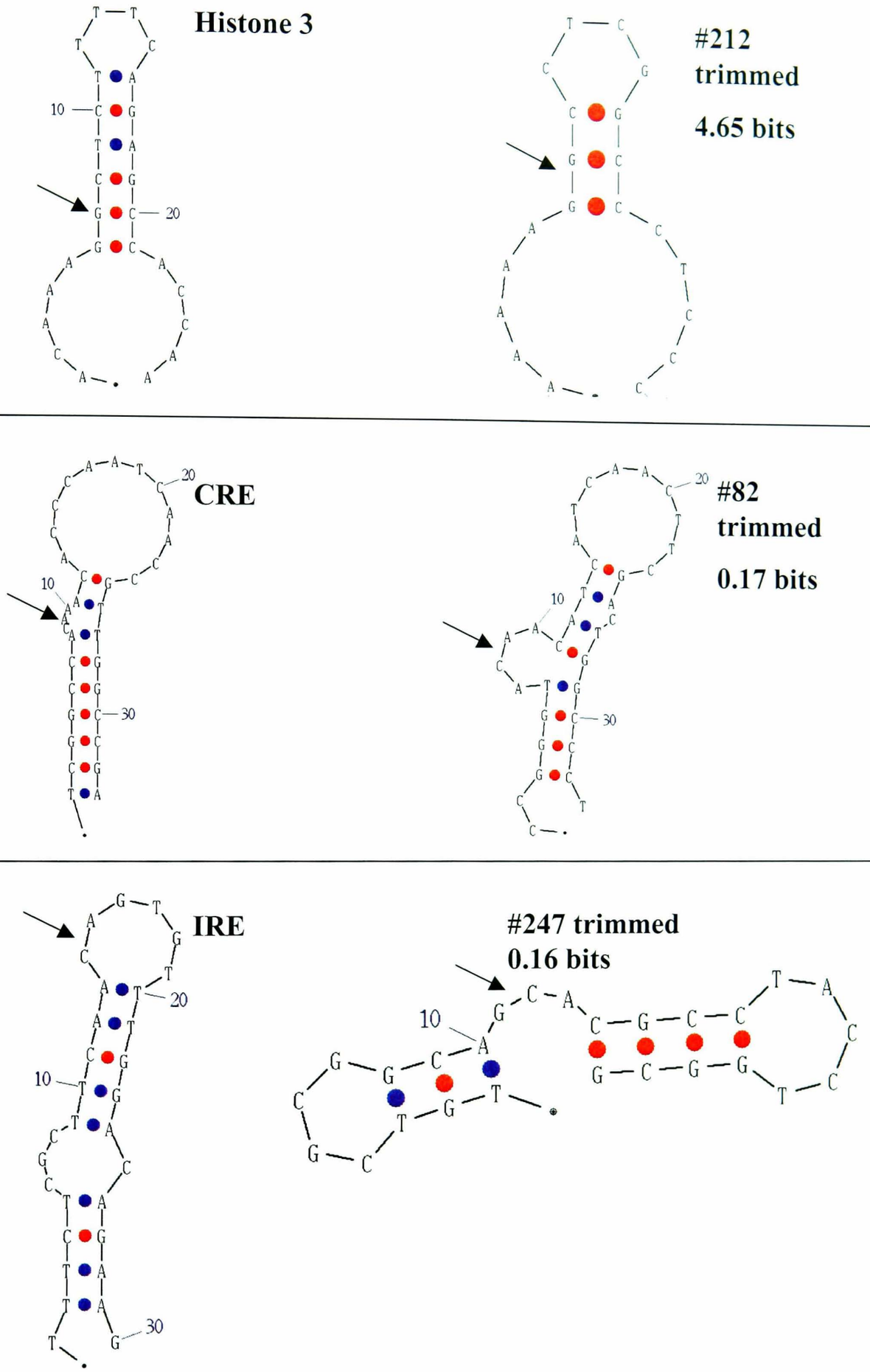


Figure 3.11 : Sequences were trimmed to encompass the region identified from the Rfam hit and RNA structures predicted as a comparison with these hits. Arrows point to areas of sequence homology to indicate small regions of structural similarity.

RNAfold program. The sequences in bold above that were analysed previously in this study (**#212, #247 and #82**) were further analysed for secondary RNA structures, i.e. these sequences were 'trimmed' to encompass only the region of sequence that was similar to the hit identified by Rfam. A comparison of the hit sequence structure with the full and trimmed input sequence is shown in fig 3.10 and reveals that there are very small similarities. For example, the CRE RNA structure contains a P-loop structure composed of bases ACAA that is also seen in the trimmed #82 RNA structure. Such small similarities were also observed for #212 and #82.

The scores for hits with each input sequence ranged from 0.06 to 4.65 bits (as shown in fig 3.10 A, B and C) was analysed to determine the significance of each hit. According to the INFERNAL manual the scores greater than the log (base two) of the input sequence length are significant i.e 4.65 bits. That is, after analysis of each input sequence length and their scores, it was concluded that these hits were not significant. These score values remained the same for the 'trimmed' sequences shown in figure 3.11.

In conclusion the RNA structures identified by Y3-H analysis were not similar to each other or significantly similar to the Rfam hits identified here, as based on the above criteria. That is, there were only ten sequences identified to be homologous to known small RNA structures from 24 input sequences and comparisons of all ten gave very low scores, rating them not significant. Also, the data from each Rfam hit was analysed and compared to the input sequence data revealing that there did not seem to be any biological similarities. And finally, the analysis of the RNA secondary structures of the Rfam hits, compared to the optimally thermodynamic RNA structures of the input sequence, had only very small similarities.

3.9 Discussion

HSV-1 sequences that bind ICP27 were found to activate gene expression when inserted into the 5'UTR of a CAT reporter plasmid. The increase in expression occurred for all plasmids (except psv300R) containing HSV-1 sequences inserted in both forward and reverse orientations.

An increase in gene expression when *cis*-acting sequences are present in either orientation has been observed at the levels of transcription, stability and translation. With regards to transcription, these sequences are called enhancers and are transcriptional controlling elements. They are composed of sequence motifs and can act independently of enhancer sequence orientation and can activate transcription over long distances either up- or down-stream of the transcription initiation site, i.e. a remote control effect (White, 2001) . The best example is that of the enhancer element of the immunoglobulin (Ig) heavy-chain gene that increases transcription from within the gene, showing the potential for enhancers to be located anywhere. Increased transcription rates occur when a specific interaction between enhancer-binding proteins and components of the transcription machinery are formed by DNA looping (Ptashne and Gann, 1997; Wang and Giaever, 1988). Such enhancer sequences have been found in viruses. The long terminal repeat (LTR) of the lymphoproliferative disease (LPD) virus of turkeys (LPDV) contains two direct repeats (DR). Enhancer elements were identified within each of the DR regions and were found to be involved in positively regulating transcription from the viral promoter (Sarid *et al.*, 1995). Similar observations have been reported with DRs found within LTRs of RSV (Laimins *et al.*, 1984a; Laimins *et al.*, 1984b) and sequence analysis revealed an homologous binding site for a transcription factor, enhancer factor III (EFIII). Regulation of HIV-1 transcription is also, in part, by enhancer elements located in the LTR. The enhancer elements identified in the LTR were cloned into a CAT vector and were shown to stimulate transcription independent of position and orientation to the promoter (Verdin *et al.*, 1990). In the study presented here, an increase in CAT activity by HSV-1 sequences in either orientation could in principle occur at the level of transcription. To confirm whether these sequences act as enhancers, further experiments would include inserting the sequences of interest upstream of the promoter or in the 3'UTR of the psvCAT vector. If these HSV-1 sequences were acting as enhancers, CAT activation would be observed in cells transfected with plasmids with inserts at all locations. A search to identify any possible transcription factor binding sites was carried out using all sequences investigated here at the TFSEARCH website (http://www.cbrc.jp/research/db/TFSEARCH.ht ml) holding a database of 1483 sequences of known transcription factors binding sites. No relevant transcription factor

binding sites were identified using this database however, this search is by no means exhaustive and further searches should be undertaken.

Another level of activity of the HSV-1 sequences could be in regulating RNA stability ultimately resulting in an increase in expression. Sequences found in the 3'UTR of a luciferase gene were found to stabilise RNA, in a sequence orientation-independent manner. The length of the 3'UTR was the regulating factor resulting in an increase in translation as the sequence length increased (Tanguay and Gallie, 1996). However, the HSV-1 sequences under investigation in this study are all located in the 5'UTR.

Sequences within the 5'UTR of ornithine decarboxylase have been reported to have a position-dependent but orientation-independent effect on translation. However, this effect is to suppress translation and is thought to be due to the sequence forming a very sTable hairpin structure thus inhibiting translation initiation (Grens and Scheffler, 1990). The presence of HSV-1 sequences, investigated in the study presented here, result in an increase of gene expression making it improbable that these sTable structures are formed.

Activation of gene expression by ICP27-binding sequences occurred when no other viral proteins were present, indicating the possible recruitment of a cellular factor(s). Cellular multifunctional proteins that have comparable functions to ICP27 should be investigated to further this proposal. Candidates for investigation might be shuttling proteins with a regulatory role that are involved in RNA binding. The Y-box proteins are one such group of multifunctional regulatory proteins. Y-box proteins are RNA-binding proteins involved in translational silencing of several maternal RNAs by exporting the transcripts to the cytoplasm and destabilising RNA secondary structures (Jiang et al., 1997). They also bind DNA via a consensus sequence (ATTGG) (the Y-box) and regulate transcription of many cellular genes (Sommerville, 1999). The coupling of the regulation of transcription and translation is a conserved feature of these proteins (Sommerville, 1999). The Y-box proteins function to silence gene expression whereas the possible involvement of a cellular protein, in this study, increases CAT gene expression suggesting that these Y-box proteins may not play a role in CAT gene

expression when HSV-1 sequences are present. The multifunctional protein hnRNP K has roles in the regulation of gene expression that includes transcription, splicing, translation and mRNA stability, comparable to HSV-1 ICP27. Generally, hnRNP K is involved in both transcriptional and translational repression, however a few reports have revealed that this multifunctional protein can also activate transcription and translation. Evans et al, (2003) described hnRNP K as a transcription factor and demonstrated its ability to increase specific RNA synthesis by interaction with an element found upstream of the *c-myc* gene. Furthermore, hnRNP K was shown to interact with the TATA-binding protein indicating hnRNP K binds a specific *cis*-acting element and interacts with the RNA polymerase II machinery to stimulate transcription (Evans et al., 2003; Michelotti et al., 1996). HnRNP K also increases translation of the c-myc protein in an IRES-independent manner (Evans et al., 2003). A further similarity of hnRNP K to ICP27 is their protein structure, both containing an RNA binding domain, three KH domains, an acidic domain and an NLS (Bomsztyk et al., 1997). This protein is a prime candidate for further research here as, not only does hnRNP K have similar functions and protein structure, it is known to bind ICP27 (Wadd et al., 1999). As wt HSV-1 infection proceeds it is not known if ICP27 recruits the functions of hnRNP K thus facilitating it through the cellular pathways. Alternatively ICP27 may be hijacking hnRNP K's role and subsequently inactivating it.

The activation of gene expression was not altered when ICP27 was introduced by expression from a transfected plasmid, indicating that other viral proteins may have been required to work in conjunction with ICP27 to give a possible effect, or perhaps ICP27 did not function in this assay. However, complementation assays identified that ICP27 expressed from the pcmv63 plasmid, used in this study, was functional resulting in wt HSV-1 titres when expressed during an ICP27-null HSV-1 infection (Leiper, 2004).

ICP27 has been known to require other viral proteins to function efficiently. ICP27 can function independently or in combination with other viral proteins. ICP27 can *trans*-activate the HSV-1 gB promoter to induce expression of CAT, however this level of expression is significantly increased in the presence of ICP0 and ICP4 (two HSV-1 IE proteins) (Rice and Knipe, 1988). However, ICP27 absolutely requires both ICP0 and ICP4 to *trans*-activate the HSV-1 TK promoter possibly forming a complex to give this

synergistic effect (Everett, 1986). The *trans*-repressor function of ICP27 was also observed when activation of expression by ICP0 was reduced in the presence of ICP27, depending on the HSV-1 promoter used (Hardwicke *et al.*, 1989; Sekulovich *et al.*, 1988; Su and Knipe, 1989). Therefore, in the investigation presented here, the inability of ICP27 alone to alter gene expression was not altogether unexpected given the requirements for other viral proteins in the regulation of other genes. Furthermore, the simultaneous reduction of host cell factors that would normally occur during HSV-1 infection may also be necessary for an effect of ICP27 to be seen. In this assay, the host cell factors involved in gene expression were almost certainly still being expressed, as the cells continued to grow, when ICP27 was introduced thus possibly masking the effect of ICP27. This may have a diminishing affect on the ability of ICP27 to bind these sequences possibly through competition with cellular RNA binding proteins, and/or the ability to activate gene expression due to the presence of cellular proteins that would normally be reduced during a wt HSV-1 infection. Moreover, perhaps ICP27 requires to be modified (e.g. phosphorylated) by another HSV-1-specified protein, thus transiently transfected ICP27 would be rendered inactive for this activating function

The sequences identified to bind ICP27 using the Y3-H system show little similarity to each other leading to the idea that perhaps there was a similarity in RNA structures. Comparisons of HSV-1 RNA structures with small RNA structures with known biology did not significantly detect any similarity with any small RNAs in the Rfam library. However, it should be noted that this search is not exhaustive as the Rfam database is relatively new and the input data are constantly being updated. Continual screens should be carried out to compare the HSV-1 sequences isolated and identified from the Y3-H study with new input sequences of the Rfam database.

Bioinformatic studies on these HSV-1 RNA secondary structure formations can be furthered by the application of several other computational methods. Programs for thermodynamic predictions and free energy calculations are continuously being developed. A further prediction method that includes sequence scanning for covariant sites alongside associated secondary structures has recently been developed. This method can produce extensive information on the formation of RNA secondary structures thus linking the identified RNA structure (e.g. a small stem loop) to an essential function of the virus (e.g. replication) (Tuplin et al., 2002; Turner et al., 2004).

Chapter 4: During HSV-1 infection, ICP27 regulates transfected CAT gene expression *via* short HSV-1 RNA sequences

4.1 Introduction

ICP27 acts to enhance viral gene expression and in some cases works in conjunction with ICP0 and ICP4. ICP27 can transactivate the expression of gC and gB without the presence of other IE proteins, however, some viral promoters require the presence of ICP4, ICP0 or both for the regulation of certain (early and late) genes, e.g. TK and VP5, HSV-1 early and late gene, respectively (Everett, 1986; Knipe, 1989; Rice and Knipe, 1988; Sekulovich *et al.*, 1988).

The inability of transfected ICP27 to activate CAT gene expression *via* the Y3-H identified binding sequences *in vivo* (Chapter 3) was not altogether unexpected. ICP27 has such a range of functions and partner proteins during an HSV-1 infection that transiently transfected ICP27 alone may not be sufficient to elicit an effect on the expression of CAT. The observation that transiently transfected ICP27 does not significantly affect CAT gene expression, along with previous work revealing that ICP27 associates with ICP0 and ICP4, suggests that perhaps other viral proteins are required to affect CAT gene expression in conjunction with ICP27. For this reason it was decided to study the effect of ICP27 on CAT activation during productive infection. This approach would introduce all viral proteins required for ICP27 interactions and furthermore lead to a productive infection, with consequent host-cell shut-off. This would allow ICP27 to carry out its functions in the presence of its partner proteins and in reduced levels of cellular factors.

The identified ICP27-RNA binding sequences did not bind to ICP27 (or binding was severely reduced) when the protein was present in mutant form (M15 and *d*4-5) in the Y3-H assay, confirming the ICP27 interaction with this range of HSV-1 RNAs

(Sokolowski *et al.*, 2003). Three ICP27 mutant viruses, 27LacZ, *d*4-5 and M15, were used in the studies presented here. Mutant 27LacZ lacks the ICP27 coding sequences, and *d*4-5 ICP27 lacks an RGG box RNA binding domain. The M15 ICP27 is unable to shuttle and the protein's transactivation function is inactivated. The effects of infection with these viruses were investigated in the CAT expression system described in chapter 3.

4.2 CAT gene expression during HSV-1 infection

BHK cells were transfected with each of the psvCAT constructs independently and incubated for 6 h to allow expression of CAT protein to commence. Subsequently, the cells were infected with wt HSV-1 to introduce all viral factors including ICP27. To optimise plasmid CAT directed gene expression during infection, transfected BHK cells were infected with wt HSV-1 for 16 h at a range of MOI from 0.1-10. The maximum level of CAT gene expression was observed at an MOI of 1, which was used throughout these experiments unless otherwise stated (fFigure 4.1A). Infections with wt HSV-1 at MOIs of 5 and 10 also elicited an increase in CAT expression levels, however, this proved to be detrimental to the BHK monolayer and led to cell death leaving very few cells for protein extraction.

To ensure transfection efficiencies were similar, constructs psv212F and 212R were co-transfected with 200ng of a β-gal plasmid (pcmvβ-gal) into BHK cells for 6h, in duplicate. Each well of transfected cells was subsequently mock or wt HSV-1 infected, harvested after a further 16h and cell lysates obtained. β-gal and CAT assays were performed using the cell lysates obtained and results identified a significant increase in CAT expression when comparing cells that were mock and wt HSV-1 infected, while β-gal values remained very similar (Figure 4.1B and Table 4.1). This preliminary data indicates transfection efficiencies between cells in each well were similar.

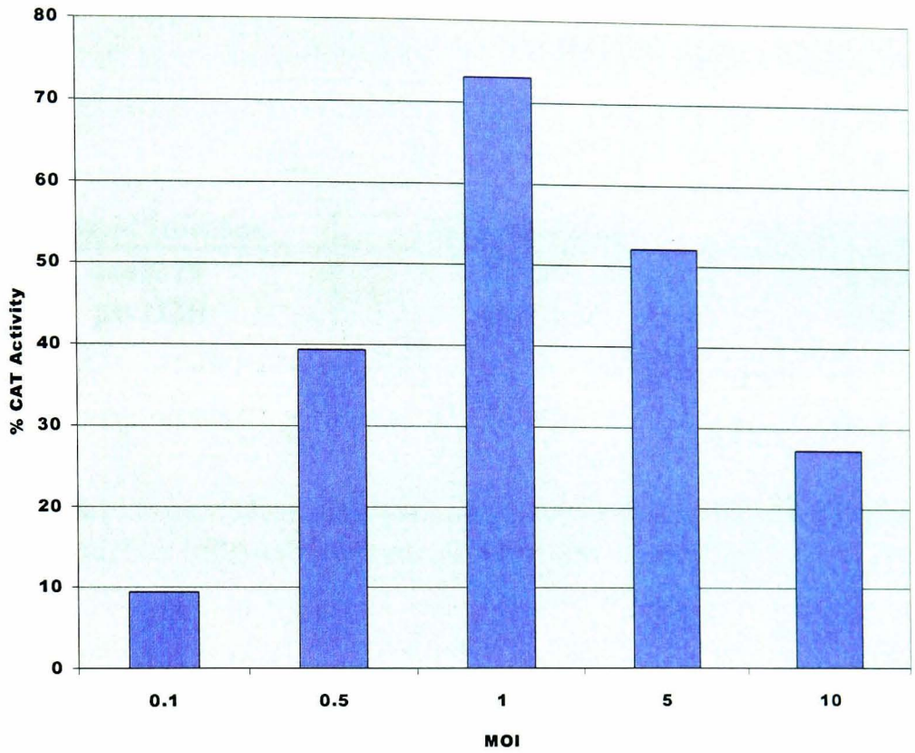


Figure 4.1A: CAT activity levels obtained following transfection of BHK cells with 300ng psvCAT DNA followed by infection with wt HSV-1 at different MOIs for 16 h.

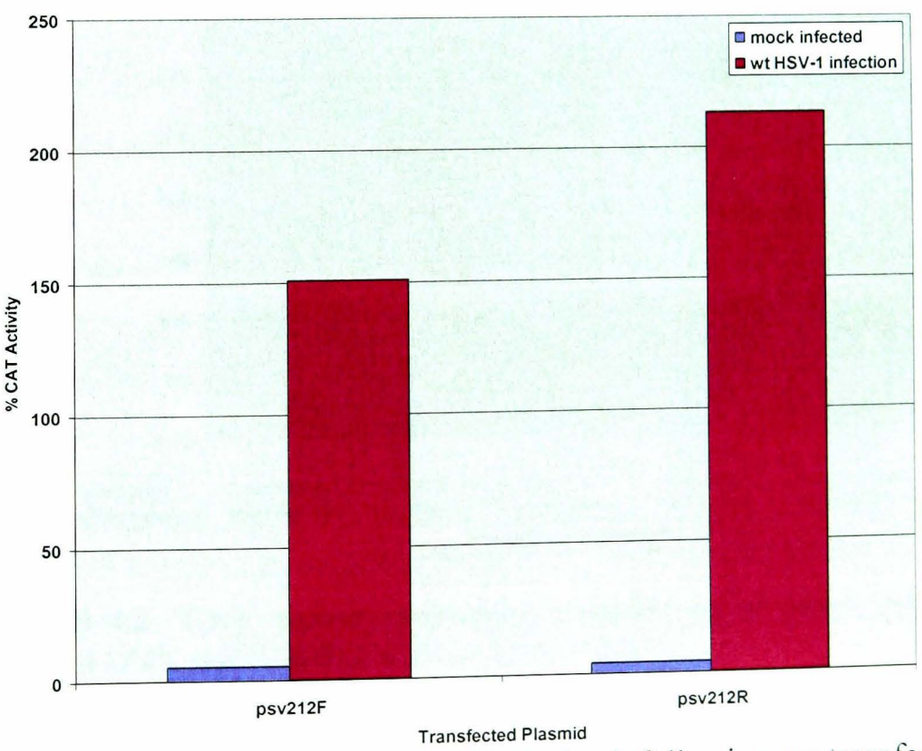


Figure 4.1B: CAT activity levels obtained following co-transfection of BHK cells with 300ng psv212 and pβ-gal DNA followed by mock or wt HSV-1 infection

Plasmid/Infection	Mock infection	Wt HSV-1 infection
psv212F	0.361	0.316
psv212R	0.337	0.341

Table 4.1: β-gal values obtained after co-transfection of psv212 and pβ-gal plasmid DNA followed by mock and wt HSV-1 infection.

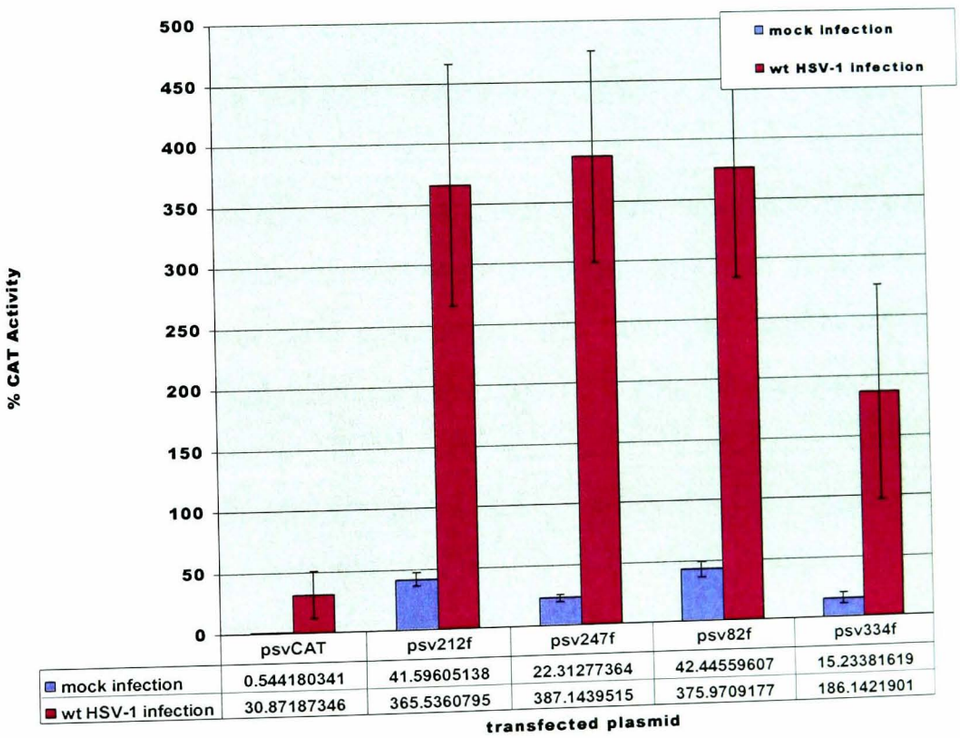


Figure 4.2: CAT activity following transfection of insert-containing plasmid DNA and infection with wt HSV-1 (n=3).

4.3 During wt HSV-1 infection, CAT gene expression is enhanced.

Plasmids psv212, psv247, psv82, psv334 with HSV-1 DNA inserts and control plasmid, psvCAT, were transfected into BHK cells and after 6 h cells were then infected with wt HSV-1 and incubated for a further 16 h. Assays carried out on the resulting cell lysates revealed an enhancement of CAT activity when HSV-1 proteins were present as compared to levels observed during mock infection (Figure 4.2). As HSV-1 infection proceeds different viral factors could possibly influence the increase in CAT expression. However, as the inserts at the 5'UTR of these psvCAT plasmids were identified as ICP27 RNA binding sequences, it would appear likely that the presence of ICP27 during HSV-1 infection might play a role in the altered CAT activation levels. For example, after mock infection of psv247 transfected cells a value of 22 ± 3 CAM units was obtained. In contrast, infection with wt HSV-1 resulted in a value of 387 ± 87 CAM units (Figure 4.2). This is a dramatic response to infection with the reporter plasmid carrying ICP27 binding sequences, for when the sequences are absent, i.e. psvCAT, and virus infection proceeds, the CAT activity only reached 30 ± 19 CAM units.. However, even though this level of activity for psvCAT is considerably lower than the levels seen with the insert-containing plasmids, the fold increase calculated from CAT activity levels reached during mock infection to those reached during wt HSV-1 infected levels is much higher when there is no insert present. That is, a 56 fold increase in CAT activity was calculated for cells transfected with psvCAT as compared to only a 8-17 fold increase for cells transfected with insert-containing plasmids, when comparing mock to wt HSV-1 infections (Table 4.2). However, it should be noted that the background levels of activity reached when no viral proteins and no insert sequences are present (i.e.mock infected cells with a psvCAT DNA transfection) are extremely low thus making it difficult to calculate an accurate fold increase in CAT activity upon HSV-1 infection. These results suggest ICP27 is having a negative effect on CAT gene expression due to the presences of the insert sequences.

Transfected Plasmid	psvCAT	psv212	psv247	psv82	psv334
Fold increase in CAT activity	56.74	8.7	17.35	8.8	12.22

Table 4.2: Fold increase in CAT activity from levels reached during mock infection to those reached during wt HSV-1 infection.

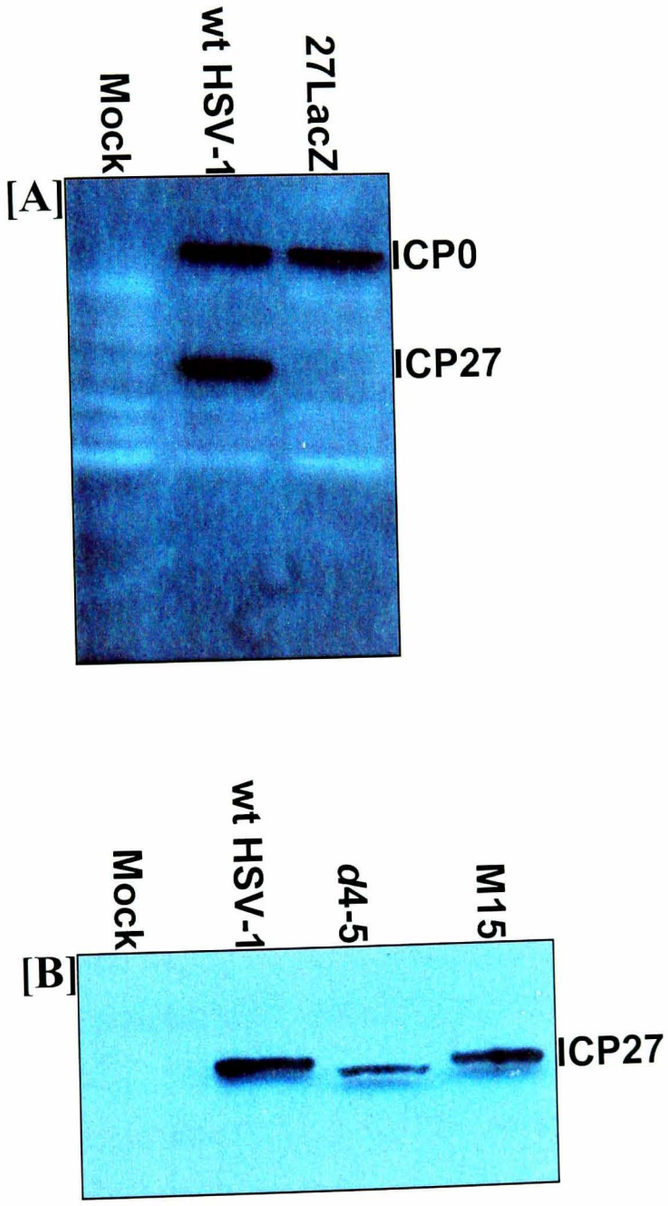


Figure 4.3: Western blot analysis of BHK cell lysates infected with wt and ICP27 null HSV-1 viruses [A] and ICP27 mutant viruses [B] to indicate the absence or presence of the immediate early protein ICP27 during these infections. The presence of immediate early protein ICP0 is also shown [A]. The ICP27 band seen during the *d*4-5 infection is slightly smaller than wt ICP27 due to the RGG box deletion.

4.4 Enhanced CAT gene expression during wt HSV-1 infection is not dependent on the orientation of the inserted sequences.

In the absence of virus, there was no detectable difference in CAT activation when forward or reverse orientations of the HSV-1 sequences were compared (Figure 3.4). However, when all viral proteins are present during infection, it was anticipated that the effect of ICP27 in regulating CAT gene expression, via these ICP27-binding sequences, would be an orientation specific effect because sequences in reverse orientation might not be recognised and would not be expected to affect CAT gene expression. Sequences #212, #247, #82 and #334 were inserted into the 5'UTR of the psvCAT plasmid in the reverse orientation and plasmids containing these sequences in both forward and reverse orientations were transfected into BHK cells separately for 6 h. Cells were subsequently infected with wt HSV-1 for 16 h and lysates were obtained for analysis. Western blot analysis confirmed that ICP27 was expressed during infection (Figure 4.3A). Unexpectedly, little difference in CAT activity was observed between cells transfected with plasmid containing the sequences in the forward or the reverse orientation (Figure 4.4). Only psv82 sequence in the reverse orientation gave a drop in value from 375 ± 91 to 79 ± 18 CAM units which was probably due to the presence of two consecutive ATGs not in-frame with the CAT ORF. One ATG codon before the initiation codon for CAT; this upstream ATG was not in frame with the CAT initiation codon. Therefore, translation initiation at the upstream ATG would not generate a protein encoding CAT. Hence, CAT enzyme activity would not be detected for plasmid psv300R. All sequences presented here contain the Kozak consensus sequence of a G at position +4 (Kozak, 1997). Again, these results suggest that the sequences in reverse orientation were also recognised by ICP27 during wt HSV-1 infection giving a reduced fold increase in CAT activity when compared to activity when no ICP27-binding sequences were present. There may be a small motif present in the sequences, in both orientations, that may be responsible for the decrease in CAT activity. To detect possible motifs present in both orientations of each insert, each sequence insert was analysed for palindromes of 7-10 nucleotides in length. Only one insert (#247) contained a palandromic sequence, leading to the conclusion that a

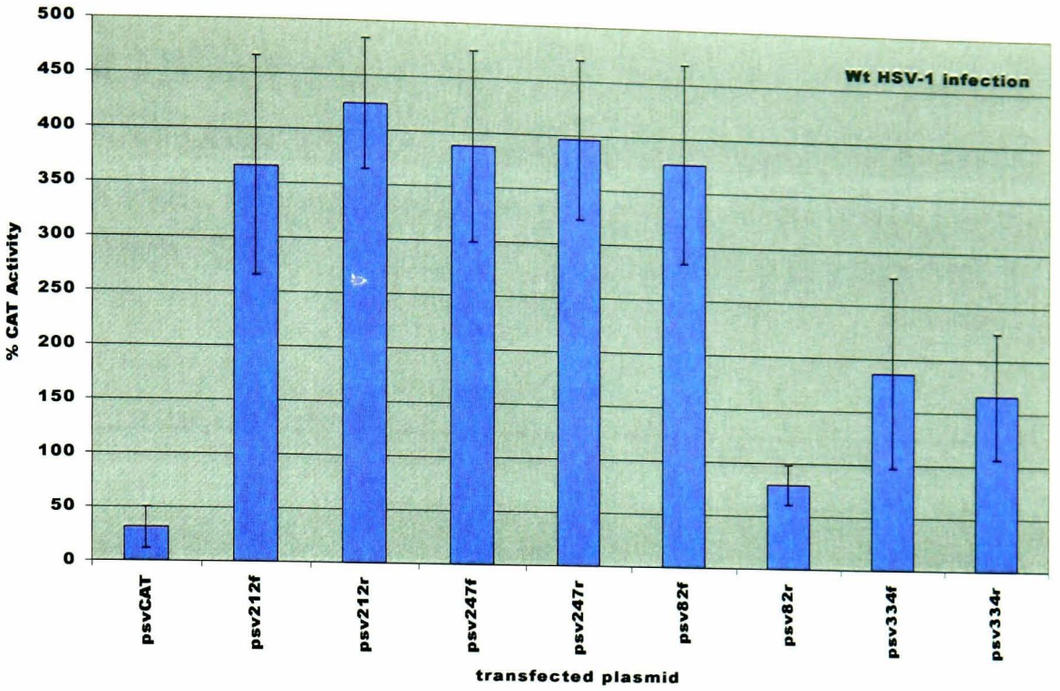


Figure 4.4: CAT gene activation following wt HSV-1 infection is not sequence orientation dependent. ICP27 binding sequences were inserted into the psvCAT plasmid in the forward (f) and reverse (r) orientation and transfected into BHK cells, and cells were infected with wt HSV-1 (n=3).

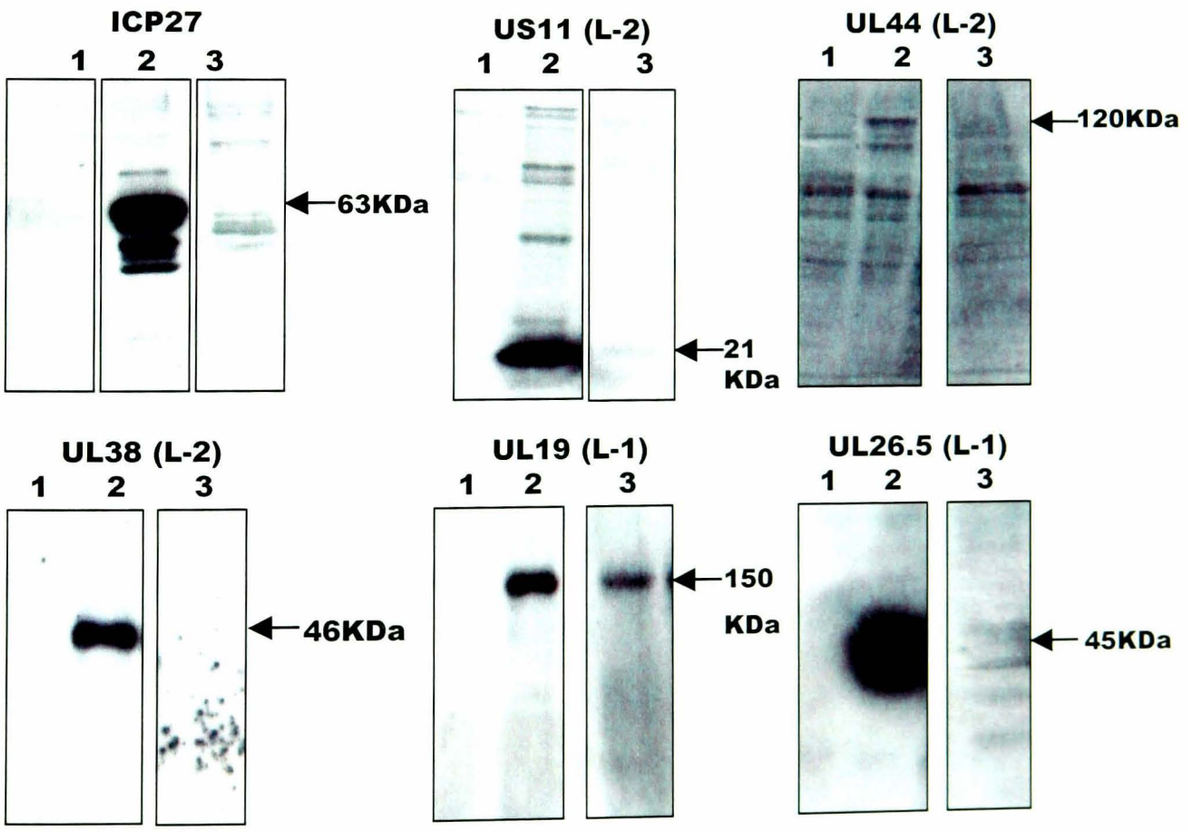


Figure 4.5: Analysis of the HSV-1 ICP27 mutant virus, 27*LacZ*.

Western blots for a range of HSV-1 proteins expressed during mock (lane 1), wt HSV-1 (lane 2) and 27*LacZ* (lane 3) infections.

sequence motif of 7-10 nucleotides in length is not responsible for the decreased enhancement in of CAT gene expression when these sequences were present in both orientations. This result could also imply the recognition of very small HSV-1 sequences or structures. These issues are investigated further and discussed later in this chapter.

4.5 Characteristics of HSV-1 ICP27 mutant viruses

To identify the role ICP27 plays in the expression of CAT when ICP27 binding sequences were present, a range of HSV-1 ICP27 mutants were used. Experiments were carried out to gain a profile of each mutant virus as compared to wt HSV-1 infections. Here, three ICP27 mutant/deletion viruses are described and a protein expression profile of each virus was gained by immunoblotting and immunofluorescence analysis.

BHK cells were infected with a particular virus at an MOI of 1 for 7 h. Extraction of total cell protein was carried out (Chapter 2.2.6.1) and protein concentration was determined using a Bradford assay. SDS-PAGE was performed using 10μg of each extracted protein sample per lane and proteins were later subjected to western blotting. Each blot was probed with antibodies raised against six HSV-1 proteins (see Materials and Methods Table 2.1). Basic properties of these six HSV-1 proteins are as follows:

1. ICP27: previously described
2. US11: a L-2 gene that encodes a 21kDa protein with DNA binding properties and is located within the nucleolus. The US11 gene product is not essential for viral growth.
3. UL44: is an unspliced L-2 gene that encodes glycoprotein C (gC). gC is a 130kDa non-essential membrane protein possibly involved in membrane attachment that is processed before incorporation into the virion and plasma membrane.

4. UL38: a L-2 gene that encodes VP19C, a minor component of the outer capsid shell that is involved in scaffold formation. It has a molecular weight of 53kDa.
5. UL19: encodes the essential viral protein VP5 (L-1), a major component of the viral capsid shell with a molecular weight of 150kDa. VP5 is not only required for capsid formation but also for the cleavage of viral DNA into unit-length genomes.
6. UL26.5: encodes a 45kDa L-1 capsid protein found in a complex with UL38 and UL19. Though not essential for viral growth, the virus progeny from a UL26.5 null mutant is severely reduced.

4.5.1 Characteristics of the HSV-1 ICP27 deletion mutant 27LacZ

The replication-defective mutant virus, 27LacZ, is a virus in which the ICP27 gene has been interrupted by the insertion of the *LacZ* gene thus no ICP27 protein is translated. The 27LacZ virus was originally used to investigate the post-transcriptional regulatory functions of ICP27 and it was found that although some late genes were transcribed, their translation was severely reduced, as also observed during other ICP27 mutant virus infections (Sandri-Goldin and Mendoza, 1992). Viral DNA synthesis does not occur during 27LacZ virus infection, which is reflected in the severely reduced expression levels of certain L-2 genes. Furthermore, after 27LacZ infection, host shutoff does not progress (Ellison et al., 2005; Hardy and Sandri-Goldin, 1994).

BHK cells were infected with 27LacZ virus, cell lysates obtained and western blotting analysis was performed. Figure 4.5 confirms that ICP27 is not present at a detectable level during infection. Analysis of RNA levels during 27LacZ virus infection using an ICP27 probe revealed no detectable levels of ICP27 RNA (data not shown) confirming that no ICP27 was present during these infections. With respect to L-2 gene expression, levels of US11 were severely reduced and expression of gC and UL38 were undetectable. Analysis of L-1 gene products revealed that the level of UL19 expression was reduced and the level of UL26.5 protein was severely reduced

(Figure 4.5). The above results reveal an overall detrimental effect on all late genes analysed here.

4.5.2 Characteristics of HSV-1 virus infection when ICP27 shuttling is inhibited (M15)

The HSV-1 ICP27 mutant virus, M15, has a two amino acid substitution at amino acids 465 and 466, in a region of the protein that is very closely conserved throughout the herpesviruses, changing amino acids 465 and 466 from PG to LE (Figure 4.6A). Infection with this virus was found to abrogate the transactivational properties of ICP27 yet leaves the transrepressor activity intact (Rice and Lam, 1994). Analysis of virus kinetics revealed that the M15 virus infection exhibited an inability to form plaques, indicating the M15 mutation is lethal to the virus and reveals that this mutation inactivated an essential function of ICP27. Furthermore, viral yield assays revealed M15 virus infection to be severely restricted for growth. Viral gene expression analysis showed enhanced immediate early gene expression (ICP4 and ICP27) and a slight enhancement of early gene expression (ICP8), as seen with the ICP27 null virus d27-1. M15 virus infection exhibits a slight reduction in the expression of L-1 genes (ICP5 and ICP25), whereas L-2 gene expression was not detected (ICP1-2 and gC). Northern blot analysis demonstrated that the M15 virus was unable to induce the expression of gC mRNA giving an almost 30-fold reduction as compared to wt HSV-1 virus(Rice and Knipe, 1990). Overall, M15 failed to repress IE and E gene expression while also failing to induce L-2 gene expression, a loss of both transactivation and transrepression functions.

Although this M15 mutation alters these important functions of ICP27 another striking effect of this mutation is that it renders the protein completely unable to shuttle, as determined by heterokaryon assays.

While the M15 defects are lethal to the virus and to the function of ICP27 it was interesting to note that this mutation does not affect viral DNA replication, which is comparable to wt levels (Rice and Lam, 1994). Again, infection with the M15 mutant virus resulted in cells that did not progress into host shutoff.

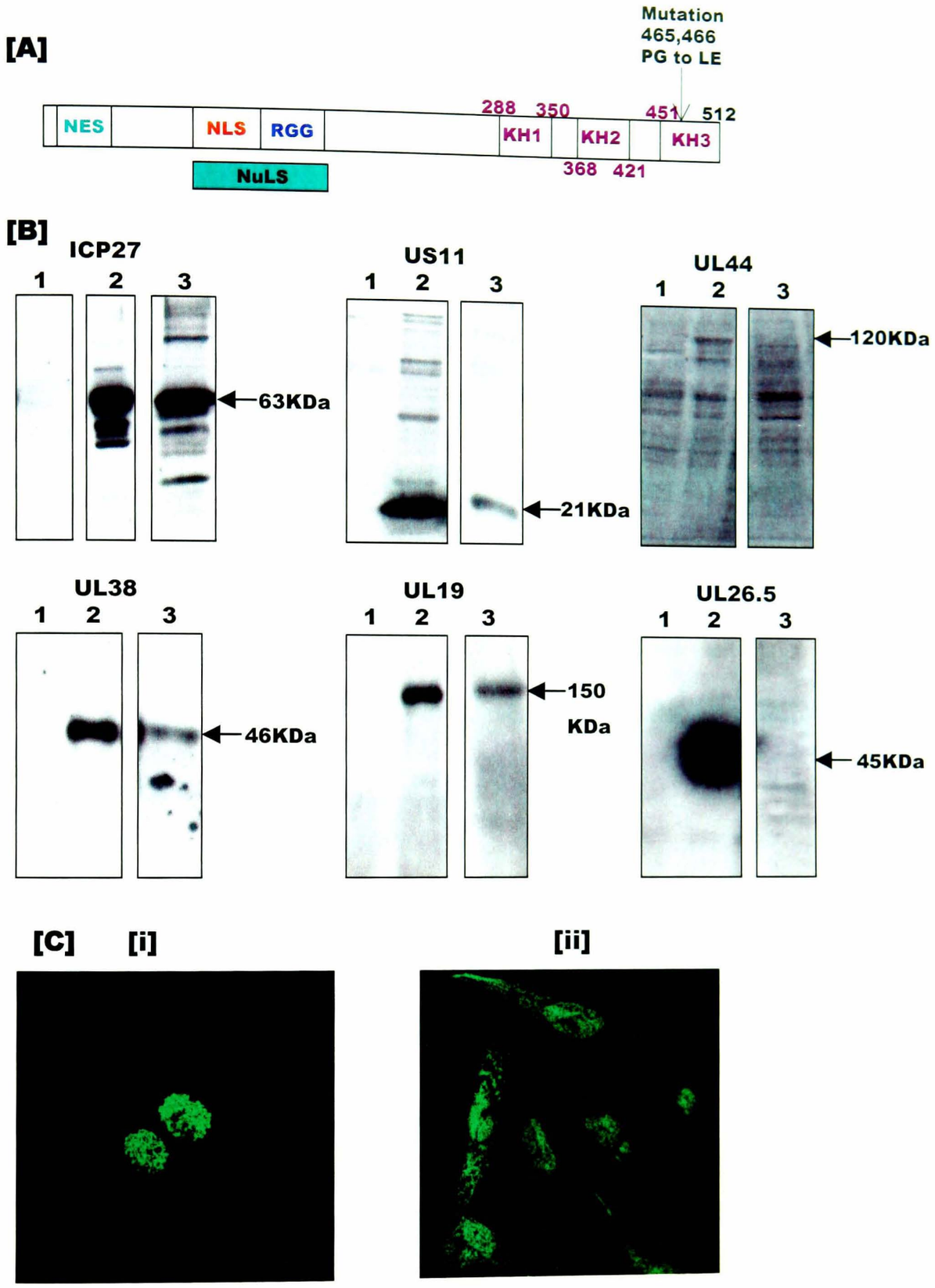


Figure 4.6: Analysis of the HSV-1 ICP27 mutant virus, M15.

A cartoon of ICP27 protein **[A]** indicating the point mutations PG-LE at a.a.465 and 466 to affect the carboxy domain of the protein. Western blots **[B]** for a range of HSV-1 proteins expressed during mock (lane 1), wt HSV-1 (lane 2) and M15 (lane 3) infections. Immunofluorescence **[C]** carried out on BHK cells infected with M15 virus **(i)** or wt HSV-1 **(ii)** revealing the localisation of ICP27.

During the investigation presented here, viral late gene expression during M15 virus infection was analysed for L-2 gene expression and revealed that UL44 was not detectably expressed and levels of US11 protein were severely reduced (Figure 4.6B). The UL38 and UL19 protein expression were also reduced and UL26.5 protein was not detectably expressed.

IF data revealed that the M15 ICP27 protein localised to the nucleus in a punctate pattern and furthermore, no ICP27 protein was detected in the cytoplasm (Figure 4.6 [C][i]). This data confirms previous reports on the location of virally expressed M15 ICP27 and indicates a defect in ICP27's ability to shuttle (Mears and Rice, 1998).

4.5.3 Characteristics of HSV-1 when the RGG-box of ICP27 is deleted (*d*4-5)

ICP27 has a nuclear import and export signal allowing nuclear shuttling of the ICP27 protein (Soliman et al., 1997). As ICP27 also affects the export of IE110 and UL15 viral transcripts (Phelan et al., 1996) it was speculated that ICP27 was involved in RNA binding. It contains an arginine/glycine-rich (R/G) domain, and such domains have been previously reported to play a role in RNA binding. This R/G- rich domain, termed the RGG box, is composed of 15 R/G residues spanning amino acids 138-152 and a direct interaction of this domain alone with RNA transcripts has been demonstrated (Mears and Rice, 1996a) (Figure 4.7A).

A recombinant HSV-1 virus containing ICP27 with the RGG box deleted was constructed (*d*4-5) which was shown to have a 58-84 fold defect in growth. This reflected the severely reduced viral DNA levels seen during this mutant virus infection. Interestingly, *d*4-5 virus infection in cells results in host shutoff (Mears et al., 1995).

BHK cells infected with *d*4-5 virus were analysed for viral late gene expression. Levels of ICP27 protein are higher in the *d*4-5 infection as compared to wt levels. Interestingly, levels of two true late proteins (gC and US11) were significantly reduced however another true late protein, UL38, gave levels slightly higher than

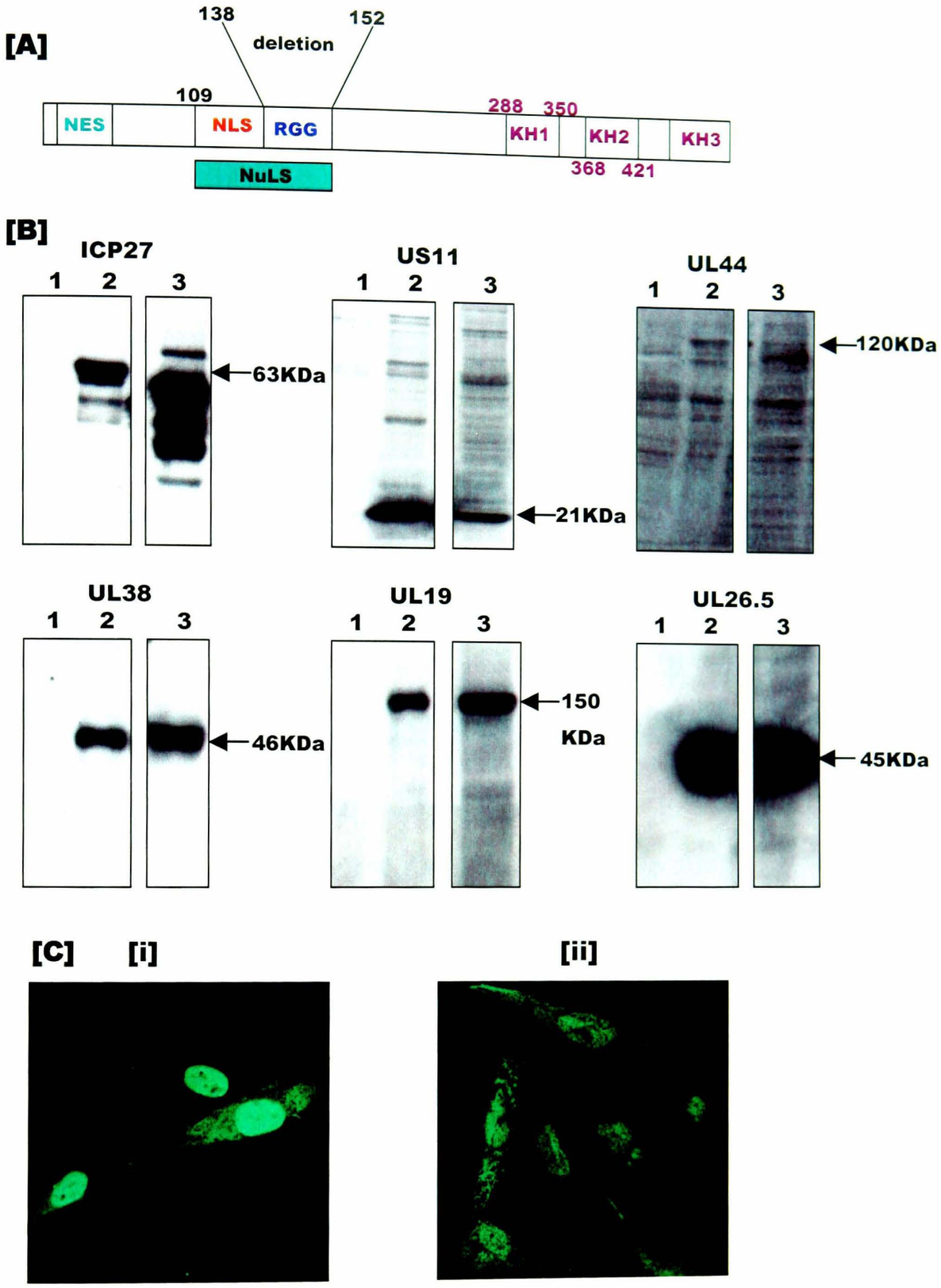


Figure 4.7: Analysis of the HSV-1 ICP27 mutant virus, *d*4-5.

A cartoon of ICP27 protein **[A]** indicating the deletion at the amino domain of the protein i.e. the RGG region a.a.139-153. Immunoblot analysis **[B]** for a range of HSV-1 proteins expressed during mock (lane 1), wt HSV-1 (lane 2) and *d*4-5 virus (lane 3) infections. Immunofluorescence **[C]** carried out on BHK cells infected with *d*4-5 virus **(i)** or wt HSV-1 **(ii)** revealing the localisation of ICP27.

those seen in wt infection. Both leaky-late protein levels remain similar to wt (Figure 4.7B).

Immunofluorescence showed that *d*4-5 ICP27 localised to the nucleus and the cytoplasm (figure 4.7 [C][i]). This pattern is similar to wt ICP27 localisation, however, there was no ICP27 detected within the nucleolus in the *d*4-5 infected cells. This was not unexpected as the RGG domain overlaps the nucleolar localisation signal (NuLS). The NuLS has been mapped to amino acid 110-152 (Figure 1.12). The loss of the NuLS may also play a vital role in the viability of the virus and should not be disregarded. Conversely, the disruption of the NuLS may have an affect on the phenotype of this virus.

4.6 Infection with an HSV-1 ICP27 deletion mutant virus does not enhance CAT gene expression when ICP27 binding sequences are present in either forward or reverse orientations.

The 27LacZ virus is an ideal candidate to confirm ICP27's role in the expression of CAT in the transfection/infection assay. During infection, 27LacZ successfully enters the cell and releases a full set of tegument proteins and continues with the synthesis of all immediate early proteins with the exception of ICP27. This includes the transcription transactivator ICP4 and the activator of gene expression, ICP0, two major regulatory proteins of the virus known to work in conjunction with ICP27. As these viral transcriptional and translational activators are present during 27LacZ infection, any change in CAT activity, as compared to wt HSV-1 infection, would give an indication of the magnitude of ICP27's role in this assay.

BHK cells were transfected with plasmids psv212, psv247, psv82 and psv334, having inserts in both forward and reverse orientations, for 6 h and subsequently infected with 27LacZ for a further 16 h. Virus infection was verified by Western blot analysis using anti-ICP27 antibody and an anti-ICP0 antibody. As all immediate early proteins

except ICP27 are expressed during 27LacZ infection, ICP0 (an immediate early protein) should be present during this infection. Western blot analysis confirmed that ICP27 was not detected during the 27LacZ infections whereas ICP0 was present at normal levels (Figure 4.3A).

Cell lysates were also assayed for CAT activity. During 27LacZ virus infection CAT activation was limited for all insert-containing plasmids and the enhancement of CAT activity as seen during wt HSV-1 infection was almost lost (Figure 4.8). For example, CAT activity for lysates of psv247 transfections reached 387 ± 87 CAM units when cells were infected with wt HSV-1 and only reached 42 ± 36 CAM units when infected with 27LacZ. The activity levels obtained with the virus lacking the ICP27 gene were very near to mock-infected levels, that is, all insert-containing plasmids gave a slight increase but remained within the experimental error (except psv212r) when compared to mock-infected cells. A certain level of increase in CAT levels was predicted upon 27LacZ infection as this virus expresses all other IE proteins including the transcription transactivators protein ICP4.

The data strongly indicate at least an indirect role for ICP27 when CAT expression is altered during wt HSV-1 infection, possibly in conjunction with cell factors. The inability to increase CAT gene expression, during 27LacZ infection, was not orientation dependent as again both forward and reverse orientations of the inserts showed little enhancement of CAT activity. It is also important to note that during the 27LacZ infection the cell does not progress into host-cell shut-off, as seen during wt HSV-1 infection, and this may suggest that the ICP27-dependent difference in CAT activation between wt HSV-1 and 27LacZ could be greater than the values discussed here. Furthermore, perhaps a cellular protein that destabilises RNA is being down regulated during wt HSV-1 infection thus leading to CAT mRNA stability.

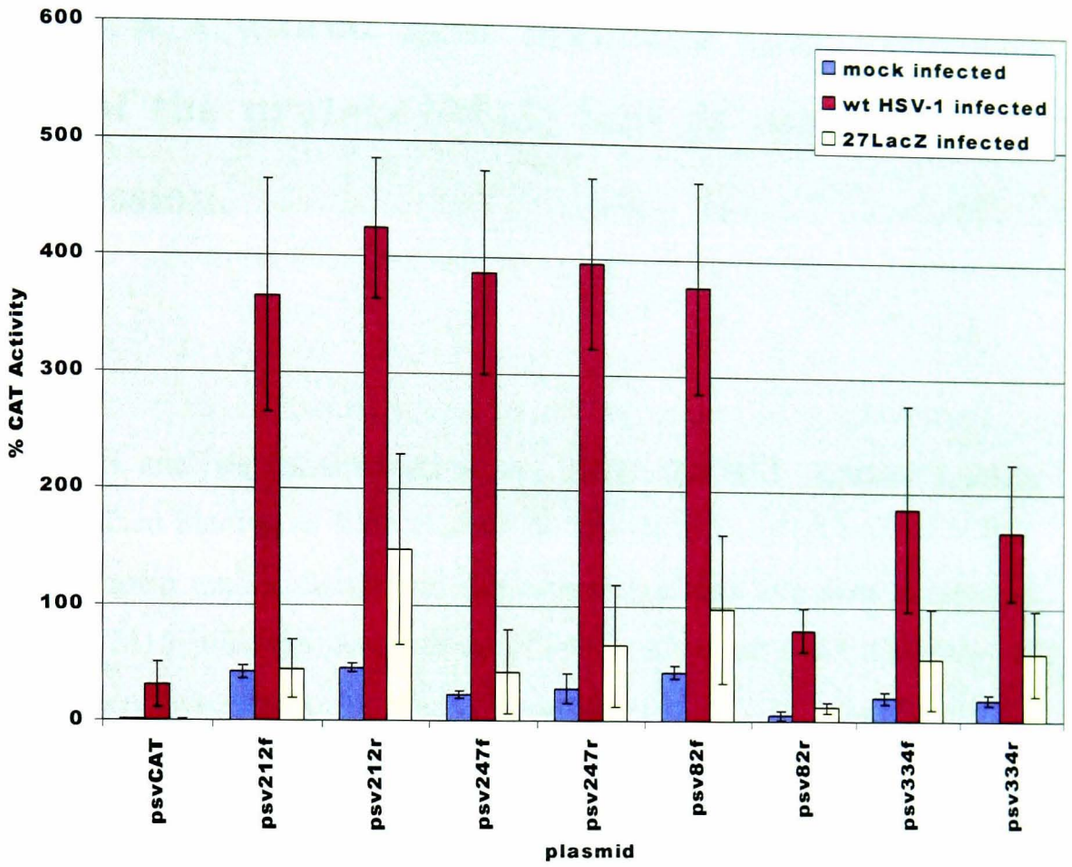


Figure 4.8: CAT levels obtained when psvCAT constructs were transfected and cells subsequently infected with a mutant HSV-1 virus that does not express ICP27, 27LacZ (n=3).

4.7 Infection with an expressing HSV-1 ICP27 mutant containing a 2 amino acid mutation that abolishes shuttling of the protein (M15) fails to enhance CAT gene expression.

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During the Y3-H analysis (Sokolowski *et al.*, 2003), the M15 mutation severely reduced or abolished binding to RNA transcripts originally found to bind wt ICP27. M15 has been shown not to shuttle and this mutation affects late gene expression. Furthermore, an M15 infection does not enter into host-cell shut-off. Therefore, to determine whether these functions affect the production of CAT enzyme from a vector with ICP27 binding sequences, infections with the M15 mutant virus were carried out.

Plasmids psvCAT, psv212f, psv247f, psv82f and psv334f were transfected into BHK cells, which after 6 h cells were infected with the M15 virus and left for a further 16 h. Western blot analysis confirmed expression of ICP27 during this infection (Figure 4.3B). Figure 4.9 shows the CAT levels obtained when the insert sequences are present and following M15 mutant virus infection. The levels of CAT activity were comparable to those seen in mock-infected cells. As with 27LacZ, cells infected with M15 are defective in host-cell shut-off. To investigate the contribution of virion host shut off in this investigation, a virus was used that expressed a defective ICP27 protein yet retained the ability to enter the cell into host-cell shut-off and produce almost normal levels of progeny virus.

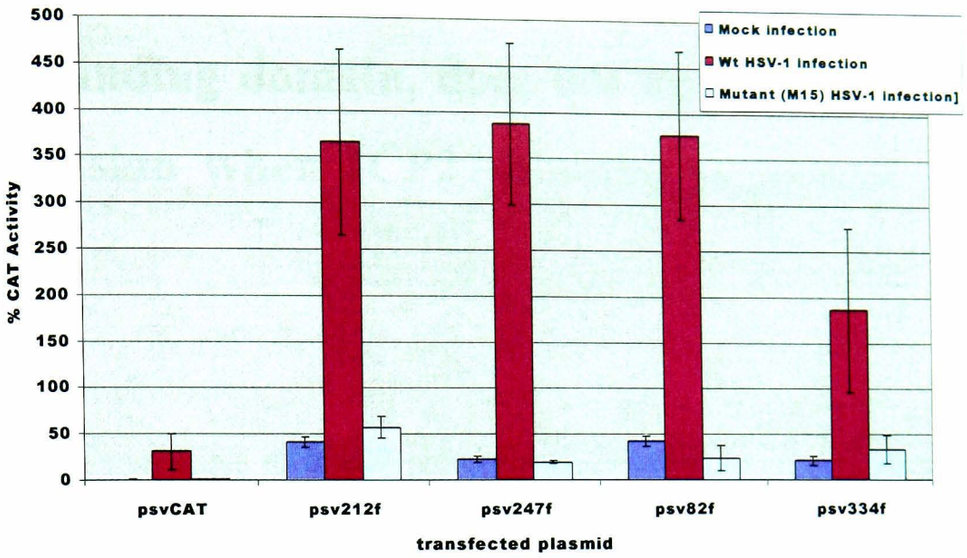

Figure 4.9: CAT activity levels obtained after cells were transfected with psvCAT plasmid DNAs and then infected with the ICP27 mutant virus M15. The levels are shown together with mock infected and wt HSV-1 infected activation levels (n=3).

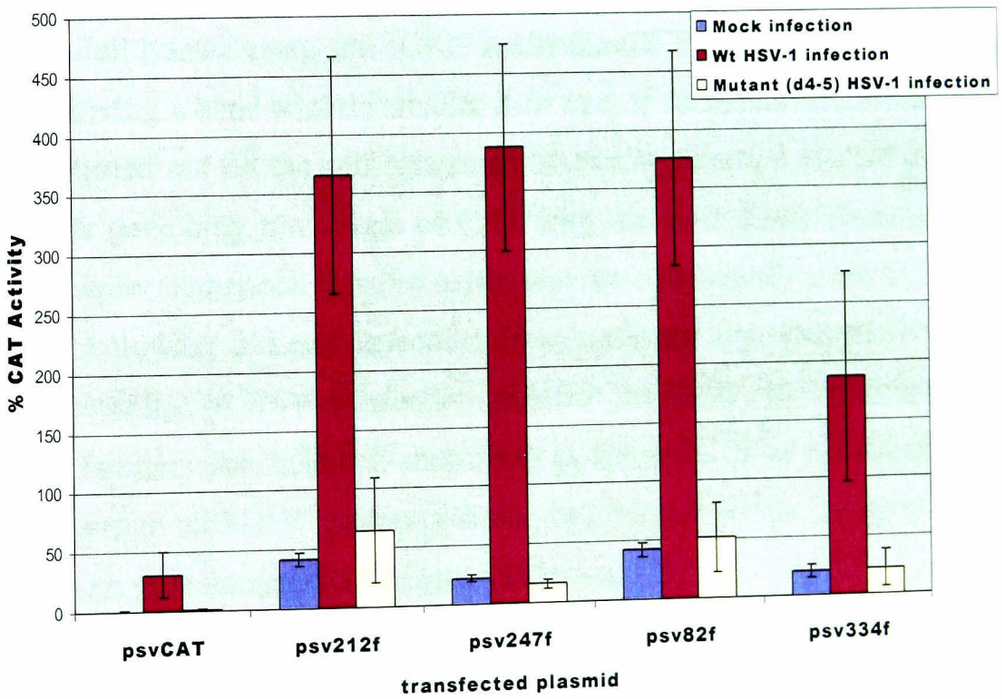

Figure 4.10 : CAT activity levels obtained when cells were infected with the ICP27 mutant virus *d*4-5, after cells were transfection with psvCAT DNAs, as compared to mock or wt HSV-1 infections (n=3).

4.8 The ICP27 mutant virus *d*4-5, that lacks the RGG box RNA binding domain, does not upregulate CAT gene expression when ICP27-binding sequences are present

Binding of HSV-1 transcripts to ICP27 protein was abolished or severely reduced when the mutant *d*4-5 ICP27 protein was used during the Y3-H assay (Sokolowski *et al.*, 2003), highlighting the importance of the RGG box at binding the viral transcripts identified. Notably, cells infected with this virus display host-cell shut-off. Thus it was decided that the *d*4-5 virus was an important control virus to use in these experiments.

BHK cells were transfected with psvCAT, psv212, psv247, psv82 and psv334 for 6 h and subsequently infected with *d*4-5 virus for a further 16 h. Western blot analysis was carried out on cell lysates using anti-ICP27 antibodies. ICP27 was present during the *d*4-5 infection giving a band slightly smaller than that of wt ICP27 (Figure 4.3B). The CAT assays carried out on the cell lysates are shown in Figure 4.10. All insert-containing plasmids gave only low levels of CAT with the *d*4-5 virus. These levels were 0.8-1.5 fold higher than mock infected levels and are comparable to the 1-3 fold increase obtained following 27LacZ infection. This indicates that enhanced CAT expression, as seen during wt HSV-1 infection, requires the RGG box RNA binding domain of ICP27. Furthermore, host-cell shut-off does not seem to be a major factor in CAT gene expression as ICP27 mutant viruses that do and do not progress into host-cell shut-off both give limited CAT expression levels.

4.9 Effects of truncations of insert #212 on CAT gene expression

RNA isolate #212 is a 63-nucleotide sequence found 46 nucleotides upstream of the UL6 (γ1) start codon. This RNA sequence is sufficient to activate CAT gene expression in the absence of any viral proteins. Also, during HSV-1 infection a dramatic increase in CAT activity, from 41 to 365 CAT units, was observed when this sequence was present in the 5'UTR of the CAT reporter plasmid. All sequences inserted into the CAT vector behaved similarly in terms of effect on CAT activity (except #82r). Plasmid psv212 plasmid transfections and consequent CAT activity analysis was repeated with this plasmid more than any other (at least 20 times), revealing a relatively consistent level of activity. Isolate #212 was therefore considered to be a suitable candidate to manipulate in order to establish the minimum sequence requirements for CAT activation.

The #212 sequence was divided into three sections; bases 1-30, 31-63 and 15-35 (Figure 4.11). Oligonucleotides were synthesised, annealed and ligated into the CAT plasmid as previously described. Sequencing verified that each oligonucleotide was inserted in the sense orientation. The psv212 truncated constructs, psv212 full length and psvCAT alone were transfected into BHK cells and after 16 h cell lysates were isolated and used to determine CAT activity levels.

When the truncated #212 inserts were present in the CAT plasmid, expression levels increased significantly as compared to psvCAT alone. CAT expression levels obtained with 1-30 and 15-35 were very similar to values obtained with the full-length sequence present (Figure 4.12). The truncation 31-63 gave a lower level of CAT conversion units (15 ± 5 units) when compared to the full-length sequence (35 ± 10 units). However, this reduced value was still significantly higher than psvCAT alone, showing that this short sequence stimulates CAT gene expression.

As transfection with truncation 15-35 elicited a good response this truncation was further cropped and inserted into psvCAT as15-24 and 25-35. The transfected

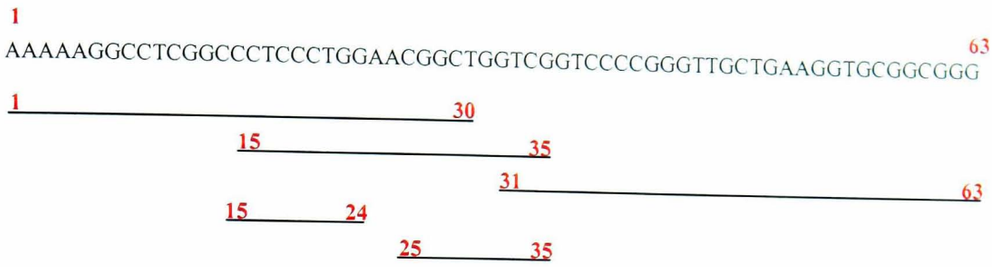

Figure 4.11: Diagram to show truncations of sequence #212 that were inserted into the psvCAT vector.

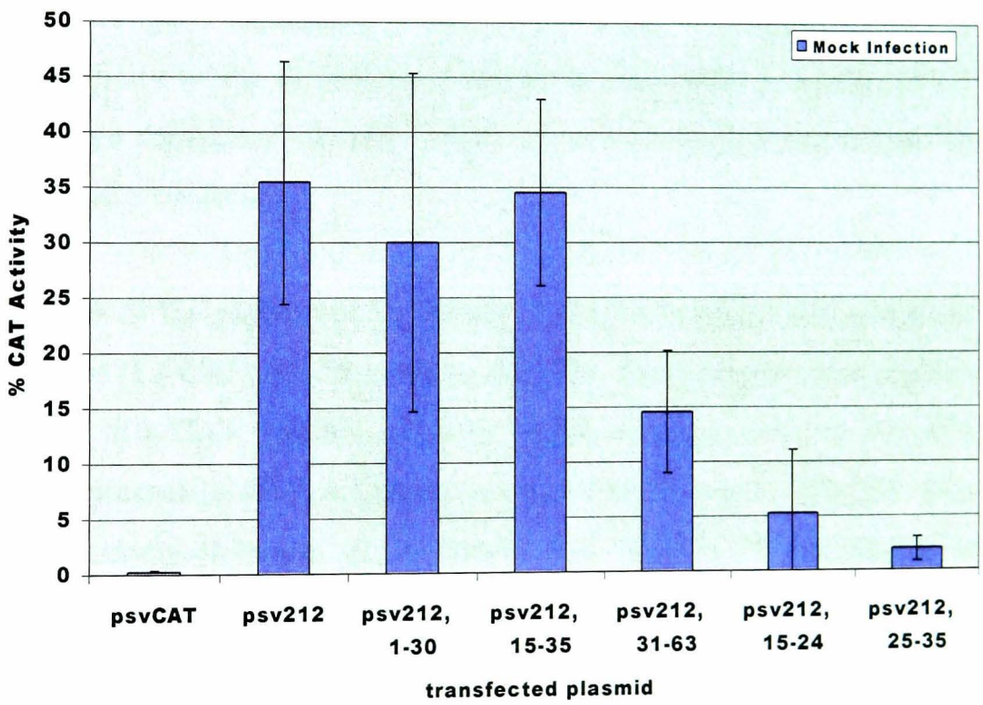

Figure 4.12: CAT activity levels obtained when truncations of the #212 isolate were inserted into the psvCAT vector and transfected into BHK cells. The numbers refer to the nucleotides present from sequence #212 (n=3).

psvCAT reporter plasmids containing these small sequences gave elevated levels of CAT activity (Figure 4.12), however, the values were severely reduced and variable when compared to full length #212. The reduction observed during transfection with both plasmids indicates that there is a possible loss of interaction between host cell factors and the inserted sequence, or perhaps the actual size of the sequence itself had an effect on expression.

4.10 Control sequences inserted into the 5'UTR of the CAT reporter plasmid identifies possible requirements for stimulation of CAT expression

When ICP27 binding sequences are present in the 5'UTR of a CAT expression vector the regulation of CAT gene expression is altered by virally expressed ICP27. To investigate the specificity of the sequences involved in these effects, a non-HSV-1 sequence, the negative regulatory element (NRE) of human papillomavirus type 16 (HPV-16), was used as a control.

HPV-16 is a member of the papovaviridae family of double stranded DNA viruses. The HPV-16 late gene (L1 ORF) 3'UTR contains the NRE and during an investigation using the L1 3'UTR in a CAT expression vector, expression was reduced 100 fold when the NRE was present in the plasmid, as compared to the control plasmid with the NRE deleted precisely (Kennedy *et al.*, 1991). The NRE is 79 nucleotides in length, and the 3' portion contains a GU rich domain. This domain binds to RNA processing factors such as $U2AF^{65}$ (Dietrich-Goetz *et al.*, 1997), HuR (Koffa *et al.*, 2000) and SF2/ASF (McPhillips *et al.*, 2004). The GU-rich sequence exerts a negative regulatory effect when expressed in the 3'UTR of the late gene and additionally in the 3'UTR of the CAT reporter gene originally used to detect the element. The NRE simultaneously forms a complex with several proteins and functions through diverse mechanisms (Cumming *et al.*, 2003; McPhillips *et al.*, 2004). The complex has a function in RNA stability (Kennedy *et al.*, 1991; Tan *et al.*, 1995), and causes nuclear retention of transcripts (Koffa *et al.*, 2000). Preliminary experiments carried out by

Jim Scott (Institute of Virology, University of Glasgow) identified that RNA transcribed from the NRE sequence did not bind to ICP27 in gel shift assays. For this reason the NRE sequence was used as a non-HSV-1, non-ICP27 binding control.

The NRE sequence was synthesised by PCR using primers (Table 2.3), that allowed the introduction of *Bam*H1 sites at both 5' and 3' ends, under the conditions as described in Methods 2.2.1. The PCR product was digested with *Bam*H1, purified and ligated with *Bam*H1 digested psvCAT. After transformation, resulting colonies were screened by restriction digestion and verified by DNA sequencing. Clones containing inserts in either orientation were obtained and used for subsequent analysis. The sequences of the NRE in the forward orientation (NRE-F) and the NRE in reverse orientation (NRE-R) are shown in Figure 4.13

Plasmids pNRE-F, psvCAT and psv212 were separately transfected into BHK cells and cell lysates were obtained after 16 h. CAT assays revealed that the NRE-F insert regulated the activation of CAT gene expression. The activation was variable and averaged 27 ± 19 CAT units (Figure 4.14). This variability may have been due to the presence of ATG's within the sequence, as shown in red in Figure 4.13. The NRE sequence inserted in the forward orientation enhanced CAT expression, thus the insert did not act as a negative regulator in this context. If this non-HSV-1 sequence enhanced the expression of CAT in this assay could it confer a response to ICP27 during HSV-1 infection? Interestingly, the NRE-R insert gave a lesser effect resulting in only 3 ± 1 CAT units, a value very close to that seen when the plasmid contained no insert (Figure 4.14).

BHK monolayers were transfected with the NRE–F plasmid and after 6 h cells were infected with wt HSV-1. CAT assays carried out on cell lysates revealed that NRE-F did respond to HSV-1 infection, albeit to a low level, with values reaching only 70 CAM units (Figure 4.15). To test whether this response was due to the presence of ICP27, cells were transfected with NRE-F and further infected with 27LacZ, *d*4-5 or M15 viruses. CAT levels during 27LacZ and *d*4-5 infections revealed less enhancement of CAT activity, reaching only some 36 CAM units when the NRE sequence was inserted in the forward orientation (Figure 4.15). However, this activity

GCTAAACGCAAAAAACGTAAGCTGTAAGTATTGTATGTATGTTGAATTAGTGTTGT
TTGTTGTGTATATGTTTGTATGTTGATCCGTCGAGATTTTCAGGAGCTAAGGAAGCT
AAAATG

NRE-R:

ACATACAAACATATACACAACAAACAACACTAATTCAACATACATACAATACTTAC
AGCTTACGTTTTTTGCGTTTAGCTGATCCGTCGAGATTTTCAGGAGCTAAGGAAGCT
AAAATG

Figure 4.13: The NRE sequences in forward (F) or reverse (R) orientations was inserted into the psvCAT vector. The presence of ATGs are highlighted in red and the final ATG is the CAT start codon.The sequence shown in blue is plasmid sequence.

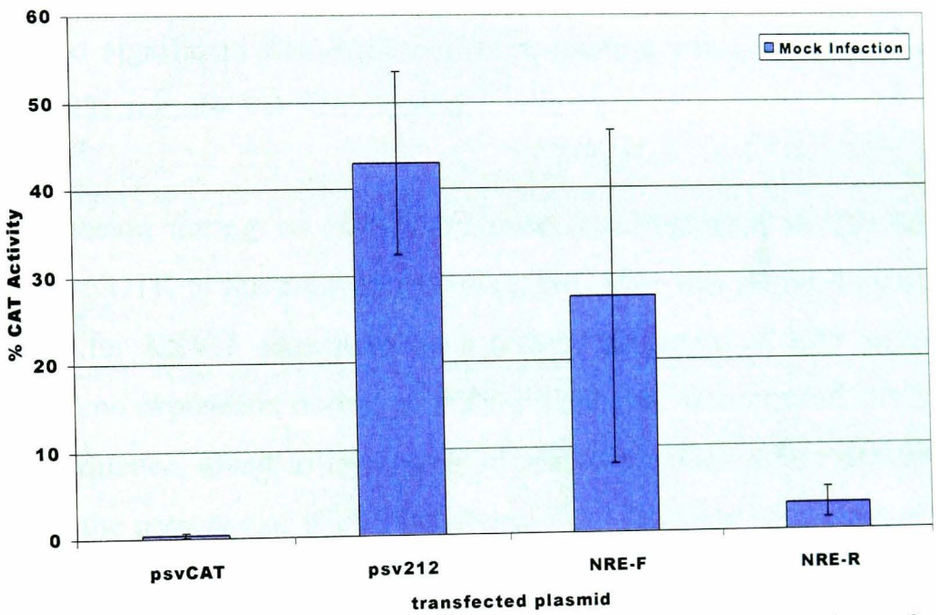

Figure 4.14: CAT activity levels obtained after transfection of psvCAT plasmid containing the NRE sequence in either forward (F) or reverse (R) orientation (n=3).

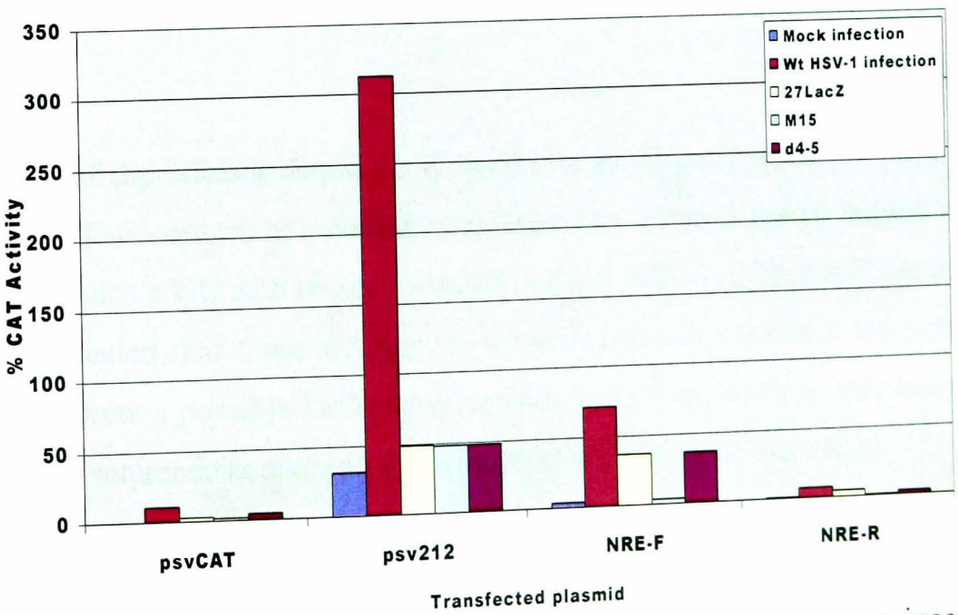

Figure 4.15: Values obtained when the NRE sequence was inserted into psvCAT plasmid in forward and reverse orientation gives the above activation profile during mock, wt or mutant ICP27 HSV-1 infections. These levels are compared to psvCAT plasmid with no insert (psvCAT) and psv212, an insert-containing plasmid already shown to enhance CAT gene expression during HSV-1 infection.

was greater than values seen with mock infection. During infection with M15, CAT activity levels were similar to those seen during mock infection. It was interesting to note that the NRE-F CAT plasmid responded poorly to HSV-1 infection and displayed little difference in CAT activity when ICP27 was absent during 27LacZ infection. When the pNRE-R plasmid was transfected into BHK cells and CAT activity analysed, the CAT levels did not increase significantly above the level seen when there was no sequence present, i.e. psvCAT, reaching only levels of 3 CAM units/μl cell lysate (Figure 4.15). The expression of CAT via the NRE-R control sequence gave no significant elevation in CAT activation when cells were infected with wt HSV-1, 27LacZ, *d*4-5 or M15 viruses.

CAT gene expression during wt HSV-1 infection was regulated *via* the sequence inserted in to the 5'UTR of the plasmid, however, the effect was shown here not to be entirely specific for HSV-1 sequences as a control sequence of HPV origin also enhanced CAT gene expression during wt HSV-1 infection, as compared to a plasmid with no insert sequence, albeit to low levels of activation. This effect was shown in part to be due to the presence of ICP27 indicating that a possible interaction of ICP27 with insert is possibly not HSV-1 specific. Interestingly, insertion of the NRE in the reverse orientation gave little to no increase in CAT activity during infection therefore the sequences were inspected in the hope of revealing possible requirements involved in ICP27 regulation of their expression and also for the increase in ICP27 independent CAT activity.

The G content of the NRE-R sequence is very low on the non-coding strand (5%), whereas the NRE-F control non-coding sequence has a considerably higher (24%) content and contains a GU rich region. Analysis of the HSV-1 sequences investigated in this study revealed that there are regions within each sequence that are rich in G residues. If this were a possible factor in enhancing gene expression in this assay then inserting a small sequence containing G residues might also give this effect.

4.11 Transfection of plasmid containing poly(G) elicited enhanced CAT gene expression during wt HSV-1 infection.

RNA binding assays using recombinant protein and RNA homopolymers immobilized on agarose beads have been used extensively for RNA-protein binding studies by many investigators (Brown *et al.*, 1998; Datar *et al.*, 1993; Fabre *et al.*, 1994; Fackelmayer and Richter, 1994; Ohno *et al.*, 1994; Siomi *et al.*, 1993b). For example, Datar *et al.* described the RNA binding properties of the fragile X mental retardation protein (FMRP) using this method. Fragile X Syndrome is caused by a triple repeat or a point mutation in the non-coding region of the FMR1 gene mutation leading to the loss of function of the FMRP protein (De Boulle *et al.*, 1993; Pieretti *et al.*, 1991; Verkerk *et al.*, 1991). The FMRP protein is an interesting example as it contains many of the same functional domains as ICP27. Both proteins contain an RGG box and KH domains, which function in RNA-binding. They both also contain NLS and NES sequences and are known to shuttle in and out of the nucleus (Abrams *et al.*, 1999; Bardoni *et al.*, 1997; Eberhart *et al.*, 1996; Fridell *et al.*, 1996; Lewis *et al.*, 1999; Pollard *et al.*, 1996). RNA binding assays carried out using FMRP protein revealed strong binding to poly(G) and weaker yet significant binding to poly(U) RNA homopolymers that was later shown to be a specific intrinsic property of the FMRP protein (Gunter *et al.*, 1998). Strikingly, Mears and Rice, using the same method, described the ability of recombinant ICP27 protein to bind moderately to poly(G) and weakly to poly(U) RNA homopolymers (Mears and Rice, 1996a). Thus various homopolymers inserted into the 5'UTR of the psvCAT plasmid could identify a nucleotide requirement for the regulation of CAT gene expression during wt HSV-1 infection.

Oligonucleotides consisting of ten A, C, G or Ts were synthesised with a *Bam*HI site introduced at each 5'end. The appropriate oligonucleotides were annealed and inserted into the psvCAT vector. Upon sequencing each construct it was possible to identify plasmids containing a run of ten A, C, G or Ts. Initial experiments were carried out to analyse CAT gene expression without virus infection. Each plasmid was transfected

into BHK cells and CAT activation was quantified by performing CAT assays on each cell lysate. The levels of CAT gene expression were significantly increased, compared with psvCAT, when the poly(G) homopolymer was present (10 ± 1 CAM units), albeit reduced compared to psv212 (20 ± 7 CAM units) (Figure 4.16). Analysis of transfections with poly (A), (C) or (T) inserts revealed limited levels of CAT activity (0.6-2 CAM units) almost comparable to plasmid containing no insert (0.4 ± 0.6 CAM units). In these experiments there was an uncharacteristically low level of expression of CAT, possibly due to the quality of the BHK cells used, however the relative amounts of activity from psvCAT and psv212 plasmids were very similar, in all experiments from this point, thus allowing a comparison of the homopolymer plasmids with them.

There was a significant increase in CAT activation with the poly(G) insert in the absence of viral factors and it was therefore of interest to study the effect of these sequences on CAT activation in the presence of virus. The plasmids were transfected and cells infected with wt HSV-1 or 27LacZ and CAT assays carried out on lysates obtained. CAT gene expression levels were dramatically enhanced to 136 CAM units with the poly(G) insert and wt HSV-1 infection whereas levels only reached 12 CAM units with 27LacZ infection (Figure 4.17). Plasmids with the other homopolymer inserts gave only slightly elevated levels of CAT expression, ranging from 2-10 CAM units, in response to infection with wt HSV-1 and also a slightly reduced level of expression, ranging from 1.8-3 CAM units, following 27LacZ infection.

The data demonstrate that gene expression was increased when poly(G) homopolymers, but not poly(A), (C) or (T), are present. This indicates that the G content of the sequences used in this study was essentially responsible for the enhanced activation observed when ICP27 was present during infection. This enhancement was severely reduced when either ICP27 was not present or carried a mutation, or the insert sequence contained few or no G nucleotides. Furthermore, it is interesting to note that there was significant ICP27-independent CAT activation when poly(G) nucleotides were present in the 5'UTR of the CAT plasmid. Again this indicates an involvement of cellular factors in the activation of CAT gene expression when this sequence is present.

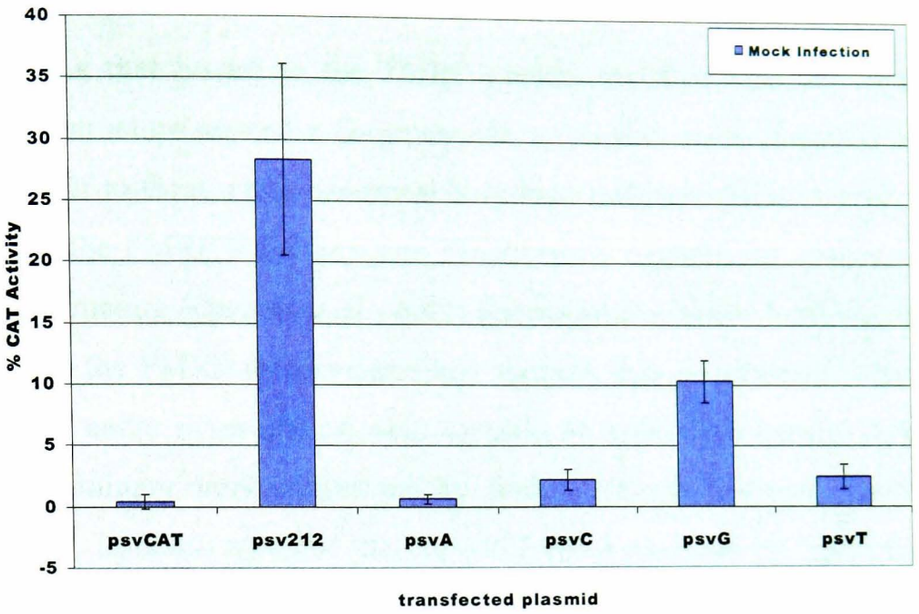

Figure 4.16: psvCAT plasmids containing a 10 mer homopolymer sequence of A, C, G or Ts were transfected and analysed for CAT expression levels (n=3).

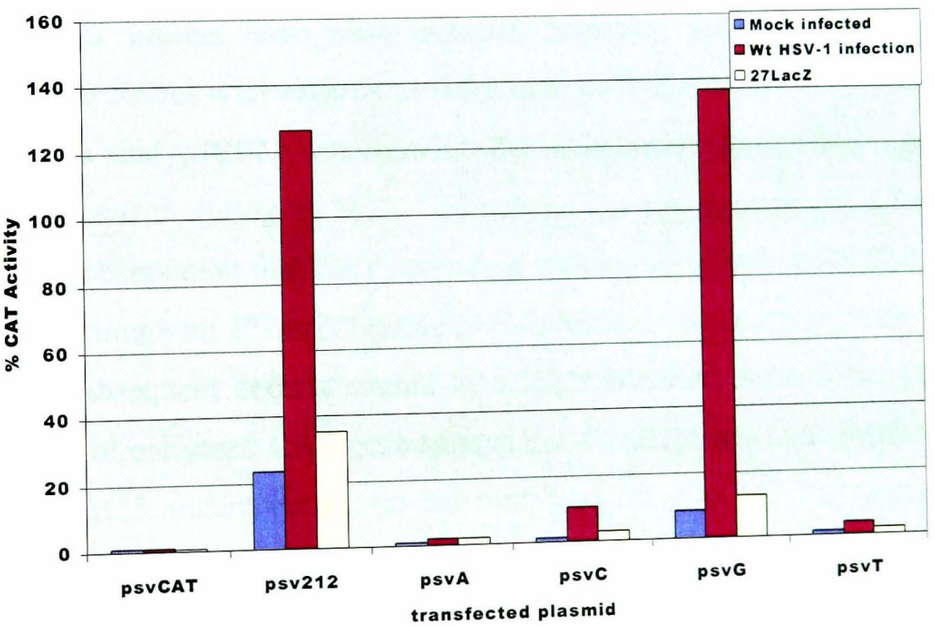

Figure 4.17: Activities obtained when homopolymer-containing plasmid DNAs were transfected then cells infected with wt HSV-1 or 27LacZ and lysates analyzed for CAT activity.

Analysis of RNAs that bound to the FMRP protein revealed that they formed a complex tertiary structure termed a G-quartet i.e. a complex stem loop element that folds back on itself to form a non-canonical base-base structure (Brown *et al.*, 2001) and furthermore, the FMRP RGG box was shown to be required for binding of this RNA G-quartet structure (Darnell *et al.*, 2001; Ramos *et al.*, 2003). As ICP27 protein behaved very like the FMRP protein described above it was of interest to find out if any of the RNAs under investigation were capable of forming G-quartet structures. Unpublished preliminary work carried out by Andres Ramos (National Institute for Medical Research, London) revealed that the #212 RNA analysed by NMR does not form a G-quartet type structure.

4.12 Discussion

ICP27 is an RNA binding protein that shuttles in and out of the nucleus. RNAs that bind to the ICP27 protein have been isolated, however, little is known of the specificity of these RNAs with regards to the function of ICP27 in the regulation of viral genes. In this study, ICP27 was shown to be involved in the positive regulation of CAT gene expression during wt HSV-1 infection. The involvement of ICP27 was confirmed by the observation that CAT activation was not enhanced in its absence, as illustrated by infection with 27LacZ. During M15 infection, the mutation in the ICP27 protein and the subsequent defects caused by such a mutation seem to be possible factors in the lack of enhanced CAT gene expression. Furthermore, cells infected with both 27LacZ and M15 mutant viruses do not enter host cell shut off. The presence of ICP27 RNA binding sequences within the 5' UTR of the CAT reporter gene during transfection and wt HSV-1 infection of BHK cells resulted in an increase of CAT activity but the enhancement was lower than that reached when no inserted sequences were present, indicating a certain level of inhibition of expression when these sequences were present. Even though there seems to be reduced activation of CAT, when these HSV-1 sequences were present, the absolute CAT enzyme levels reached were much higher than when no insert sequences were present. Thus enhanced expression as determined by CAT activity was demonstrated when ICP27 binding

sequences were present during wt HSV-1 infection. Any change in sequence size, sequence composition etc of the insert, consistently gave a reduction in expression levels. Furthermore, expression levels reached when no ICP27-binding sequences were present were at levels, which were very difficult to detect. This problem could skew the activation levels, leading to inaccuracies in the extent of increases directed by HSV-1 infection. Taking all these factors into consideration, it is possible, that during transfection and wt infection when insert sequences were present, there could be levels of CAT approaching a maximum level of expression.

Experiments using bacterially expressed ICP27 showed direct binding to RNA (Ingram *et al.*, 1996). However, this binding was not specific for a target RNA as ICP27 bound equally efficiently to RNA fragments from early and late HSV-1 genes, fragments from exon-1 of a late HSV-1 gene and also to two RNA fragments from an adenovirus late gene, where one contained a splice site (Ingram *et al.*, 1996).

Further investigations using bacterially expressed ICP27 also showed that ICP27 did not bind to target RNAs specifically and identified that the R/G rich region of ICP27 was responsible for RNA binding (Brown *et al.*, 1995). Furthermore, RNA was found to bind the RGG box alone of ICP27 (Mears and Rice, 1996a). The ICP27-RNA interaction was confirmed by the observation that RNA binding was abolished when using the *d*4-5 deletion mutant (Mears and Rice, 1996a). This interaction was not specific for an RNA target sequence as full length ICP27 and the RGG box region alone both bound to two control probes i.e. an SV40 RNA, previously shown not to bind full length ICP27 (Brown *et al.*, 1995) and the IFN-β antisense RNA. Therefore, bacterially expressed ICP27 and RGG box bind to non-specific RNA transcripts via the RGG box. However, using a eukaryotic system, ICP27 bound RNA transcripts specifically. Sandri-Goldin was able to UV cross-link HSV-1 infected cells and showed that ICP27 bound to a selected group of intronless RNAs e.g. ICP27, ICP4, TK, ICP8, and not to intron-containing RNAs e.g. ICP0 and UL15. This *in vivo* UV cross-linking study shows the specificity of ICP27 binding to intronless HSV-1 RNA, albeit only eight transcripts were analysed (Sandri-Goldin, 1998a). Furthermore, ICP27 transcribed and translated *in vitro* in rabbit reticulocyte cell lysates binds to poly (G) and poly (U) RNA homopolymers but not to poly (A) and poly (C)

homopolymers (Mears and Rice, 1996a). Therefore, eukaryotically expressed ICP27 showed some binding specificity to target RNAs.

In the study described here, a lack of enhancement of CAT activation was also seen with ICP27 mutants lacking an RGG box irrespective of whether or not HSV-1 sequences were present in the CAT reporter plasmid. The CAT expression levels observed during the *d*4-5 infection provide possible evidence for a direct role for ICP27 in enhancing CAT gene expression during wt HSV-1 infection. That is, high CAT activation levels are reached when ICP27 is present during wt HSV-1 infection and these high levels are severely reduced when ICP27 is mutated to prevent RNA binding. Given that ICP27 is present during the *d*4-5 infection, and that viral replication and growth proceeds, the prevention of enhanced CAT gene expression suggests an essential role for ICP27 in the expression of this reporter gene. Furthermore, as infection progresses with the *d*4-5 virus, the cells enter into host-cell shut-off, thus this event did not influence the enhanced CAT gene expression seen during wt HSV-1 infection.

Transfection of plasmid without any HSV-1 sequences (psvCAT) gave an increase in CAT expression during wt HSV-1 infection suggesting that viral factors can cause an increase in gene expression in the absence of viral sequences. Taking this into account, alongside the orientation independent effect of the sequences, offers the possibility that the HSV-1 enhancement of expression could be the product of two separate non-specific effects. That is, an increase in CAT expression due to the presence of cloned sequence, or the global increase in gene expression caused by viral trans-acting factors. However, the cloned sequences, in the forward orientation, are known to bind ICP27 and not to mutant ICP27 (Y3-H) and, alongside the activation data here, this would strongly suggest that ICP27 is responsible, at least in part, for enhancing gene expression during wt HSV-1 infection.

In an attempt to identify a minimal sequence requirement the #212 sequence was truncated. One truncation almost abolishes enhanced CAT gene expression when no viral proteins were present. EMBOSS (European Molecular Biology Open Software Suite) was used to analyse the frequency with which these small sequences existed within the HSV-1 genome. It was evident that each sequence appeared only once

within the genome. Upon analysis of the two #212 truncated sequences it was not evident what the activating sequence was except that they were HSV-1 sequences. The insertion of a non-HSV-1 sequence into the psvCAT plasmid might therefore indicate whether this enhanced gene regulation was specific to HSV-1 sequences or not. This experiment needs to be extended to analyse the effect observed during HSV-1 infections with and without ICP27, and also to include a more detailed analysis of sequence length requirements.

Further indication of sequence requirements for CAT activation were gained by using the NRE-R control sequence which failed to enhance CAT activity either in the absence of presence of virus. The most obvious difference between this sequence and the activating ones is the difference in the content of the G-residues. The enhanced activation of CAT gene expression was not detected during wt HSV-1 infection when the inserted sequence contained only four randomly spaced G nucleotides, leading to the idea that multiple runs of G nucleotides are important for the enhanced activation of gene expression when ICP27 is present.

RNA homopolymer assays have been a useful tool in identifying binding efficiencies, and to some extent specificities, of RNA binding proteins. Indeed, the classification of the hnRNP protein RNA-binding properties was originally based on their ability to bind RNA homopolymers e.g. hnRNP C is a poly(U) binding protein and hnRNP K and J are poly(C) binding proteins, whereas hnRNP E, F and M are poly(G) binding proteins. Interestingly, hnRNP A bound all four ribonucleotide homopolymers and furthermore, hnRNP P bound well to both poly(A) and (G). That is, both hnRNP A and P proteins are not absolutely base-specific binding proteins (Swanson and Dreyfuss, 1988) yet a degree of binding specificity is retained to the particular homopolymers mentioned above.

The RNA-dependent RNA polymerase encoded by the hepatitis C virus NS5B gene was shown to bind strongly, but not exclusively to poly(G) RNA homopolymers (Johnson *et al.*, 2000; Lohmann *et al.*, 1997). The vaccinia virus processivity factor VP39 was shown to bind avidly to poly(A) and poly(U) homopolymers, however weak binding was also observed with poly(C) and (G) (Johnson and Gershon, 1999). The pseudorabies virus UL54 (which has an interesting 41% homology to ICP27)

expresses an early protein that contains an R/G–rich region at the N-terminus. This protein binds poly(G) RNA homopolymers, however, no other nucleotides were tested to identify binding efficiencies or specificities (Huang and Wu, 2004). The FMRP protein, previously described here, binds efficiently to poly(G) and weakly to poly(U) indicating that the FMRP protein has binding affinities to G/U-rich sequences (Siomi *et al.*, 1993b).

ICP27 binds preferentially, albeit moderately, to poly(G) RNA homopolymers and weakly to poly(U), however absolutely no binding was observed to poly(A) or (C). The RGG box of ICP27 was required but not absolutely essential for efficient poly(G) binding (Mears and Rice, 1996a). It is evident from the analysis of RNA binding assays described above that very few RNA binding proteins exhibit specific binding of a particular ribonucleotide, making the information gained from these assays very difficult to interpret in terms of absolute specificity. Many RNA binding proteins show a preference for certain homopolymer ribonucleotides and ICP27 is no exception. In this investigation, the function of ICP27, with regard to the presence of homopolymer sequences inserted into the 5'UTR of a CAT gene was analysed to observe any nucleotide specificity. The ICP27 activation data obtained here, together with the reports demonstrating preferential direct binding with poly(G) RNA to ICP27, and to ICP27 RGG box alone (Mears and Rice, 1996), strongly indicate that ICP27 possesses specific RNA binding efficiencies to G-rich sequences and that an interaction between ICP27 and these G-rich sequences leads to an increase in gene expression. Interestingly, the 68% GC-rich HSV-1 genome has 1,103 runs of 5 G residues or more. Furthermore, the CAT gene has no runs of 5 Gs or more.

The previously identified HSV-1 RNA sequences seemed not to carry any obvious specific sequence motifs responsible for the binding to ICP27 (Sokolowski *et al.*, 2003), except for the specific requirement of a G-rich sequence. The ICP27 RGG box alone binds RNA, however this binding was also not specific as this domain was also shown to interact with two control RNAs, that were previously shown not to bind full length ICP27 (Brown *et al.*, 1995). Non-specific binding to ribonucleotides has also been reported for a domain within the RNA-binding protein nucleolin (Ghisolfi *et al.*, 1992a; Ghisolfi *et al.*, 1992b; Ginisty *et al.*, 1999). Nucleolin is involved in the early stages of ribosome assembly within the nucleolus. Nucleolin contains an RGG

domain at the C-terminus of the protein that was shown to bind different RNA homopolymers with similar affinities. Nucleolin also contains a central domain known to bind specifically to the 5' external transcribed spacer of the pre-rRNA. The RGG domain of nucleolin was also shown to bind this region non-specifically. Further analysis of this non-specific binding was carried out using circular dichroism spectroscopy, a method that gives a measurement representing the structural behaviour of RNA within an RNA-protein complex. Interestingly, this investigation revealed that a change within the RNA occurs during the binding of the RGG domain to it. The changes in behaviour indicated an unstacking of bases and an unfolding of secondary RNA structures, possibly revealing the ability of nucleolin to bind and unwind RNA. It was therefore postulated that this efficient non-specific binding of the RGG domain of nucleolin allowed the RNA to be unstacked and unfolded thus allowing the specific binding of the central domain of the nucleolin protein to the unwound RNA (Ghisolfi *et al.*, 1992a; Ghisolfi *et al.*, 1992b; Ginisty *et al.*, 1999). Nucleolin is a multifunctional protein that exhibits properties similar to ICP27 including RNA-binding, transcription repression and shuttling between nucleus and cytoplasm. In addition, both proteins are phosphorlylated by CKII. Furthermore, the proteins' structural similarities include an acidic domain, an NLS and an RGG box. Obviously, there are many differences in the roles of the proteins, however, taking these similarities above into consideration alongside the binding properties of the RGG box and the functional data gained here, it is feasible that the RGG box of ICP27 could behave similarly to the RGG domain of the nucleolin protein. That is, allowing non-specific binding of RNA transcripts leading to the specific binding of the RNA to a different domain of the protein. Further investigation into these matters is required.

The enhancement of gene expression could be due to any of the known functions of ICP27. ICP27 can transactivate the transcription of certain HSV-1 genes (Rice and Knipe, 1988) possibly through an effect on initiation (Jean *et al.*, 2001) or by the phosphorylation of viral and/or cellular proteins involved in, and thus promoting, transcription (Xia *et al.*, 1996). Furthermore, ICP27 is known to associate with RNA polymerase II complexes (Jenkins and Spencer, 2001; Zhou and Knipe, 2002). ICP27 increases RNA 3' processing at inherently weak poly (A) sites by increasing processing efficiencies (McGregor *et al.*, 1996b) and, furthermore, it stabilises RNA

by binding to the AU-rich elements found within the 3'UTR of labile RNAs (Brown *et al.*, 1995). ICP27 protein contains an NES and an NLS thus allowing the protein to shuttle in and out of the nucleus, and both are necessary for its role in RNA nuclear export. It is possible; therefore, that ICP27 regulates some viral gene expression via transcriptional processes, while regulating others by post-transcriptional mechanisms. Most of the functions mentioned above could be responsible for the increase in gene expression observed in this investigation. ICP27 enhances the expression of UL24 (an HSV-1 leaky late gene) (Pearson *et al.*, 2004) and VP16 (an HSV-1 tegument protein) (Ellison *et al.*, 2005). During ICP27-null virus infection the protein levels of UL24 are reduced by 70-fold. This was shown to be due to a decrease in cytoplasmic accumulation of UL24 mRNA, not a decrease in total mRNA, suggesting a post-transcriptional regulation of gene expression at the level of nuclear export (Pearson *et al.*, 2004). Furthermore, in the absence of ICP27, most cytoplasmic VP16 mRNA molecules do not associate with actively translating ribosomes but co-sediment with the 40S ribosomal subunit, suggesting a defect at the level of initiation of translation resulting in a 9-80 fold decrease in protein yield (Ellison *et al.*, 2005). Therefore, ICP27 has been shown to stimulate gene expression post-transcriptionally at two distinct levels; enhancing the export of UL24, and the interaction of VP16-specific mRNA with polysomes thus leading to the stimulation of their translation.

ICP27 associates with components of the cellular initiation translation machinery including eIF3 and eIF4G (see below) and also with the poly (A) binding protein (PABP) indicating a possible role in the stimulation, or the repression, of translation at the initiation level (Fontaine-Rodriguez *et al.*, 2004). This association with the translation machinery was confirmed when ICP27 was shown to co-sediment with polyribosomes, (as did PABP) and was present in the 80S ribosome and polyribosome fraction (Larralde *et al.*, 2006). A more direct indication of ICP27's ability to affect gene expression at the level of translation was investigated using a tethered function assay. Here, ICP27 was shown to directly stimulate the translation of mRNAs that bound to it in the absence of any other viral protein (Larralde *et al.*, 2006).

An increase in translation could also be possible without the influence of ICP27. The 5'UTR can influence the translation of downstream ORF's and represents a level of control of gene expression. Regulation influenced by elements of the 5'UTR usually

occurs at the level of translation initiation. Briefly, the 43S pre-initiation complex is composed of the 40S ribosomal subunit bound to eukaryotic initiation factors (eIFs), (including eIF3) and a ternary complex comprising tRNA and eIF2 that is coupled to GTP. This 43S pre-initiation complex is thought to bind RNA via the 5' cap-binding complex, eIF4F. Components of the eIF4F complex are eIF4E, that binds to the 5' cap structure, and the eIF4G, a scaffold protein that binds many initiation factors including eIF4E and eIF3. Thus, a competent scanning initiation complex is formed. Another possible reason for an increase in translation during wt HSV-1 infection could be due to the presence of the viral protein ICP34.5. This protein prevents host shutoff by ensuring the dephosphorylation of eIF2 and therefore allowing protein synthesis to continue. However, the expression of ICP34.5 should be analysed during 27LacZ, M15 and *d*4-5 virus infections as these viruses do not progress into host shutoff and enhanced CAT gene expression is not observed during these infections.

Control elements that determine translation efficiency that are located in the 5'UTR include internal ribosomal entry sites (IRESs), upstream AUGs (uAUGs) and upstream open reading frames (uORFs), 5'UTR sequence length, secondary structures and also binding sites for regulatory proteins. (Gray and Wickens, 1998; Mignone *et al.*, 2002).

The IRES is a secondary structure found within the 5'UTR and is most commonly associated with picornavirus translations (Jackson and Kaminski, 1995). Most initiation factors, including eIF4F, are required for IRES-mediated translation (Jackson and Kaminski, 1995) along with other *trans*-acting factors such as polypyrimidine tract-binding protein (Hellen *et al.*, 1993; Kaminski *et al.*, 1995) and the La autoantigen (Meerovitch *et al.*, 1993; Svitkin *et al.*, 1994a). These *trans*-acting factors are possibly required to hold the IRES in position (Svitkin *et al.*, 1994b).

uORFs and uAUGs in the 5'UTR can regulate the translation of the main ORF. The first AUG scanned by the ribosome is selected as the site of initiation (Kozak, 1983; Kozak, 1987), whereas uORF can function together to regulate translation (Hinnebusch, 1996). Both types of interruptions to the reading of the main ORF leads to the repression of translation. However, uORF can also directly provide a termination site before the main ORF thus allowing a degree of secondary initiation

from the main ORF, that is less inhibitory than the uAUGs (Kozak, 1984; Liu *et al.*, 1984).

Long 5'UTRs allow for the formation of stable secondary structures that lead to repression of translation initiation by hindering the scanning ribosome, a process that is dependent on the stability of the RNA structure. A transcript containing a 5'UTR of 870nt (TGF-β1) leads to an increase in translation efficiencies when compared to a larger 5'UTR of 1.1kb (TGF-β3) i.e. two of the three transcripts of the TGF-β proteins differ in their abilities to regulate translation due to the variable lengths of the 5'UTR. Interestingly, all of these 5'UTRs are GC rich. The distance of the secondary structure from the 5' cap is also a determinant of translational regulation as the closer the secondary RNA structure is to the 5' cap the more likely the 43S pre-initiation complexes will not bind to the RNA thus translation initiation is inhibited. Hairpin structures that are located further away from the cap can also inhibit translation as these structures can be particularly stable rendering the helicase component (eIF4A) of the initiation complex unable to unwind the RNA in the 5'UTR thus preventing the 43S complex from binding to the AUG codon.

Interestingly, many mRNAs that contain highly structured 5'UTRs are implicated in cell death or cell growth suggesting that their translation is tightly controlled by 5'UTR elements. An over-expression of the initiation factors (e.g. eIF4E) involved here results in the loss of translational repression and can ultimately lead to tumorigenesis (De Benedetti, *et al.*, 1994). A severe reduction in 5'UTR length (less than 12nt) impairs translation initiation, and moderately increasing the length increases translation efficiency possibly by increasing the number of interactions of the 43S pre-initiation complexes (Kozak, 1991a; Kozak, 1991b). As the length of the 5' leader sequence increases translation initiation is enhanced, however, as the sequence increases further in length and secondary structures are formed there is a decrease in the efficiency of translation initiation.

An example of a small structural element within the 5'UTR (not an IRES) is the iron response element (IRE), a stem loop structure of approximately 30nt in length. The iron regulatory proteins (IRP) regulate RNAs that contain an IRE, in response to iron

levels in the cell. Under conditions of iron deprivation the IRP binds to the IRE thus blocking the binding of the 43S pre-initiation complex. When iron is plentiful in the cell the IRP proteins are either inactivated or degraded (Tong and Rouault, 2000). The regulatory function of the IRPs are also determined by the distance of the IRE to the 5' cap resulting in the inhibition of initiation factors that bind the 40S ribosomal subunit which increases when the IRE is proximal to the 5' cap. However, as the IRE moved distally to the cap another inhibitory function occurs i.e. inhibiting the scanning of the 40S ribosomal unit. The positioning of these binding sites reflects an ability of the regulatory protein to either down or up regulate translation rather than to switch it on or off (Gray and Hentze, 1994).

Overall, the diversity with which the 5'UTR can regulate translation is evident leading to roles in enhancing, reducing or even eliminating an element(s) of the translational regulatory machinery depending on the sequence length, position or structure. These aspects of translation control should be taken into consideration in this investigation as alternative pathways of enhancing gene expression. The HSV-1 sequences studied in this investigation have been inserted into the 5'UTR of the CAT reporter gene and this range of possible routes of translational control makes it difficult to determine a possible effect of these sequences with regards to their position within the 5'UTR. The increase in length could be a possible factor for the increase in CAT gene activation as this is a common factor between all of the constructs containing HSV-1 sequences. However, the exception here is that a sequence of similar length that was inserted into the 5'UTR of the CAT gene as a non-HSV-1 control sequence failed to activate CAT gene expression. Analysis of this RNA did not reveal an inhibitory structure and there was no change in sequence length, however the nucleotide content was severely deficient in G residues.

CHAPTER 5: Enhanced CAT gene expression, during HSV-1 infection, is at the level of translation.

5.1 Introduction

ICP27 is known to act at both transcriptional and post-transcriptional levels (as previously discussed). When ICP27-binding sequences were present, CAT production, from a transiently transfected reporter construct was enhanced during wt HSV-1 infection and was not enhanced during HSV-1 ICP27 mutant infections.

During M15 mutant virus infection there is a disruption of the transcriptional transactivational properties of ICP27 and also a decrease in the expression of several L-2 genes e.g. gC. The lack of enhanced CAT production, as observed during the investigation presented here, may be due to a disturbance in this transactivational role of ICP27. However, the ability of M15 ICP27 to shuttle in and out of the nucleus is also abolished and this may have an additional affect on gene expression in this assay. Therefore, it is possible that the altered expression of CAT in M15 infections could be either transcriptional or post-transcriptional.

The RGG box deletion within ICP27 (*d*4-5) severely reduces the binding of RNA transcripts with the Y3-H assay. The disruption of the ability of ICP27 to bind RNA may be the reason no enhancement of the CAT gene expression was observed during the *d*4-5 infections investigated here. Furthermore, the transcriptional activity of this mutant protein has not been investigated.

To identify whether enhanced gene expression occurred at the level of transcription RNA quantification and the comparison of RNA levels was undertaken.

During the previous transfection/infection assays discussed in chapter 5, transfections/infections were performed in duplicate in order to isolate RNA using the TRIZOL method of extraction. Each RNA sample was DNase treated and the amount of total RNA was quantified. RNA was added to the Omniscript mastermix and reverse transcription was carried out (2.2.2.7) to generate cDNA. It is in this state that the amounts of CAT transcripts were compared using real time quantitative PCR (2.2.2.8).

5.2 Introduction to Real-Time Quantitative PCR

Real-time PCR allows the detection and therefore the monitoring of the progress of the PCR as it occurs. Measuring the kinetics throughout the PCR process allows the reactions to be characterised, specifically during the early phase of the reaction i.e. the time-point during amplification when the target is first detected. The point at which the target DNA is first detected is during the exponential phase. During this phase there is an exact doubling of the product DNA at every cycle, thus, the reaction is specific and precise making the exponential phase the optimal point for analysing data.

In order to quantify the PCR product during this phase the PCR is carried out in the presence of SYBR Green I dye, a highly specific, double-stranded DNA binding dye. When SYBR Green I dye is added to the PCR reaction it immediately binds to all double-stranded DNA and the intensity of the fluorescent emissions increases. As the reaction proceeds the SYBR Green I dye binds to each new copy of double-stranded DNA sequence, thus, as progression through the exponential phase of the PCR is made, an increase in the fluorescence intensity will be proportional to the amount of product produced.

A major problem using the SYBR Green I dye is that it binds to all double-stranded (ds) DNA. The dye could bind to an unwanted dsDNA product and proceed to give a high intensity of fluorescence and therefore a false reading of the intensity of the dye bound to the desired DNA product. To ensure this problem is overcome it is important

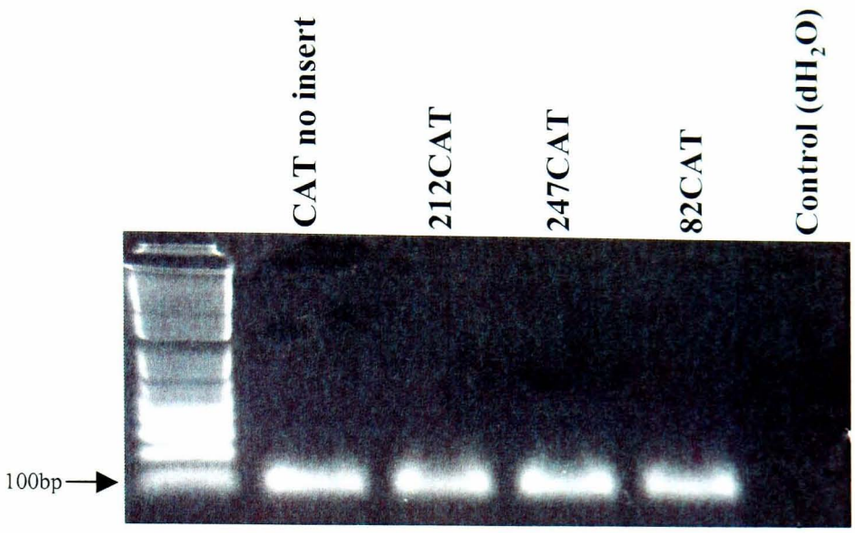

Figure 5.1: Q-PCR products were analysed by agarose gel electrophoresis. **Lane 1**: DNA marker ladder; **Lane 2-5**: cDNA samples; **Lane 6**: sample control (dH_2O).

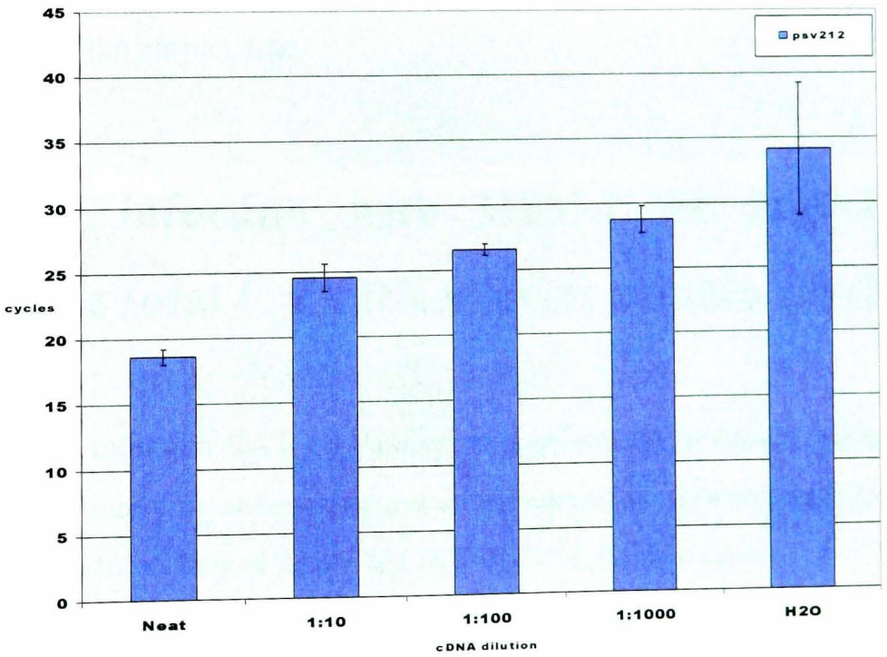

Figure 5.2: Number of cycles reached when the cDNA of sample 212CAT was serially diluted and Q-PCR performed.

to make certain only one product is synthesised. Initial experiments to identify the products being synthesised, and therefore quantified, were performed. RT was carried out on 4 different RNA extraction samples, plus a control sample (dH_2O), and 1µl was added to a PCR reaction. After cycling ended, each cDNA sample was run on an agarose gel alongside a DNA marker. Each sample collected after Q-RT-PCR indeed had only one product of the correct size (104bp).

PCR was then carried out on serial dilutions of each cDNA sample. The dilutions were: neat, 1:10, 1:100 and 1:1000. The Q-RT-PCR, performed in triplicate, gave an approximately linear response to each dilution indicating the synthesis of only one product, and that pipetting techniques were accurate. During Q-RT-PCR with the control sample (no cDNA), no product was observed until cycles 30-40, strongly indicating that no primer-dimers are formed and furthermore there was no contamination between samples.

In summary, these control/preliminary experiments confirmed the specificity of the primers giving no by products, no primer-dimer and resulting in a product of the target gene area of the correct size.

5.3 During infection with HSV-1 wt and ICP27 mutant virus total CAT RNA levels remain similar.

During wt HSV-1 infection CAT expression was enhanced, however, during ICP27 mutant virus infections this enhancement was not observed. It was possible that this may be reflected in the different levels of CAT RNA in the two cases.

BHK cells were transfected with psvCAT, psv212, psv247 or psv82 and subsequently infected with wt HSV-1, *d*4-5 or M15 virus, cells were harvested and RNA extracted. Using a 1:100 dilution of RNA, real time-RT-PCR was performed, in triplicate, to determine RNA levels of each sample. No significant differences were found in CAT specific RNA levels between cells infected with wt HSV-1, *d*4-5 or M15 (Figure 5.3). Unfortunately, cycle numbers reached during this assay were too low to give an

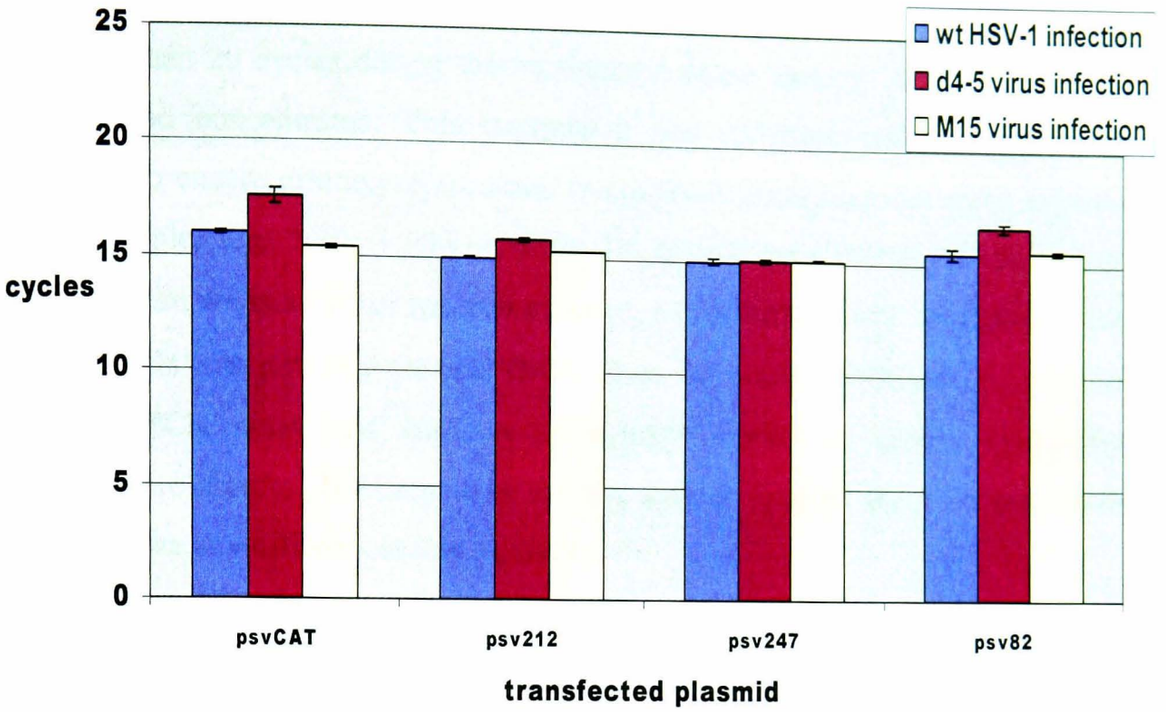

Figure 5.3: CAT RNA levels reached during transfection of psvCAT plasmids, with and without ICP27-binding sequences, during wt HSV-1, *d*4-5 or M15 infection (n=3).

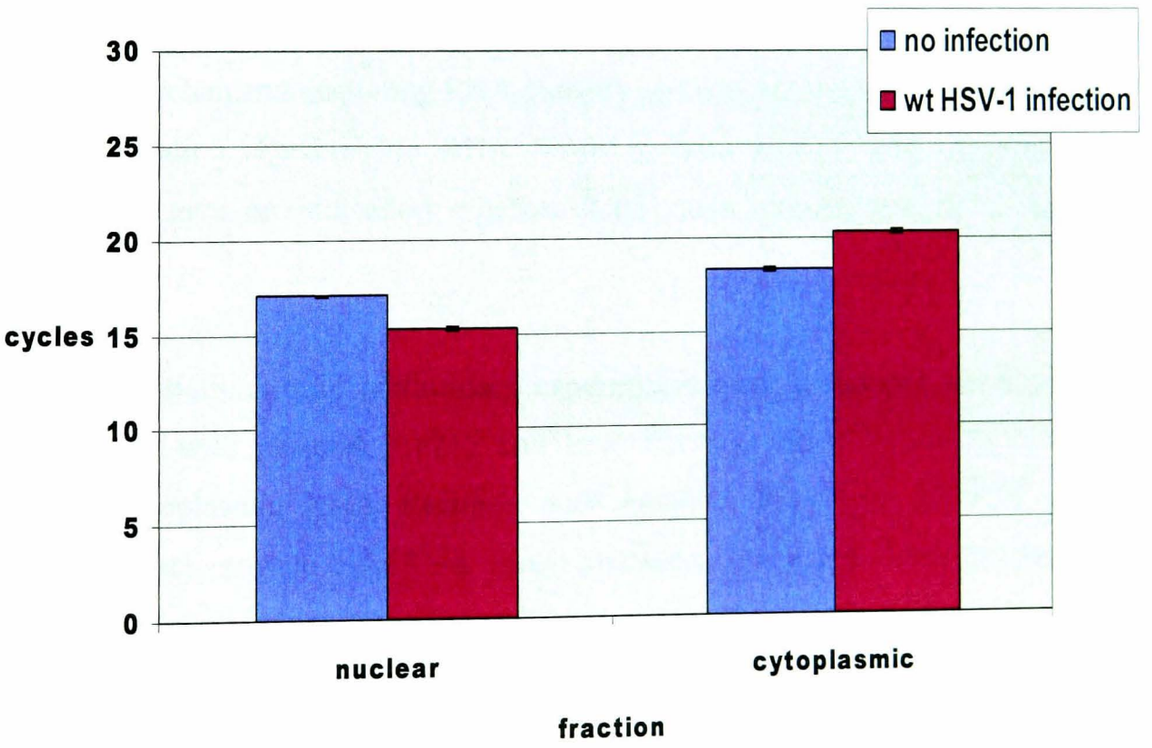

Figure 5.4: RNA levels analysis after Q-RT-PCR using RNA extracted from the nucleus and the cytoplasm of psv212 transfected cells with or without wt HSV-1 infection (n=3).

accurate interpretation of these results. That is, according to the preliminary data using the serial dilution, cycles of around 25 should have been reached. Any sample giving less than 20 cycles during the exponential phase strongly indicates the RNA sample is too concentrated. This suggests a few technical problems should be addressed. To ensure dilution is accurate, future work should include three dilutions of each sample, e.g. 1:10, 1:100, 1:1000. To ensure transfection efficiencies are similar between wells a further reporter plasmid, e.g. γ-actin, should be co-transfected into BHK cells with psvCAT plasmid DNA. Thus, the γ-actin RNA can be quantified during the PCR assay and used as an internal control to ensure transfection efficiencies are similar. If efficiencies are not similar original RNA stocks can be adjusted to take any differences into account.

5.4 Q-PCR analysis of nuclear and cytoplasmic extracted RNA fractions

ICP27 is known to act at the post-transcriptional level leading to an increase in gene expression by mechanisms including RNA stability and export, and has been linked to a role in translation. Quantifying RNA levels in both nuclear and cytoplasmic fractions would give an indication whether ICP27 acts transcriptionally or post-transcriptionally.

Due to time restrictions only preliminary experiments were performed. BHK cells were transfected with plasmid psv212 and later mock or HSV-1 infected before nuclear and cytoplasmic RNA fractions were isolated. Real time-RT-PCR was performed for each sample and RNA levels analysed. There was little difference observed in the levels of nuclear CAT RNA, extracted from cells transfected with psv212, whether wt HSV-1 infection had occurred or not (Figure 5.4). The 2 cycle increase in nuclear RNA levels does not account for the 9-fold increase in CAT expression when psv212 was transfected and infected with wt HSV-1, as compared to no infection.

There was a 2 cycle decrease in cytoplasmic RNA levels from psv212 transfections during HSV-1 infection, as compared to no infection. The levels of cytoplasmic RNA remained relatively constant regardless of whether an increase in gene expression had occurred, indicating again that RNA stability is not affected during wt HSV-1 infection. Again, these results could not be accurately analysed, as the cycle numbers reached was less than 20, thus indicating too much RNA in the original sample. Future experiments would include the use of PCR standards to allow RNA quantification. This would be highly beneficial as it would give an accurate analysis of amounts of CAT RNA transcribed. Also, co-transfection of the psvCAT constructs with a reporter plasmid into BHK cells would ensure transfection efficiencies were similar.

5.5 Discussion

The ability of ICP27 to transactivate transcription is well documented where ICP27 has been shown to transactivate viral promoters including gB, ICP5 and gC, albeit most cases required the co-expression of ICP4 and ICP0 (Everett, 1986; Rice and Knipe, 1988; Rice and Lam, 1994; Rice *et al.*, 1993; Sekulovich *et al.*, 1988). ICP27, however, was shown to have the ability to transactivate the gB promoter on its own (Rice and Knipe, 1990). Deletion analysis of ICP27 revealed that the C-terminal domain (between amino acids 406 and 512) of the ICP27 protein was involved in the transactivation (Rice *et al.*, 1989). The ICP27 mutant plasmid M15 has a two amino acid mutation within this activation domain (PG465,466LE). Co-transfection of the M15 plasmid with the gBCAT plasmid (as used in the Rice, 1990, investigation) did not stimulate the expression of CAT, indicating a disruption in the transactivation activity of ICP27. The M15 virus was constructed and during infection it was observed that the gC L-2 protein was not produced. Upon further inspection it was revealed that the inability to express gC was at the mRNA level, as demonstrated by northern blot analysis. However this was only shown for RNA levels detected from the cytoplasmic fraction of M15 infected cells thus the requirement of ICP27 for the transactivation of transcription of gC was only postulated (Smith et al., 1992). Other investigations have described the need for ICP27 during late gene expression and

showed a decrease or lack of mRNA of certain viral late genes during ICP27 mutant virus infections (Soliman et al., 1997). However, the effect of ICP27 on viral DNA synthesis, during these infections, was shown to play a key role in the reduction in transcription (McCarthy *et al.*, 1989; Smith *et al.*, 1992). One ICP27 mutant virus with a deletion at the C-terminal domain, n504, was shown to have an effect on late gene expression yet DNA replication levels remained normal (Rice and Knipe, 1990). This virus was later shown to promote the transcription of two L-2 genes, gC and UL47, as established by in vivo RNA labelling assays (Jean *et al.*, 2001).

To attempt to determine if ICP27 affects the rates of transcription, when ICP27-binding sequences are present, real time-RT-PCR experiments were performed to establish the RNA levels within the cell during infections with wt HSV-1 or ICP27 mutant viruses.

After transfecting psvCAT plasmids into BHK cells, total RNA levels did not alter significantly when the cells were infected with wt HSV-1, *d*4-5 or M15 viruses. However, the analysis was inaccurate as the concentration of RNA in the original samples was too high. Thus during real time PCR, the samples were overloaded and cycles numbers reached were too low. This was unfortunate as a further dilution of the samples would most likely have given at least some preliminary data. If further dilution of these RNA samples (by 1:10,1:100 and 1:1000), revealed no difference in RNA levels it would strongly indicate that transcription is not affected during the psv transfection assays. This would lead to a hypothesis for a post-transcriptional role for ICP27 in the assays conducted.

ICP27 has been reported to regulate gene expression post-transcriptionally by acting at various levels including splicing, polyadenylation, export and translation. Further real time-RT-PCR analysis was carried out to determine the levels of RNA within the nucleus or cytoplasm of non-infected and infected cells, to give an indication of the level ICP27 is acting at. Due to technical difficulties, these results could not be successfully analysed. However, if RNA levels did not change between samples this would indicate that the changes in CAT gene expression during mock and wt HSV-1 infection was not due to an effect at the level of export, and is therefore unlikely to be at the level of splicing and polyadenylation. This would lead to a hypothesis that

ICP27 is acting at the level of translation. Conversely, ICP27 could be functioning at the level of RNA export and would be identified by a change in levels of RNA between nuclear and cytoplasmic fractions. ICP27 is a known shuttling protein with a role in RNA export, aiding in the increase of viral gene expression, thus this hypothesis could be valid.

Unfortunately, due to time restriction, nuclear and cytoplasmic extracts were not isolated for cells that were infected with the ICP27 mutant viruses and these experiments should be performed to include the transfection of all the psvCAT clones and infections should also include *d*4-5 and M15 to further determine whether ICP27 has a role in export in these assays. These technical problems make it very difficult to link ICP27 to an effect on translation here; however, it has previously been linked to a role in translation. ICP27 associates with polysomes during infection and furthermore the protein has been shown to directly enhance mRNA, using a tethered function assay in Xenopus oocytes. Briefly, this assay involves injecting oocytes with two different RNA transcripts, one containing a luciferase reporter gene, binding sites for the bacteriophage MS2 coat protein, and the other a MS2/ICP27 fusion. Interaction of the MS2 coat protein with its binding site would therefore bring ICP27 into close proximity to the RNA and gene expression was analysed by carrying out luciferase assays on cell lysates. MS2/ICP27 was shown to increase luciferase gene expression, whereas, MS2/M15 or MS2/*d*4-5 abolished this increase in gene expression. Thus wt ICP27 directly stimulated the translation of mRNAs to which it was bound (Larralde *et al.*, 2006). ICP27 has also been implicated in regulating translation at the initiation step through its interaction with eIF3 and eIF4G (Fontaine-Rodriguez *et al.*, 2004). In the study presented here, the change in CAT expression levels could be due to an ICP27 mediated effect on translation. To determine whether ICP27 has an effect on translation of the CAT gene during infection further work should be carried out.

Previous work investigating the expression of the VP16 gene, which is transcribed yet protein synthesis is severely reduced during an ICP27-null virus infection, revealed that the mRNA distribution changes from the actively translating large polyribosomal subunit during wt HSV-1 infection to the 40S ribosomal subunit during ICP27-null virus infection. VP16 expressed during a wt HSV-1 infection was shown to be associated with actively translating complexes by treating the infected cells with

puromycin, which causes ribosomes to disrupt, thus releasing the transcripts from the ribosome. Another method to confirm this association is to disrupt the ribosome using EDTA. A shift in wt HSV-1 VP16 to the top of the sucrose gradient was shown using both ribosomal disruption methods (Ellison *et al.*, 2005). This data provided strong evidence that ICP27 is required for translation of VP16 mRNA and is of interest in the investigation presented here, as sequences #334 is located within VP16 gene. Further fractionation assays have shown that ICP27 associates with the 80S ribosome and polyribosome fraction and, furthermore, M15 ICP27 was found to be absent from the polyribosome fraction (Larralde *et al.*, 2006). Both of the above investigations should be considered when furthering the investigation presented here.

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Future work would entail an investigation into the ribosomal distribution of the CAT transcripts during mock, wt HSV-1 or ICP27 mutant virus infected cells. Polysomes would be fractionated into polyribosomes, monoribosomes and uncomplexed ribosomal subunits by sucrose gradients. RNA should be extracted from each fraction and then analysed on northern blots to determine the distribution of the CAT mRNA across the gradient. UV absorbance profiles would distinguish the position of the polysomes in each fraction would then be correlated with the northern blot results to determine where the CAT transcript is located during mock, wt HSV-1 or ICP27 mutant virus infections. A change in the distribution of the CAT transcript during wt HSV-1 of ICP27 mutant virus infection would strongly indicate a role for ICP27 in the translation of these transcripts.

Conclusion

The results obtained in the study presented here are discussed in the relevant chapters, therefore this section will summarise the main findings of the investigations.

ICP27 is an RNA binding protein that has the ability to shuttle in and out of the nucleus thus a hypothesis was born suggesting that these two properties of ICP27 were responsible for an increase in viral gene expression. This is believed to be the property of ICP27 that contributes to the switch from early gene to late gene expression during a wt HSV-1 infection. However, with recent reports connecting ICP27 to the translation machinery it was postulated that ICP27 might play a further role to promote this 'switch'. With these reports it became fundamentally important to investigate RNAs that bind to ICP27, to identify any functional properties of these sequences when ICP27 is present, which would further the understanding of this multifunctional protein.

Presented here are the results of the first investigations and analyses into the properties of ICP27-binding HSV-1 sequences and ICP27's role in gene expression when these sequences are present. A CAT assay was developed which enabled measurement of activity in cells transfected with a CAT reporter plasmid when an ICP27-binding sequence was inserted into the 5'UTR. This system was used to demonstrate that CAT activity increased when these sequences were present, in an orientation independent manner. Sequence analysis did not identify a consensus 'activation code' or RNA structure that could be responsible for this increase in expression. It was also of interest to note that no viral proteins were present in this assay, which indicated cellular proteins were possibly responsible for this increase in CAT expression when ICP27-binding sequences were present. It would be of interest to identify these cellular proteins and investigate any possible similarities they have to ICP27.

CAT activity was further enhanced following wt HSV-1 infection. This was shown to be ICP27-dependent as there was little to no enhanced CAT activation upon infection with ICP27-null or mutant viruses. However, ICP27 alone was insufficient to enhance

CAT activation, strongly indicating ICP27's requirement for other viral proteins. This is most likely, as ICP27 has, on many occasions, been reported to require both ICP0 and ICP4 for its transcriptional function. Further investigations to identify the viral protein(s) in this reaction should be pursued.

The introduction of a non HSV-1 sequence in the reverse orientation into the 5'UTR of the CAT reporter plasmid resulted in the identification of a possible sequence requirement for enhanced activation during wt HSV-1 infection. A sequence with a high G content was necessary to elicit enhanced activity and this was confirmed when using the CAT reporter plasmid with homopolymer sequences as 5'UTR inserts. Interestingly, ICP27 was previously known to bind poly (G) homoploymers suggesting that ICP27 binds to G-rich sequences and aids to increase their expression. HSV-1 sequences are 68% GC-rich making an RNA-binding protein that binds to G-rich sequences and enhances gene expression beneficial, if not crucial, to the virus. The level at which ICP27 aids gene expression was investigated by quantifying CAT RNA in the nucleus and cytoplasm of uninfected and wt HSV-1 infected cells. It is hypothesised that ICP27 binds HSV-1 G-rich sequences to aid in their translation. This is plausible, as it has been demonstrated that ICP27 can directly enhance translation and has also been shown to be present at active translation sites. Furthermore, ICP27 interacts with two translation initiation factors as well as PABP. Further investigations into the involvement of this important viral protein at the level of translation should ultimately lead to the discovery of the mechanism(s) involved, possibly at the initiation stage, and it is this information that may lead to human intervention of the HSV-1 infection.

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