UNIVERSITY OF ALBERTA

CHARACTERIZATION OF THE EFFECTS OF MUTATION OF THE ICP27 PROTEIN ON HSV-1 GENE EXPRESSION

by

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LIST OF ABBREVIATIONS

C	Carboxy
cdc	Cyclin dependent kinase
CMV	Cytomegalovirus
CTD	Carboxy-terminal domain
E	Early
EBV	Epstein-Barr virus
EMCV	Encephalomyocarditis virus
$\gamma 1$	Leaky-late
$\gamma 2$	True-late
g	Glycoprotein
HCF	Host cell factor
HCMV	Cytomegalovirus
HHV-6	Human herpesvirus 6
HHV-7	Human herpesvirus 7
HHV-8	Human herpesvirus 8
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
Hve	Herpesvirus entry mediator
ICP	Infected cell polypeptide
IE	Immediate-early
IFN-α	Alpha interferon
IFN-β	Beta interferon
kb	Kilobases
kDa	Kilodalton
KSHV	Kaposi's sarcoma-associated herpesvirus
L	Late
LMB	Leptomycin B
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
N	Amino
ND10	Nuclear domain 10
NES	Nuclear export signal
NLS	Nuclear localization signal
nt	Nucleotides
Oct-1	Octamer transcription factor 1
PAA	Phosphonoacetic acid
PABP	Poly (A) binding protein
PML	Promyclocytic leukemia protein
RRE	Rev Response Element
RNAP II	RNA polymerase II
TAF	TBP-associated factor

TBP	TATA-binding protein
TNF	Tumour necrosis factor
ts	Temperature-sensitive
UV	Ultra-violet
VP16	Virion protein 16
VZV	Varicella-zoster virus

Chapter 1: Introduction

1.1 Introduction to Herpesviruses

Herpes simplex viruses type 1 and 2 (HSV-1 and HSV-2) are common human pathogens that cause infections manifested as dermatologic disorders, notably causing visible lesions on skin or mucosal epithelium that appear as blisters. Infection by HSV-1 has been recorded as early as ancient Greek times. Hippocrates described herpes simplex lesions in the 5th century B.C and the name herpes is derived from the Greek word 'herpein' – to creep or crawl – in reference to the spreading nature of the skin lesions. Herpesviruses are large, double-stranded linear, DNA viruses with an icosahedral nucleocapsid approximately 100-110nm in diameter. The nucleocapsid is surrounded by a protein layer referred to as the tegument, and a spherical viral envelope. Viral glycoproteins appear as spikes on the envelope and are evenly distributed across the surface.

The herpesviruses are distinguished by their biological properties; such as site of replication and assembly, host-range, and establishment of latency. The current classification defines three subfamilies within the herpesviridae family: alpha-, beta- and gammaherpesvirinae.

1.1.2 Alphaherpesvirinae

The alphaherpesvirinae subfamily includes HSV-1 and HSV-2, varicella-zoster virus (VZV- the causative agent for chickenpox and shingles), pseudorabies virus (PRV), and equinine herpesvirus type 1 (EHV-1). Alphaherpesvirinae are characterized by a broad and variable host range, short reproductive cycle, rapid spread in cell culture, destruction of infected cells and the capacity to establish a latent infection in sensory ganglia (Roizman 1996) (Roizman and Sears 1996) (Mettenleiter 1994).

1.1.3 Betaherpesvirinae

The Betaherpesviruses include human cytomegalovirus (HCMV) and human herpesviruses 6 and 7 (HHV-6 and HHV-7). The Betaherpesvirinae are characterized by a restricted host range, long reproductive cycle, enlargement of infected cells (cytomegalia), as well as the capacity to establish a latent infection in secretory glands, lymphoreticular cells, and kidney (Roizman 1996) (Mocarski and Kemble 1996) (Roizman and Sears 1996).

1.1.4 Gammaherpesvirinae

The Gammaherpesvirinae subfamily consists of viruses with a narrow host range, infecting specifically B or T cells (Roizman 1996). Examples of viruses in this subfamily are Epstein Barr virus (EBV), herpesvirus saimiri (HVS) and human herpesvirus 8 (HHV-8).

1.2 General Epidemiology and Pathology of HSV-1

Primary HSV-1 infection occurs when the virus encounters a mucosal surface or skin abrasion. This occurs most frequently at an oral-facial site for HSV-1 and genitally for HSV-2, although HSV-1 has been shown to produce genital lesions (Corey and Spear 1986). The course of primary infection and reactivation is generally similar for HSV-1 and HSV-2. However, the onset of primary infection differs as HSV-1 primary infection usually occurs in early childhood, while HSV-2 has a later onset corresponding to the onset of sexual activity (Corey 1994). Viral replication occurs at the site of infection resulting in tissue damage that manifests as vesicular lesions, the hallmark of herpesvirus infection. The virus is transported to nearby sensory ganglia, where it establishes a latent infection (Stevens 1989). HSV-1 latency is characterized by lack of viral DNA replication and production of progeny virus, with only limited gene transcription. During latency the HSV-1 genome remains in the nucleus of the neuron as circular extra-chromosomal DNA (Rock and Fraser 1985) (Mellerick and Fraser 1987). This state of quiescence allows the virus a permanent residence through out the life span of the host. Periodically, HSV-1 may reactivate from its latent state. Upon reactivation, virus particles travel along sensory neurons to mucosal sites to re-initiate a lytic replication cycle. This results in recurrent disease episodes. Symptomatic or asymptomatic shedding of HSV-1 at mucocutaneous sites allows for transmission to new hosts (Koelle and Wald 2000) (Mertz, Coombs et al. 1988). Episodic reactivations normally involve a much more limited extent of affected tissues and recovery times are shorter than with primary infection. The signals and mechanisms involved in reactivation are poorly understood but this phenomenon has been associated with damage to trigeminal ganglia, UV light, stress, illness, menses, lactation, malnutrition, and extensive fatigue (Steiner and Kennedy 1993) (Steiner 1996).

While there is little evidence that the host immune response is involved in promoting the latent state, the immune response is known to be important in determining whether the virus from the reactivation of a neuron will produce an infectious lesion. Cytotoxic T lymphocytes (CTLs) are involved in controlling HSV-1 infection. As such, HSV has evolved several mechanisms to avoid this immune response. HSV-1 infected cells can avoid CTL detection by interfering with the transporter associated with antigen processing (TAP) and shutting down the TAP-dependent transport of viral peptides into the endoplasmic reticulum, impairing the assembly of MHC class I molecules (Koelle, Tigges et al. 1993; Fruh K and Y. 1995; Hill, Jugovic et al. 1995). HSV-1 gene products have also been shown to inhibit CTL-induced apoptosis of infected cells (Jerome KR 1998; Jerome KR 2001). Additionally, HSV-1 infected fibroblasts have been shown to inactivate CTLs thereby reducing the cytotoxic effector function of the CTLs through the involvement of US3, an HSV-1 viral protein kinase (Sloan DD 2003). The immune competence or ability of the host to limit viral replication and spread is more of a determining factor in the frequency of reactivation than the viral strain with which the host is infected (Meignier, Martin et al. 1990).

Although, HSV-1infections are most commonly associated with mucosal lesions (oral or genital), HSV-1 can also infect a wide range of organs and tissues. HSV-1 is a major cause of blindness (Walpita, Darougar et al. 1985), can cause facial paralysis, and in rare cases acute encephalitis (Corey and Spear 1986). HSV-1 infection in newborns or immunocompromised individuals can result in severely disseminated disease (Kimberlin, Lin et al. 2001).

Studies have shown that world- wide approximately 50 - 85% of adults are seropositive for HSV-1 (Pebody, Andrews et al. 2004). HSV-2 is less prevalent than HSV-1 with 4 - 24% of European adults seropositive.

1.3 HSV-1 virions

A mature HSV-1 particle contains four structural elements, the virion core, the nucleocaspid, the tegument layer and the envelope. The virion core contains the single, double-stranded, linear DNA genome of approximately 150 kilobase pairs (Kb) (Becker, Dym et al. 1968) (Kieff, Bachenheimer et al. 1971). The double-stranded DNA genome is associated with protein within the core (Zhou, Chen et al. 1999). The precise arrangement of DNA is unknown. However, the ends of the genome probably share some proximity as a small fraction of the packaged DNA appears circular (Poffenberger and Roizman 1985). HSV-1 contains 68% G+C and HSV-2 contains 69% G+C (Kieff, Bachenheimer et al. 1971).

The capsid is composed of 162 capsomers (Wildy, Russell et al. 1960) arranged in icosahedral symmetry. Herpesviridae capsids show very little variation in size and are approximately 100-110 nm in diameter. HSV-1 capsids contain 7 proteins: VP5, VP19C, VP21, VP22a, VP23, VP24, and VP26 (Tatman, Preston et al. 1994). Four of these proteins compose the outer shell of the capsid (VP5, VP19C, VP23, VP26), while VP21 and VP22a are thought to act as scaffolding proteins. VP21/VP24 has protease activity, which cleaves autoproteolytically to produce VP21 and VP24, as well as to cleave preVP22a into VP22a (Gibson and Roizman 1974) (Liu and Roizman 1991) (Newcomb and Brown 1989) (Newcomb and Brown 1991). VP19C binds DNA and may act to attach viral DNA to the capsid (Braun, Batterson et al. 1984).

The HSV-1 tegument is a structured protein layer found between the envelope and the nucleocapsid. It contains multiple proteins including VP16, VP11/12 (UL46), VP13/14 (UL47), vhs (UL41), US11 and VP1/2 (Read and Frenkel 1983) (Batterson and Roizman 1983). The formation of tegument is still a poorly understood process. Tegument proteins may be capable of self-assembly as the over-expression of VP16 and VP22 causes the formation of novel protein structures or 'tegument bodies' in the absence of other HSV-1 proteins (Elliott, Mouzakitis et al. 1995). Additionally, formation of the tegument

does not seem to depend on the presence of a capsid or envelope (McLaughlin, Addison et al. 1992).

The viral envelope is a membrane lipid bilayer required for virus infectivity. Acquired from the cell during viral egress, the envelope contains multiple viral proteins, 10 of which are glycosylated. These glycoproteins are gB, gC, gD, gE, gG, gH, gI, gK, gL, and gM. These glycoproteins appear as spikes on the envelope and are non-randomly distributed (Stannard, Fuller et al. 1987). It is unclear how HSV-1 glycoproteins are specifically directed to the envelope but many have been shown to interact with tegument proteins such as VP16 (Zhu and Courtney 1994) (Johnson, Wittels et al. 1984) (Reynolds, Ryckman et al. 2001), suggesting a possible method for their organization. These glycoproteins mediate attachment to the host cell and interact with immune system components (Johnson, Frame et al. 1988) (Friedman, Cohen et al. 1984) (Johnson and Feenstra 1987).

1.4 The HSV-1 genome

The dsDNA genome of HSV-1 is about 150kb in size and consists of two segments of unique DNA. These segments, called unique long (UL) and unique short (US) regions, are flanked by inverted repeats (RL and Rs). The UL region is approximately 108kb in size and contains 58 genes. The US region is 13 kb and contains 14 known genes (Roizman and Knipe 2001). A short 'a' sequence is present at the junction of UL and US, as well as both termini of the genome. The structure of the 'a' sequence is highly conserved, consisting of a variable number of repeats (Locker and Frenkel 1979) (Mocarski and Roizman 1981) (Wadsworth, Jacob et al. 1975) (Roizman 1979). The 'a' region is an essential cis-acting element required for cleavage and packaging of viral DNA and isomerization of the viral genome (Deiss, Chou et al. 1986) (Dutch, Bruckner et al. 1991) (Smiley, Duncan et al. 1990) (Vlazny, Kwong et al. 1982) (Varmuza and

Smiley 1985) (Nasseri and Mocarski 1988) (Sarisky and Weber 1994) (Smiley, Lavery et al. 1992). The UL and US regions of the genome can invert relative to one another, to yield four linear isomers. DNA from wild-type virus infected cells contains equimolar concentrations of the four predicted isomers (Delius and Clements 1976) (Hayward, Jacob et al. 1975).

HSV-1 genes are named based on their location in the genome (e.g., US11, UL30). The gene products are identified by multiple naming schemes for example, the UL48 gene of HSV-1 encodes one protein with several designations: VP16 (virion protein 16), alpha TIF (alpha gene trans-inducing factor), ICP25 (infected cell protein 25) and VMW65 (virion protein molecular weight 65 kDa).

1.5 HSV-1 Lytic cycle

1.5.1 Attachment and Entry

HSV-1 entry into cells begins with the attachment of the virion to the cell surface. This attachment is mediated through an interaction of gC and/or gB with heparin sulphate proteoglycans (Shieh, WuDunn et al. 1992) (WuDunn and Spear 1989). Following cellular attachment, gD can interact with one of several cell surface proteins. The first identified of these proteins is a member of the tumor necrosis factor (TNF) receptor family – Herpesvirus entry mediator (HVEM) (Montgomery, Warner et al. 1996) (Whitbeck, Peng et al. 1997) renamed HveA (herpesvirus entry mediator A) upon the discovery of a second receptor (Warner, Geraghty et al. 1998). Other receptors for gD binding include HveB (Warner, Geraghty et al. 1998), HveC (Geraghty, Krummenacher et al. 1998) and 3-0-sulphated heparin sulphate (Shukla, Liu et al. 1999). The third and final step in HSV-1 entry is the fusion of the viral envelope with the plasma membrane of the cell (Morgan, Rose et al. 1968). Virus-cell fusion requires gB, gD, and gH-gL

hetero-oligomers (Ligas and Johnson 1988) (Forrester, Farrell et al. 1992) (Sarmiento, Haffey et al. 1979) (Little, Jofre et al. 1981). HSV-1 capsids have been shown to bind dynein, a microtubule motor protein through UL34 (Sodeik, Ebersold et al. 1997) and it is thought that this interaction promotes the transport of viral capsids to the nucleus.

1.5.2 HSV-1 Gene Expression

During the lytic cycle, HSV-1 gene expression proceeds in a tightly regulated temporal cascade (Roizman and Furlong 1974) (Roizman, Kozak et al. 1975). Based on their temporal expression, HSV-1 genes are classified into three kinetic classes, immediate early (IE) genes, early (E) genes and late (L) genes. IE genes are expressed first and are defined as genes whose expression occurs in the absence of de novo protein synthesis. IE genes regulate the expression of the E genes that encode metabolic and regulatory proteins, as well as DNA replication machinery. Some of these E genes are required for viral DNA replication. Viral DNA replication stimulates the expression of the final class of genes, the late (L) genes, which encode viral structural proteins. HSV-1 L genes can be further divided into true late (γ1) and leaky late (γ2) based upon the stringency of their dependence on viral DNA replication for expression (Roizman and Knipe 2001).

1.6 IE gene expression

IE gene expression is activated by VP16 through a common regulatory element upstream of each IE promoter – TAATGARAT (Mackem and Roizman 1981) (Whitton and Clements 1984) (Gaffney, McLauchlan et al. 1985). VP16 binds a cellular protein called host cell factor (HCF) (Katan, Haigh et al. 1990) (Kristie, Lebowitz et al. 1989) (Stern and Herr 1991) (Xiao and Capone 1990).

This protein duplex proceeds to form a complex with a second cellular protein, Oct-1, that is bound to viral DNA (Stern, Tanaka et al. 1989) (Gerster and Roeder 1988) (O'Hare, Goding et al. 1988) (Preston, Frame et al. 1988). Binding specificity is conferred by Oct-1 (Hayes and O'Hare 1993). Five IE genes exist: ICP0, ICP4, ICP22, ICP47 and ICP27. All but one (ICP47) have important roles in regulating gene expression.

1.6.1 ICP22

ICP22 is a 68 kDa protein that is required for optimal virus replication in human embryonic lung (HEL), Rat1 and BHK cells, and is considered non-essential for viral replication in Vero cells (Sears, Halliburton et al. 1985) (Poffenberger, Raichlen et al. 1993). ICP22 is phosphorylated by UL13 and Us3 viral protein kinases (Purves and Roizman 1992) (Purves, Ogle et al. 1993), and nucleotydylated by casein kinase II (Mitchell, Blaho et al. 1997) (Blaho, Mitchell et al. 1994). ICP22 and UL13 have been shown to be required for the enhanced expression of a subset of L genes including US11, UL41, and UL38 (Ogle and Roizman 1999) (Purves, Ogle et al. 1993). ICP22 is required for inducing an altered phosphorylation of the largest subunit of RNAP II (Rice, Long et al. 1995) (Long, Leong et al. 1999). This modification correlates with the repression of host cell transcription (Spencer, Dahmus et al. 1997).

ICP22 contains two independent nuclear localization signals (Stelz, Rucker et al. 2002) and localizes to the nucleus of infected cells. Within the nucleus, ICP22 co-localizes with UL4 and UL3 in small dense nuclear bodies (Markovitz and Roizman 2000) (Jahedi, Markovitz et al. 1999). In HSV-1 infection, ICP22 and UL13 activate and stabilize cdc2 kinase (Advani, Weichselbaum et al. 2000). Cdc2 cell cycle kinase is a member of a family of cyclin-dependent serine/threonine protein kinases involved in the progression of cell cycle. Cdc2 regulates entry into M phase (King, Jackson et al. 1994). The

activity of cdc2 kinase is tightly regulated requiring an interaction with its cyclin partners A and B, as well as activation by active cdc25C (Booher, Holman et al. 1997) (McGowan and Russell 1995). Negative regulators of cdc2 are the kinases wee1 and myt1 (Wells, Watanabe et al. 1999). In HSV-1 infection, ICP22 and UL13 are required for the post-translational modification (potential activation) of cdc25C phosphatase. This modification of cdc25C phosphatase is required to dephosphorylate (activate) cdc2 (Advani, Weichselbaum et al. 2000). Additionally implicating ICP22 in cdc2 function, experiments with a dominant negative form of cdc2 exhibited a block in the expression of L gene US11, reminiscent of the requirement of ICP22 for enhanced expression of several late genes (Advani, Weichselbaum et al. 2000). Potentially suggesting a mechanism for this role in gene expression, UL42, the DNA polymerase processivity factor, has been shown to physically interact with, and be phosphorylated by, cdc2 (Advani, Weichselbaum et al. 2001).

1.6.2 ICP47

ICP47 is a small polypeptide about 12kDa in size. This protein does not have a role in gene regulation and is non-essential for replication in cell culture (Mavromara-Nazos, Ackermann et al. 1986). ICP47, however, does play a key role in the inhibition of antigen presentation to CD8+ T cells, preventing the lysis of virus infected cells by cytotoxic T-lymphocytes (CTLs) (Koelle, Tigges et al. 1993) (Posavad and Rosenthal 1992; York, Roop et al. 1994). ICP47 binds to the transporter associated with antigen processing (TAP) and shuts down the TAP-dependent transport of viral peptides into the endoplasmic reticulum, impairing the assembly of MHC class I molecules (Hill, Jugovic et al. 1995) (Fruh, Ahn et al. 1995) (Tomazin, van Schoot et al. 1998). This enables the virus to be undetectable to the immune system, and thus replicate longer in the infected cell.

1.6.3 ICP4

ICP4 an essential regulatory protein of 175kDa encoded by the RS1 gene, which is present in two copies on the viral genome (Roizman and Sears 1996). ICP4 is required for viral replication in all experimental systems and to induce transcription of most of the HSV-1 genes (Watson and Clements 1978). In the absence of ICP4, E gene expression is greatly reduced, while IE genes are over-expressed. This is thought to be at least partially due to ICP4's role in repressing the expression of several viral genes including itself (DeLuca, McCarthy et al. 1985) (Dixon and Schaffer 1980) (O'Hare and Hayward 1985). This protein contains several discrete functional domains associated with transactivation, dimerization, nuclear localization, repression and DNA binding (Samaniego, Wu et al. 1997) (Everett, Paterson et al. 1990) (Paterson and Everett 1988) (DeLuca and Schaffer 1988). ICP4 binds directly to specific DNA binding sites located near the start of transcription (Michael and Roizman 1993) (Leopardi, Michael et al. 1995). These binding sites appear to be required for repression (Koop, Duncan et al. 1993) but not transactivation (Smiley and Duncan 1992) (Gu and DeLuca 1994). The mechanism responsible for repression is not a steric block to transcription but rather involves specific ICP4 interactions with TATA-binding protein (TBP) and TFIIB (Gu, Kuddus et al. 1995) (Kuddus, Gu et al. 1995). Specific ICP4 DNA binding sites do not appear to be required for ICP4 mediated transactivation (Smiley and Duncan 1992) (Gu and DeLuca 1994). Transactivation is mediated through the ICP4 interaction with TBP-associated factor 250 (TAF250) (Carrozza and DeLuca 1996), which mediates an interaction with TBP-containing general transcription factor TFIID (Gu and DeLuca 1994). ICP4 is modified by phosphorylation, poly (ADP)-ribosylation and nucleotidylation (Blaho, Michael et al. 1992) (Blaho and Roizman 1991) (Preston and Notarianni 1983).

1.6.4 ICP0

ICP0 is a primarily nuclear, 110KDa IE protein. ICP0 was first described as a "promiscuous" transactivator of viral gene expression due to its ability to increase expression driven by a wide variety of transfected promoters in the absence of specific promoter sequences (Cai and Schaffer 1992) (Everett 1991) (Mosca, Bednarik et al. 1987) (O'Hare and Hayward 1984) (Perry, Rixon et al. 1986) (Quinlan and Knipe 1985). ICP0 is encoded by the IE1 gene of the long unique repeat. ICP0 contains two introns, being one of the few HSV-1 genes that is spliced. ICP0 deletion mutants exhibit an unusual multiplicity of infection (MOI) dependent phenotype. Viruses with deletions in ICP0 grow poorly in cultured cells at low MOI, but at a normal rate at high MOI (Sacks and Schaffer 1987) (Stow and Stow 1986). It is thought that ICP0 influences the balance between lytic infection and latency. Infection in the absence of ICP0 (at low MOIs) results in the quiescence of viral genomes which can be reactivated by the expression of ICP0 (Cai, Aster et al. 1993) (Preston, Mabbs et al. 1997) (Samaniego, Neiderhiser et al. 1998) (Marshall, Lachmann et al. 2000).

Several key functional domains have been identified in ICP0. These include an NLS (Everett 2000), a RING finger domain (Everett 1988), a USP7 binding sequence (Everett, Meredith et al. 1997), and a sequence required to localize to ND10 bodies (Everett 1987) (Everett 1988). ICP0 has been shown to interact with multiple proteins, both viral and cellular. These include ICP4 (Yao and Schaffer 1994), translation elongation factor delta 1 (EF-δ1) (Kawaguchi, Bruni et al. 1997), cyclin D3 (Kawaguchi, Van Sant et al. 1997), transcription factor BMAL1 (Kawaguchi, Tanaka et al. 2001), and the ubiquitin-specific protease USP7 (Everett, Meredith et al. 1997). ICP0 has also been shown early in infection to co-localize with nuclear structures referred to as ND10 or

promyelocytic leukemia (PML) nuclear bodies and then to cause their disruption (Everett and Maul 1994) (Maul and Everett 1994) (Maul, Guldner et al. 1993).

ND10 are nuclear domains that contain multiple cellular proteins including the PML protein and Sp100, which are modified by the small ubiquitin like protein SUMO-1 (Muller and Dejean 1999) (Duprez, Saurin et al. 1999). Adenovirus, SV40 and HSV-1 have been shown to start their transcription and replication at ND10 sites (Maul, Ishov et al. 1996) (Ishov and Maul 1996) (Tang, Bell et al. 2000), which could suggest that ND10s present some advantage to the virus. The proteins of ND10 are interferon upregulated, have repressive properties and play a role in the host anti-viral repression system (Xu, Ahn et al. 2001).

Multiple observations link ICP0 to the ubiquitin-proteosome pathway. First, ICP0 was found to bind ubiquitin specific protease 7 (USP7) (Everett, Meredith et al. 1997) (Xu, Ahn et al. 2001). Second, MG123 a proteosome inhibitor interferes with ICP0 function (Everett, Orr et al. 1998). Third, ICP0 contains a RING finger domain that is required for its function (Everett 1988). Proteins with RING finger domains have been shown to function as E3 ubiquitin ligases (Joazeiro and Weissman 2000), which are required for the process of ubiquitination.

Ubiquitination is involved in a process of specific protein degradation. Attachment of the small protein ubiquitin to proteins, targets them for degradation by the proteosome. E1 and E2 enzymes assemble ubiquitin protein chains which are then attached to the target protein by an E3 enzyme (reviewed in Yang and Yu 2003). ICP0 has been reported to induce the accumulation of polyubiquitin chains in vitro in the presence of E1 and E2 enzymes (UbcH5a, UbcH6), and to act as an E3 ubiquitin ligase during viral infection (Boutell, Sadis et al. 2002) (Hagglund and Roizman 2002). ICP0 has been shown to induce the proteosome–dependent degradation of several proteins. These include two major components of ND10, PML and Sp100 (Chelbi-Alix and de The 1999) (Everett 2000) (Everett, Freemont et al. 1998) (Muller and Dejean 1999), a

subunit of DNA protein kinase (Parkinson, Lees-Miller et al. 1999), and two centromere proteins, CENP-C and CENP-A (Everett, Earnshaw et al. 1999) (Lomonte, Sullivan et al. 2001).

ICP0 mutants are hypersensitive to interferon (IFN) as determined by their ability to form plaques (Mossman, Saffran et al. 2000). Mossman (Mossman, Macgregor et al. 2001) showed that ICP0 mutants, unlike wild-type HSV-1, fail to accumulate viral mRNAs in IFN treated Vero cells, implying a defect in transcription, RNA processing, or stability. ICP0 appears to inhibit the induction of IFN stimulated genes in a proteosome-dependent manner by dispersing PML and to overcome an antiviral repression by IFNα and β (Harle, Sainz et al. 2002) (Mossman and Smiley 2002).

1.6.4 ICP27

ICP27 is a 63kDa, phosphorylated, predominantly nuclear and essential regulatory protein required through the productive replication cycle (Sacks, Greene et al. 1985) (Roizman and Furlong 1974). It has roles in post-transcriptional processing, transcription regulation, and nuclear mRNA export. ICP27 has also been shown to prevent apoptosis in cells infected with HSV-1 (Aubert and Blaho 1999) and to play a role in blocking cell cycle in G1 phase (Song, Yeh et al. 2001). Additionally, it has been reported to bind ICP8, brokering an association with cellular RNA polymerase II holoenzyme (Zhou and Knipe 2002), and in high concentrations to alter the localization of ICP0 and ICP4 (Zhu, DeLuca et al. 1996).

Studies with ICP27 temperature sensitive (ts) mutants and null mutants demonstrated that this protein is involved in the down regulation of viral IE and E genes and host cellular genes, and is required to up-regulate the expression of L genes. ICP27 defective mutants were shown to be unable to up regulate

expression of E and L genes (McCarthy, McMahan et al. 1989) (McGregor, Phelan et al. 1996) (Rice and Knipe 1990) (Sacks, Greene et al. 1985) (Uprichard and Knipe 1996), and to down regulate IE and E genes at late times post-infection (McCarthy, McMahan et al. 1989) (Sacks, Greene et al. 1985) (Rice and Knipe 1990). These mutants are unable to efficiently replicate their genome (McCarthy, McMahan et al. 1989) (Rice and Knipe 1990) (Uprichard and Knipe 1996). The ability of ICP27 to stimulate viral DNA replication is believed to be an indirect result of its ability to activate E gene expression (McCarthy, McMahan et al. 1989) (McGregor, Phelan et al. 1996) (Uprichard and Knipe 1996). ICP27 mutants are also defective in host-shut-off (Sacks, Greene et al. 1985).

As with many other multi-functional proteins, ICP27 contains multiple functional domains or motifs. These include elements that promote nuclear export, nuclear localization, RNA binding and a zinc-finger motif (Figure 9). These domains and their roles in ICP27 function will be reviewed thoroughly in later sections.

Numerous studies have suggested that the C-terminal half of ICP27 is critical to its function. Mutagenesis of the C-terminal portion of this protein disrupts its ability to modulate the expression of reporter genes in transfection assays (Rice, Su et al. 1989) (McMahan and Schaffer 1990) (Hardwicke, Vaughan et al. 1989). It is therefore likely that the C-terminal portion of ICP27 plays a fundamental role in one or more of its essential regulatory functions. There is, nevertheless, one report of an ICP27 frame-shift mutation that abolishes the C-terminal portion of ICP27 but remains viable in cultured cells in the presence of an extragenic compensatory mutation (Bunnell and Rice 2000). In keeping with its complex role in HSV-1 infection, ICP27 has been shown to interact with multiple cellular and viral proteins. ICP27 partners include itself (Zhi, Sciabica et al. 1999), ICP0 (Mullen, Gerstberger et al. 1995), ICP4 (Panagiotidis, Lium et al. 1997), Aly/REF (Koffa, Clements et al. 2001), hnRNPK

(Wadd, Bryant et al. 1999), snRNPs (Sandri-Goldin and Hibbard 1996), p32 (Bryant, Matthews et al. 2000), casein kinase 2 (Wadd, Bryant et al. 1999), RNA polymerase II and ICP8 (Zhou and Knipe 2002), SRPK1 and SAP145 (Bryant, Wadd et al. 2001). Discussion of several of these interactions will occur in detail in later sections.

Lytic infection with HSV-1 results in the repression of most host cell protein synthesis (Fenwick and McMenamin 1984) (Fenwick and Clark 1982) (Fenwick 1984) (Schek and Bachenheimer 1985) (Kwong and Frenkel 1987) (Kwong, Kruper et al. 1988) (Nishioka and Silverstein 1977) (Nishioka and Silverstein 1978). The inhibition of host protein synthesis is a two-phase process. Shut-off is initially triggered by the viral host shut-off (vhs) protein, which causes disaggregation of polyribosomes (Fenwick and Everett 1990) (Sydiskis and Roizman 1966) and degradation of host mRNA (Schek and Bachenheimer 1985) (Read and Frenkel 1983). A second stage of host shut-off reduces the remaining level of protein synthesis and requires viral gene expression (Kwong and Frenkel 1987) (Read and Frenkel 1983) (Fenwick and Walker 1978) (Nishioka and Silverstein 1978). ICP27 contributes to the shut-off of host cell gene expression, as temperature sensitive and deletion mutants of ICP27 are defective for host protein shut-off (Sacks, Greene et al. 1985). This shut-off phenomenon is thought to be an indirect consequence of a more direct function of ICP27, the modulation of post-transcriptional processing of mRNA (Hardwicke and Sandri-Goldin 1994). ICP27 regulates gene expression post-transcriptionally by promoting polyadenylation at weak 3' viral poly (A) sites (McGregor, Phelan et al. 1996) (McLauchlan, Simpson et al. 1989) and by affecting host pre-mRNA splicing.

1.6.4.1 ICP27: Splicing and host shut-off

The role of ICP27 in HSV-1 lytic infection was deemed in the past largely that of a transcriptional regulator. Both trans-activation and trans-repression functions were attributed to this protein (Hardwicke, Vaughan et al. 1989) (McCarthy, McMahan et al. 1989) (Sekulovich, Leary et al. 1988).

A seminal work by Sandri-Goldin in 1992 (Sandri-Goldin and Mendoza 1992) was the first to suggest that ICP27's role might in fact not be transcriptional, but post-transcriptional. Transfection experiments were performed with the cloned ICP27 gene as an effector plasmid, and target plasmids consisting of HSV-1 promoter sequences fused to the chloramphenicol acetyl-transferase (CAT) gene (Sekulovich, Leary et al. 1988). Interestingly, ICP27 was shown to function as a regulator in a promoter-independent manner. Further studies revealed that the activation function correlated with weaker poly (A) sites, and the repressor function correlated with the presence of a 5' or 3' intron (Sandri-Goldin and Mendoza 1992). This led to the hypothesis that ICP27 inhibited splicing and altered the use of poly (A) sites. These observations initiated a flurry of studies that have resulted in strong support to a model in which ICP27 modulates RNA processing post-transcriptionally. However, there is recent report of ICP27 being directly required for the transcriptional activation of a specific viral L gene - gC (Jean, LeVan et al. 2001).

It has become increasingly apparent that ICP27 is involved in splicing. ICP27 has been reported to decrease the accumulation of several spliced host mRNAs (Hardwicke and Sandri-Goldin 1994), as well as two viral spliced transcripts (Hardy and Sandri-Goldin 1994), and promote the nuclear retention of intron containing transcripts (Phelan, Dunlop et al. 1996). Consistent with a role in splicing, ICP27 has been shown to cause a redistribution of splicing factors (Phelan, Carmo-Fonseca et al. 1993) (Sandri-Goldin, Hibbard et al. 1995) and co-immunoprecipitate with anti-Sm antisera (Sandri-Goldin and Hibbard 1996), indicating that ICP27 binds one or more snRNPs. The punctuated nuclear staining pattern of ICP27 is a result of the co-localization of ICP27 with snRNP

clusters (Phelan, Carmo-Fonseca et al. 1993) (Sandri-Goldin, Hibbard et al. 1995).

Studies have shown a direct interaction between ICP27 and proteins that have a role in splicing, one of which is the cellular protein p32 (Bryant, Matthews et al. 2000). p32 regulates RNA splicing by inhibiting ASF/SF2 RNA binding and phosphorylation (Petersen-Mahrt, Estmer et al. 1999). p32 has primarily a mitochondrial distribution (Matthews and Russell 1998) (Muta, Kang et al. 1997) but can also be found in the nucleus (Matthews and Russell 1998). p32 has been shown to interact with multiple proteins, both viral and cellular, including transcription factor TFIIB (Yu, Loewenstein et al. 1995), adenovirus polypeptide V (Matthews and Russell 1998), and the HIV proteins Rev and Tat (Yu, Loewenstein et al. 1995) (Luo, Yu et al. 1994) (Tange, Jensen et al. 1996). Based upon both its cellular localization and its interactions with multiple proteins it has been suggested that p32 has a role not only in splicing (Petersen-Mahrt, Estmer et al. 1999) (Luo, Yu et al. 1994) (Tange, Jensen et al. 1996) (Yu, Zhang et al. 1995), but also in nucleo-cytoplasmic transport to and from the mitochondria (Matthews and Russell 1998) (Jiang, Zhang et al. 1999).

ICP27 was found to interact with SR protein kinase I (SKPKI) (Sciabica, Dai et al. 2003) and SAP145, a splicing protein (Bryant, Wadd et al. 2001). The interaction between these proteins is thought to inhibit splicing prior to the first catalytic step (Bryant, Wadd et al. 2001). Several studies have also demonstrated by in vitro splicing assays that ICP27 causes the increased accumulation of unspliced and partially spliced viral transcripts (Sandri-Goldin and Mendoza 1992) (Lindberg and Kreivi 2002) (Bryant, Wadd et al. 2001). ICP27 therefore may interact directly with the splicing machinery of the host cell, causing an inhibition of pre-mRNA splicing.

While the global shut-off of RNA splicing is an attractive model given that the bulk of the HSV-1 genes do not contain introns, there is strong evidence to suggest that the ability of ICP27 to inhibit host RNA splicing is perhaps not an

indiscriminant phenomenon. HSV-1 encodes four genes that do contain introns. One of these is UL15, a gene that encodes an essential L protein required for packaging of DNA. The UL15 intron contains the UL16 gene, which encodes a non-essential capsid associated protein, and the UL17 gene, which encodes an essential tegument protein required for the cleavage and packaging of DNA (Roizman and Knipe 2001). This seems to preclude the possibility of a global splicing shut-off. Additionally, work done by our lab demonstrated that the cellular alpha-globin gene, normally silent in non-erythroid cells, is activated by HSV-1 infection and that ICP27 induces the cytoplasmic accumulation of unspliced and spliced alpha-globin pre-mRNA (Cheung, Ellison et al. 2000). This would be impossible if splicing was completely inhibited.

1.6.4.2 ICP27: mRNA export

The structure of HSV-1 genes poses a dilemma. How do viral intron-less RNAs access the cytoplasm? For cellular transcripts, splicing is an integral component of nuclear mRNA export. Many viruses use unique mechanisms for bypassing splicing. Simple retroviruses such as the type D retroviruses contain cis-acting RNA export elements (constitutive transport elements or CTEs) (Bear, Tan et al. 1999) that function by interacting directly with TAP (Kang and Cullen 1999) (Gruter, Tabernero et al. 1998), thus eliminating the need for REF. The HSV-1 TK gene contains multiple cis-acting elements that interact with hnRNP L, an alternate export adaptor protein (Liu and Mertz 1995). The HIV Rev protein provides one of the best-characterized examples of a viral RNA export protein (Figure 2). Rev shuttles between the nucleus and the cytoplasm (Meyer and Malim 1994) (Richard, Iacampo et al. 1994), and promotes the export of unspliced and partially spliced transcripts (Fischer, Meyer et al. 1994) by binding to a secondary RNA structure within the transcript, the rev response element (RRE) (Malim, Hauber et al. 1989). Rev contains a Nuclear export signal (NES)

consisting of a leucine rich stretch of amino acids (Fischer, Huber et al. 1995) that complexes with the cellular exportin Crm-1. Crm-1 (chromosome maintenance region 1) functions as an export receptor (Fornerod, Ohno et al. 1997) (Stade, Ford et al. 1997) and has been shown to bridge the interaction between Rev and the nuclear pore complex (Neville, Stutz et al. 1997). In uninfected cells, the Crm-1 export pathway mediates export of 5S rRNA, U snRNAs, and some cellular proteins such as kinase A inhibitor (Fischer, Huber et al. 1995) (Fridell, Bogerd et al. 1996) (Fritz and Green 1996).

ICP27 has been proposed to function as a transport factor for the splicing-independent nuclear export of viral mRNAs (Sandri-Goldin 1998) (Koffa, Clements et al. 2001) (Phelan, Dunlop et al. 1996). ICP27 proposed function in RNA export is based on several observations. First, ICP27 contains all key domains for nuclear export. It contains a strong nuclear localization signal (NLS) (Mears, Lam et al. 1995), a leucine rich nuclear export signal (NES) (Sandri-Goldin 1998) that is similar to the NES of the HIV protein Rev (Meyer and Malim 1994), and an RGG-box type RNA binding domain (Mears and Rice 1996). ICP27 may also contain a second type of RNA binding domain in addition to the RGG-box in the form of three KH-like domains (Soliman and Silverstein 2000). Second, ICP27 has been reported to shuttle between the nucleus and the cytoplasm (Mears and Rice 1998) (Sandri-Goldin 1998) (Phelan and Clements 1997) (Hibbard and Sandri-Goldin 1995), bind viral RNA (Mears and Rice 1996) (Sandri-Goldin 1998) (Brown, Nakamura et al. 1995) (Ingram, Phelan et al. 1996), and effect the nuclear export of several intronless viral transcripts (Sandri-Goldin 1998). Lastly, ICP27 interacts with heterogeneous nuclear ribonucleoprotein K (hnRNPK) (Wadd, Bryant et al. 1999). hnRNPs belong to a family of mRNA binding proteins that functionally link mRNA metabolism in the nuclear and cytoplasmic compartments. hnRNPs coat pre-mRNA in the nucleus and are involved in multiple nuclear processes, including transcriptional regulation, telomere length maintenance, Ig class switching, alternate splicing,

and processing of the 3' end of pre-mRNA (reviewed in Nakielny and Dreyfuss 1999). Members of this family of proteins accompany mRNA to the cytoplasm and are involved in mRNA localization, translation, and turnover.

As ICP27 contains the domains expected for an export factor, shuttles between the nucleus and the cytoplasm, and binds proteins involved in mRNA metabolism, it was hypothesized that ICP27 was probably involved in nuclear export. The protein organization and domain content of ICP27 is remarkably similar to HIV Rev, and by analogy to Rev, ICP27 was proposed to export viral unspliced mRNA out of the nucleus through an interaction with Crm-1 (Soliman and Silverstein 2000) (Chen, Sciabica et al. 2002) (Figure 3).

1.6.4.2.1 Leptomycin B a specific inhibitor of Crm-1

Leptomycin B (LMB) is an anti-fungal compound isolated from *Streptomyces* (Hamamoto, Gunji et al. 1983) that inhibits Crm-1-mediated nuclear export (Kudo, Matsumori et al. 1999) (Figure 2). LMB has been shown to covalently and selectively bind to the sulfhydryl group of a single cysteine residue (Cys-529) of Crm-1, causing an alteration of its three dimensional structure that leads to its inactivation (Kudo, Matsumori et al. 1999) (Figure 4).

LMB is believed to be, and is widely utilized as, a selective and specific inhibitor of Crm-1 based on several lines of study. LMB was first connected to Crm-1 in 1994 through a genetic screen in *Schizosaccharomyces pombe* that identified mutations in Crm-1 as the cause of decreased susceptibility to LMB (Nishi 1994). LMB, while toxic to *S. pombe*, has no effect on *Saccharomyces cerevisiae* (Hamamoto, Gunji et al. 1983). However, a single amino acid change (Threonine to cysteine) in Crm-1 at position 539 renders *S. cerevisae* sensitive to LMB (Neville M 1999). LMB has a similar effect, and produces a similar phenotype (i.e. a decrease in NES mediated transport,) in strains harboring this mutation as what has been observed in other systems. Additionally, LMB has

been shown to have no effect on Xenopus early embryogenesis until Crm-1 becomes functional during the gastrula-neurula transition, which coincides with a change in Crm-1 nuclear localization (Callanan, 2000). To further lend credence to the specificity of LMB as a selective inhibitor of Crm-1, Koster et al 2003 have isolated several compounds in the ratjadone family from myxobacterium strain *Sorangium cellulosum* (Koster and Kjems, 2003). These compounds inhibit the formation of Crm-1-NES complexes and have activity in the same concentration range as LMB. Based on these observations, LMB is used routinely to inhibit Crm-1 nuclear export in multiple systems. HIV Rev protein function is regularly assessed using LMB (Wolff, Sanglier et al. 1997).

1.6.4.2.2 ICP27 mRNA export via Crm-1

Studies by Soliman and Silverstein to elucidate the mechanism of ICP27 observed a decreased cytoplasmic accumulation of several viral RNAs as well as several late proteins in the presence of LMB (Soliman and Silverstein 2000). Additionally, the authors also report LMB blocked ICP27 shuttling. The authors concluded that LMB inhibited ICP27-mediated Crm-1 dependent RNA export. Interestingly, Koffa et al reported in 2001 that in contrast to the claim of Soliman and Silverstein, ICP27 neither shuttles nor exports viral mRNAs via the Crm-1 pathway (Koffa, Clements et al. 2001). They found that ICP27 bound directly to REF and recruited TAP to this nucleoprotein complex to stimulate export of viral mRNA (Figure 5). Chen et al further provided evidence that the leucine-rich NES of ICP27 does not require Crm-1 for activity (Chen, Sciabica et al. 2002). Chen confirmed the interaction between ICP27 and REF/Aly, and additionally demonstrated that the export of ICP27 was not sensitive to LMB but was sensitive to a dominant negative TAP deletion mutant. In light of this new evidence, it now appears that ICP27 promotes viral RNA export by recruiting Aly/REF to intron-less RNAs. However, there is still a great deal of controversy

surrounding ICP27 and RNA export as well as the relative importance of the NES versus the REF binding domain. As the scope of my thesis was to further examine this issue, a more detailed discussion follows in section 1.10.

1.7 E gene expression and DNA replication

IE genes encode protein products that up regulate the expression of the E genes. E genes encode the viral DNA replication machinery as well as metabolic and regulatory proteins. Seven viral E proteins are required for viral DNA replication (Challberg 1986) (Wu, Nelson et al. 1988). The seven gene products are the viral DNA polymerase (UL30), its processivity factor (UL42), a ss-DNA binding protein (ICP8), an origin binding protein (UL9), and the helicase-primase complex (UL8, UL5, UL52). The expression of UL30 and UL42 RNA and protein is examined in this study as prototypic markers of early gene expression and therefore a more detailed discussion of these gene products follows.

1.7.1 DNA polymerase (UL30)

The virally encoded DNA polymerase is required for HSV-1 DNA replication (Wu, Nelson et al. 1988). UL30 is a 136kDa polypeptide (Gibbs, Chiou et al. 1985) that shares homology with other DNA polymerases (Gibbs, Chiou et al. 1988) and contains an intrinsic 3'-5' exonuclease proofreading activity. Measurement of the transcription rates for UL30 mRNA established that pol mRNA is synthesized at a rate comparable to thymidine kinase (TK) and ICP8 mRNA (Zhang and Wagner 1987). However, UL30 protein is much less abundant than TK or ICP8, indicating regulation at the level of translation or RNA

stability (Yager and Coen 1988) (Yager, Marcy et al. 1990). The kinetics of UL30 protein synthesis are also quite different from those of TK, for which protein synthesis parallels mRNA abundance. UL30 protein synthesis peaks before mRNA levels reach a maximum and shuts-off when mRNA is still abundant (Yager, Marcy et al. 1990). UL30 mRNA accumulation, and perhaps protein synthesis, is dependent on viral DNA synthesis (Wobbe, Digard et al. 1993).

1.7.2 The processivity factor (UL42).

The HSV-1 DNA polymerase holoenzyme is a complex between the UL30 protein and the 65kDa UL42 protein (Crute, Tsurumi et al. 1989) (Gottlieb, Marcy et al. 1990). UL42 binds directly and stably to dsDNA (Marsden, Campbell et al. 1987) (Powell and Purifoy 1976), and can associate with UL30 even in the absence of DNA. The ability of UL42 to bind both UL30 and DNA is critical for its function as a processivity factor. It has been demonstrated that UL42 increases the DNA binding specificity of the polymerase and decreases the rate of dissociation from the primer-template without reducing the elongation rate (Weisshart, Chow et al. 1999).

The crystal structure of UL42 was recently solved (Zuccola, Filman et al. 2000) and UL42 was shown to be structurally similar to proliferating cell nuclear antigen (PCNA), a cellular processivity factor. PCNA has been shown to bind cdk2 and it has been proposed that PCNA may bring cdk2 together with DNA replication proteins and act as an adapter (Koundrioukoff, Jonsson et al. 2000). These observations lead to a more thorough analysis of the UL42 ORF and identified a degenerate cyclin box motif (Advani, Weichselbaum et al. 2000). Studies have shown that UL42 interacts with cdc2 cyclin dependent kinase and is phosphorylated by this kinase (Advani, Weichselbaum et al. 2001). Phosphorylation occurs in the carboxy-terminal of the UL42 protein (Advani, Weichselbaum et al. 2001). Activation of cdc2 kinase in HSV-1 infected cells

appears to be required to enhance the expression of a subset of L genes and this newly observed interaction between UL42 and cdc2 may shed some light on this phenomenon. Multiple proteins associated with viral DNA replication contain phosphorylation sites for cdc2 kinase. These include UL30, the DNA polymerase, UL52, the helicase/primase, and ICP8 (Advani, Weichselbaum et al. 2001). Additionally, phosphorylation of ICP0 at late times post-infection is blocked by roscovitine, an inhibitor of cdc2,cdk2,cdk7, and cdk5 (Advani, Weichselbaum et al. 2001). Based upon these observations, the current model proposed is that UL42 binds cdc2 kinase and facilitates an interaction between the kinase and several viral protein substrates, to allow their phosphorylation by cdc2 kinase (Advani, Weichselbaum et al. 2001).

1.7.3 Viral DNA replication

Early work to elucidate the mechanism of HSV-1 DNA replication produced several observations upon which the classical model for HSV-1 replication is based. It was observed that terminal fragments decreased while DNA lacking terminal fragments (endless DNA) increased relative to what is seen in packaged DNA (Jacob, Morse et al. 1979). This observation of "endless DNA" seemed to suggest a circularization of the genome but was also consistent with concatemer formation and integration into the host genome. Other studies also suggested that HSV-1 DNA does circularize. Electron microscopy was utilized to observe some unit length circles (Jacob and Roizman 1977) (Shlomai, Friedmann et al. 1976) (Friedman, Costa et al. 1976). Additionally, circularization of the genome was suggested by studies utilizing an HSV-1 mutant unable to undergo L-S inversion (Poffenberger and Roizman 1985), as well as studies relying on the ability of pulsed-field gel electrophoresis to separate the different DNA configurations (Garber, Beverley et al. 1993). As a requirement for the mechanism of theta replication is circularization of the input linear genome, the

classical model proposes that HSV-1 DNA replication begins with theta replication (Challberg and Kelly 1989).

Closely preceding the onset of viral DNA replication, the appropriate E proteins assemble in pre-replicative sites near ND10 (Ishov and Maul 1996). Once viral DNA replication has begun, structures called replication compartments form where replication occurs (Quinlan, Chen et al. 1984). The first step of HSV-1 DNA replication is the binding of UL9, the origin binding protein, to the ori sequences. UL9 unwinds the DNA allowing ICP8 (ssDNA binding protein) to bind to the ssDNA. Next recruited are the helicase-primase complex (UL8, UL5, and UL52) and the polymerase holoenzyme (UL30, UL42). These seven proteins form a complex, and initial theta-form replication occurs (Severini, Morgan et al. 1994) (Zhang, Efstathiou et al. 1994). Replication is proposed to quickly switch to rolling-circle replication (ori-independent) as progeny HSV-1 DNA is in the form of head-to-tail concatemers (Jacob, Morse et al. 1979) (Jongeneel and Bachenheimer 1981).

Recent studies by Jackson and DeLuca however, have brought this model into question by providing evidence that genome circularization is not a prerequisite for replication and may in fact be responsible for the establishment of latency in vivo (Jackson and DeLuca 2003). Early studies of HSV-1 DNA replication observed an increase in the genomic "end-joints" before the onset of infection (Poffenberger and Roizman 1985) (Deshmane, Raengsakulrach et al. 1995) (Garber, Beverley et al. 1993). This was interpreted as the circularization of the genome and led to the model of theta-form replication even though no theta-form intermediates were ever observed (Lehman and Boehmer 1999). Several other possibilities could account for this increase in joint regions. For example, they could stem from the formation of branched structures as well as intermolecular ligation or recombination (Jackson and DeLuca 2003). In this study, Jackson and DeLuca observed circularization of HSV-1 genomes only in the absence of viral gene expression that mimicked a latent state. During

productive infection, genomes were not circular. ICP0 inhibited the formation of circular genomes. Jackson and DeLuca propose that the inhibition of circularization by ICP0 may be an additional mechanism for how ICP0 regulates the balance between latent and lytic infection.

1.8 Late gene expression

Viral DNA replication signals the onset of the final stage of viral gene expression, activation of the L genes. The L genes encode mainly structural proteins, as well as those associated with assembly and egress. HSV-1 L genes can be subdivided into true late and leaky late based upon the stringency of their dependence on viral DNA replication for expression (Weinheimer and McKnight 1987) (Knipe and Smith 1986). Leaky late (γ1) expression occurs in the absence of viral DNA replication but augments upon the onset of replication. True late genes (γ2) expression does not occur until replication.

1.9 Viral capsid assembly and egress

The synthesis and accumulation of L proteins leads to the self-assembly of viral capsids (Newcomb, Homa et al. 1994). During capsid formation, VP5, the major capsid protein, VP22a, the scaffolding protein, and the triplex proteins VP19c and VP23 condense to form the procapsid (Roizman and Knipe 2001). In vitro studies have demonstrated that procapsid assembly requires only VP5, VP19C, scaffolding protein VP23, either preVP22A (UL26.5 gene) or VP21 (UL26 gene), and no cellular proteins (Thomsen, Roof et al. 1994) (Tatman, Preston et al. 1994) (Newcomb, Homa et al. 1999). DNA is packaged into mature icosahedral capsids. DNA cleavage from the concatomer form and subsequent packaging are thought to be coupled events that are dependent on cis-acting packaging sequences (pac1 and pac2 sequences) present within the

viral genome (Varmuza and Smiley 1985) (Deiss, Chou et al. 1986). The progeny molecules are fed into the capsid while the scaffolding proteins VP21 and VP22a are simultaneously displaced (Thomsen, Newcomb et al. 1995) (Gibson and Roizman 1972) (Tengelsen, Pederson et al. 1993) (Gao, Matusick-Kumar et al. 1994). Encapsidation of the viral DNA requires the viral gene products of UL6, UL15, UL25, UL28, UL32, UL36 and UL37 (Roizman and Sears 1996).

Following packaging of DNA, the viral nucleocapsid acquires a tegument layer and a lipid envelope. It has been suggested that interactions between the tegument proteins, such as VP16, and the envelope glycoproteins are required for envelopment at the nuclear membrane (Weinheimer, Boyd et al. 1992) (Zhu, Cai et al. 1994) (Eisenberg, Long et al. 1985) (Campadelli-Fiume, Stirpe et al. 1990). The virion protein UL11 may contain an envelopment signal (MacLean, Clark et al. 1989) and may influence the rate of capsid envelopment (Baines and Roizman 1992). Recently, UL11 has been shown to associate with several infected cell proteins including UL16 (Loomis, Courtney et al. 2003). Other proteins have also been shown to play a role in this process, including UL34 and UL31 (Reynolds, Ryckman et al. 2001) (Roller, Zhou et al. 2000). UL31 and UL34 have been reported to form a complex that is localized to the nuclear membrane and is required for the envelopment of nucleocapsids (Reynolds, Ryckman et al. 2001) (Roller, Zhou et al. 2000). The process of envelopment appears to require several tegument proteins that are necessary for binding the envelope to the tegument layer. These include VP22 and VP16, which bind gE/gM and gH/gD, respectively (Johnson, Wittels et al. 1984) (Fuchs, Klupp et al. 2002) (Farnsworth, Goldsmith et al. 2003).

The prevalent model for the origin of the viral envelope involves an envelopment-deenvelopment-reevelopment pathway in egress. The viral capsid acquires an envelope at the inner nuclear membrane, passes through the outer nuclear membrane, buds into the ER/Golgi apparatus, and is released through a

membrane bound vesicle (Johnson and Spear 1982) (Torrisi, Di Lazzaro et al. 1992) (Campadelli-Fiume, Farabegoli et al. 1991). The tegument and lipid envelope are acquired when the capsid buds into the Golgi apparatus. Skepper et al directly demonstrated this via immunogold electron microscopy (Skepper, Whiteley et al. 2001). Cells infected with a mutant gD (specifically targeted to the ER) display a heavy labeling at the inner and outer nuclear membranes but very little on extracellular virions. This would indicate that HSV-1 virions must acquire an envelope from a post-ER cytoplasmic compartment and follow an envelopment-deenvelopment-reevelopment pathway in egress (Skepper, Whiteley et al. 2001).

1.10 ICP27: The controversy

As anticipated in section 1.6.4.2, the role of ICP27 in nuclear export is still under debate. The relative contribution of the NES vs. the REF binding site is unclear. By analogy with HIV Rev, researchers hypothesized that ICP27 exported mRNAs via the cellular exportin Crm-1. Further support for this hypothesis came from studies by Soliman and Silverstein (2000). Key in their study was the use of leptomycin B (LMB), a potent antifungal antibiotic isolated from a *Streptomyces* sp. (Hamamoto, Gunji et al. 1983) that specifically inhibits Crm-1 mediated export by covalently modifying Crm-1 (Kudo, Wolff et al. 1998) (Kudo, Matsumori et al. 1999). LMB completely blocks nuclear export by Rev in HIV infection (Wolff, Sanglier et al. 1997). Cells were infected with HSV-1 in the presence of LMB and the cytoplasmic accumulation of several late RNAs was measured. The cytoplasmic accumulation of RNA encoding the IE gene ICP4 and the late gene Vp22 was not altered by the presence of LMB, while RNAs encoded by the late genes Vp16, UL17, UL18, UL35, gC, and gH had a reduced cytoplasmic accumulation. Interestingly, among the RNAs that were affected, there was variation in the degree to which they were affected; Vp16 RNA was

reduced to 42% of wt, while gC was reduced to 28% of wt. Soliman also observed changes in viral protein expression by LMB. The levels of IE and E proteins were unchanged in the presence of LMB. The effect on L protein accumulation varied, being most severe for gC and least for Vp16. Additionally, the authors reported what they believed to be a block in the shuttling of ICP27 by LMB. The authors therefore concluded that some transcripts are exported in an ICP27-mediated, Crm-1 dependent manner, while others are not. Interestingly, no corresponding nuclear accumulation of these transcripts was observed. The authors speculated that these transcripts were quickly degraded in the nucleus. This is in direct contrast to earlier studies (Sandri-Goldin 1998) (Phelan, Dunlop et al. 1996) that were able to demonstrate a nuclear accumulation of certain viral transcripts in the absence of ICP27. The authors also concluded that this decrease in protein levels was a direct result of inhibiting ICP27-mediated Crm-1 dependent RNA export. However, no direct role for ICP27 in viral mRNA export was demonstrated in these studies. The authors assumed that ICP27 was the only target of LMB.

In our view, the observations of Soliman and Silverstein seem inconsistent with a block of nuclear export. If nuclear export was completely abrogated, one would expect a more striking decrease in gene expression. Also peculiar is that no nuclear accumulation of the affected transcripts was observed. If transcripts were strictly limited to the nucleus in the presence of LMB, then why do they not accumulate in this compartment? Additionally, if all RNA export is blocked by inhibiting a primary nuclear export pathway, then the effects on gene expression should vary less between genes. If two RNAs are dependent on ICP27-mediated nuclear export then when export is blocked both of these RNAs should demonstrate an equal inhibition. Taken together, these observations seem to us to be more reminiscent of a block in viral DNA replication. A severe decrease in the cytoplasmic accumulation of true late transcripts, a minor decrease in leaky late transcripts (with no corresponding nuclear accumulation), and no effect on IE

transcript accumulation could all be predicted to be a result of inhibiting viral DNA replication.

Studies on ICP27 mediated RNA export by Koffa (Koffa, Clements et al. 2001) called into question ICP27 mediated export through Crm-1. Using microinjection into *Xenopus laevis* oocytes, Koffa reported that, in contrast to previous studies, ICP27 neither shuttles nor exports viral mRNAs via the Crm-1 pathway. ICP27 binds directly to REF and recruits TAP to this nucleoprotein complex to stimulate export of viral mRNA. Chen et al (Chen, Sciabica et al. 2002) provided further evidence that the leucine-rich NES of ICP27 does not require Crm-1 for activity. Chen confirmed the interaction between ICP27 and REF/Aly and additionally demonstrated that the export of ICP27 was not sensitive to LMB but was sensitive to a dominant negative TAP deletion mutant. Based upon these studies, the current consensus is that ICP27 mediates export of RNA through its interaction with REF and TAP, and does not function through Crm-1, making it insensitive to LMB.

Around the same time, there was a similar debate about the EB2 protein of Epstein-Barr virus. EB2 is the EBV homolog to the HSV-1 ICP27 protein. It too was originally described as a promiscuous transcription factor (Lieberman, O'Hare et al. 1986), but was later found to increase the cytoplasmic accumulation of unspliced RNAs (Buisson, Hans et al. 1999). EB2 displays the properties of an RNA export protein, shuttling between the nucleus and the cytoplasm in addition to binding RNA (Semmes, Chen et al. 1998). Additionally, EB2 was found to contain a leucine-rich region (LRR) that resembled the leucine rich NES consensus (Semmes, Chen et al. 1998). In 1999, Boyle et al reported that the EB2/Sm protein of Epstein-Barr virus associated with Crm-1 to mediate the export of intronless viral RNAs. In contrast, Farjot et al (Farjot, Buisson et al. 2000) demonstrated that EB2-mediated export of RNA was insensitive to LMB. Additionally, they reported that the putative NES of EB2 was not required for

EB2-mediated export of RNA. These contradictions are oddly reminiscent of the debate about ICP27 and seem to preclude a simple explanation.

The current evidence seems to preclude the possibility that ICP27 functions to export RNA through Crm-1. However, this view is difficult to reconcile with several additional observations. The first is that, as reported by Murata 2001 (Murata, Goshima et al. 2001), LMB reduces viral titres by 100-fold. This would imply a Crm-1 function in HSV-1 infection. Second, a viral mutant has been isolated with a single point mutation in the acidic region of ICP27 that renders the virus resistant to LMB (Murata, Goshima et al. 2001). Third, LMB treatment of HSV-1 infected cells was reported to inhibit the shuttling of activity of ICP27 (Soliman and Silverstein 2000), although another group has failed to reproduce this finding (Koffa, Clements et al. 2001). Fourth, when the NES is deleted from ICP27, it becomes restricted to the nucleus (Lengyel, Guy et al. 2002), which is consistent with a defect in the shuttling function of ICP27. Fifth, NES deletion mutants show a 5-30 fold defect in viral replication as compared to wt HSV-1 (Lengyel, Guy et al. 2002). In contrast, a deletion in ICP27 of the putative site of interaction with REF (aa 104-138) that abolishes interaction with REF (Koffa, Clements et al. 2001) had very little effect on viral replication (Lengyel, Guy et al. 2002). The apparently more deleterious effect of deleting the NES than the REF site calls seriously into question the relevance of the interaction between REF and ICP27.

It is clear that there are still many unanswered questions about the role of ICP27 in RNA export. How does LMB inhibit viral replication? If ICP27 does function through Crm-1, why would a mutation in ICP27 render the virus insensitive to this drug?

The scope of my thesis was to undertake a detailed study of the effect of LMB on HSV-1 gene expression, nuclear/cytoplasmic localization of viral RNA and viral DNA replication in the context of several ICP27 mutants.

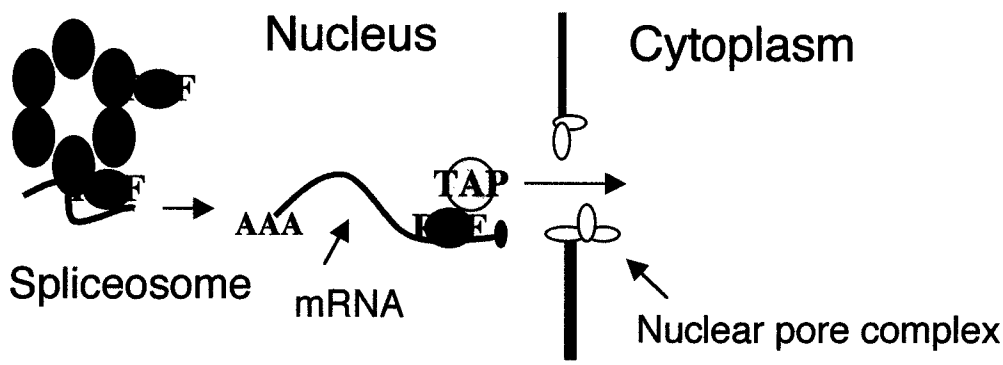


Figure 1: Cellular export of mRNA through the TAP pathway. REF accumulates in splicing factories and is deposited on mRNA during splicing. REF recruits TAP, a cellular export factor that interacts directly with the nuclear pore. RNA is then shuttled Between the nucleus and the cytoplasm. REF (RNA export factor), TAP (Transporter for antigen processing)

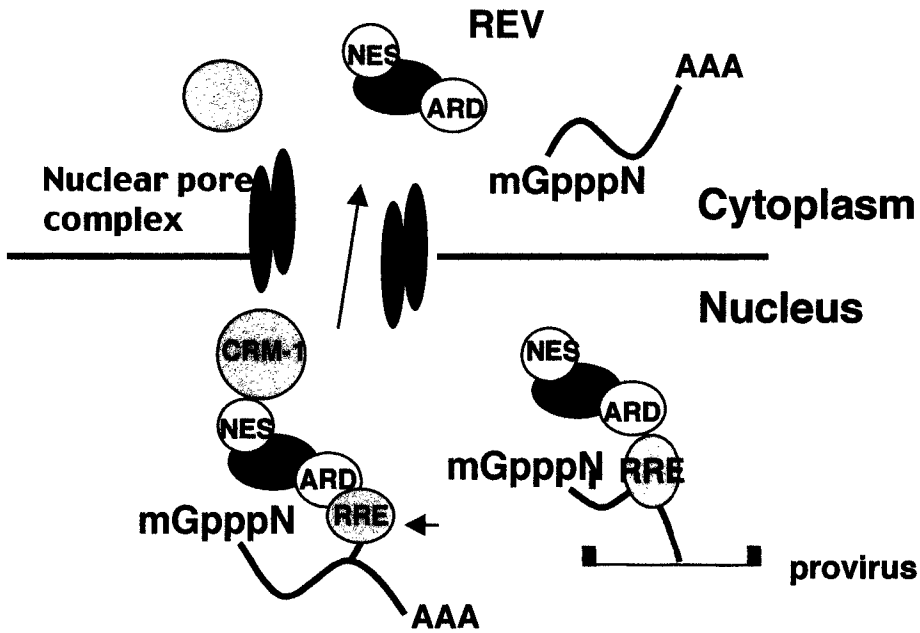


Figure 2: Viral RNA export mediated by the HIV Rev protein.
Rev binds a secondary structure within the RNA, the Rev response element (RRE). The nuclear export sequence (NES) of Rev interacts with Crm-1 (exportin-1), an export receptor that interacts with the nuclear pore complex. Rev shuttles RNA cargo from nucleus to the cytoplasm. Upon entry into the cytoplasm the complex disassociates.

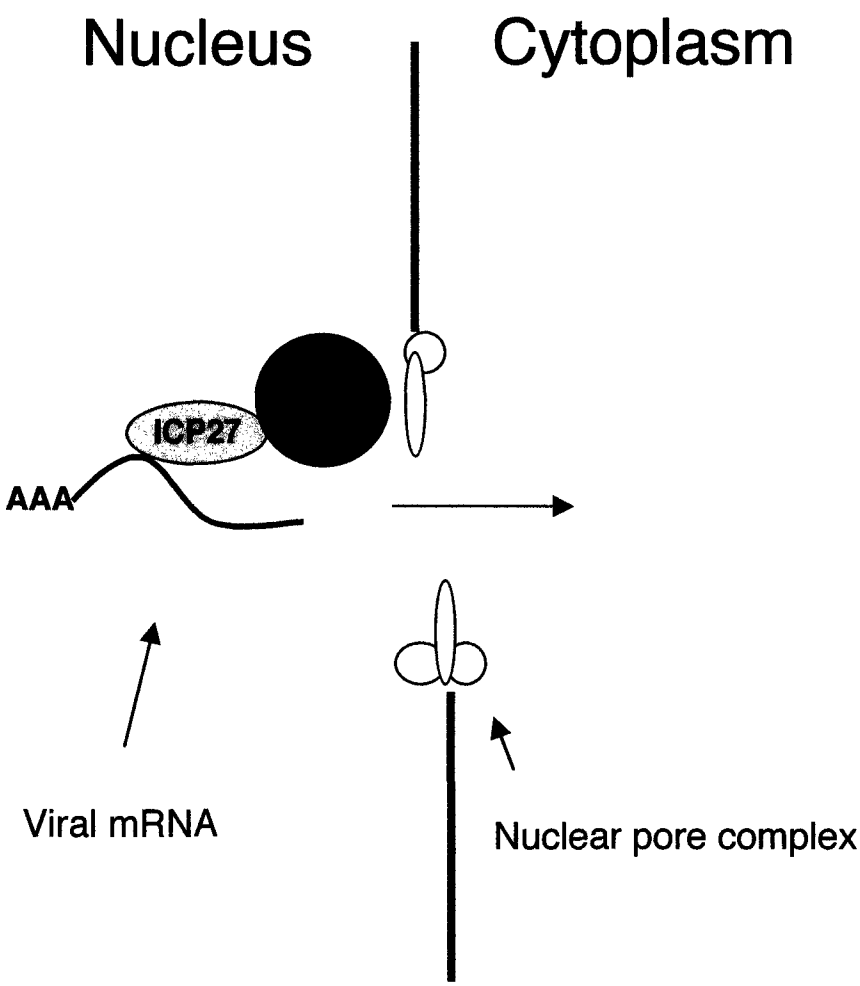


Figure 3: Proposed model for RNA shuttling via ICP27. ICP27 protein binds intronless viral RNA through an Arginine rich domain (ARD) and interacts with Crm-1 (exportin-1), an export receptor that interacts with the nuclear pore complex to mediate export of unspliced viral transcripts.

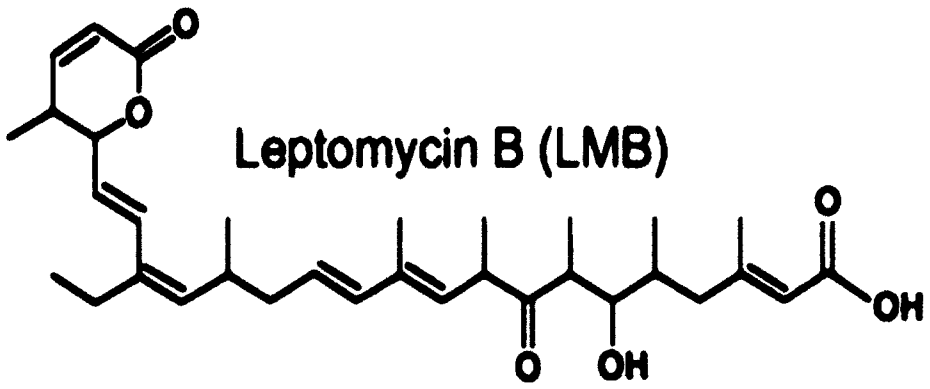


Figure 4: Chemical structure of LMB.
An anti-fungal antibiotic isolated from Streptomyces that specifically and covalently modifies Crm-1 inhibiting Crm-1 mediated nuclear export.

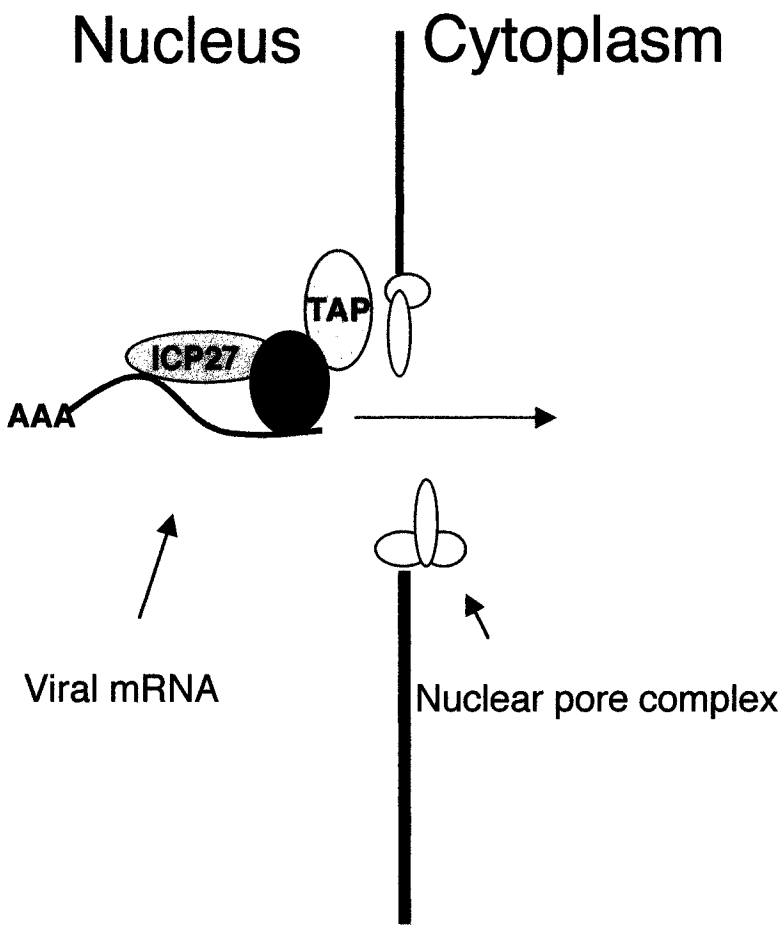


Figure 5: New model for RNA shuttling via ICP27.
ICP27 protein binds intronless viral RNA through an Arginine rich domain (ARD) and interacts directly with REF an export receptor. REF binds TAP which can interact with the nuclear pore complex to mediate export of unspliced viral transcripts.

Chapter 2: Materials and Methods

2.1 Cells, viruses and plasmids

Vero (African Green monkey kidney) cells and derivatives were utilized in this study. V27 cells, a derivative of Vero cells containing an integrated copy of ICP27, were used to propagate stocks of d27-1 (ICP27 null) virus. Vero cells were obtained from the American Type Culture Collection and were propagated in Dulbecco modified Eagle medium (DMEM) containing 5% fetal bovine serum, 50 units/mL penicillin and 50µg/mL streptomycin at 37°C. Medium for V27 cells additionally contained gentimycin at 200µg/mL.

KOS 1.1 was the wild-type strain of HSV-1 in these studies. d27-1 and dLeu viruses have been previously described (Rice and Knipe 1990) (Lengyel, Guy et al. 2002). Briefly, d27-1 contains a deletion in the ICP27 gene, preventing the expression of any functional ICP27 protein, and dLeu is an ICP27 mutant lacking residues 6-19 of the NES. M50T was engineered with two point mutations that convert a methionine to a threonine at position 50 of ICP27. This mutation has been previously shown to render HSV-1 resistant to leptomycin B (LMB) (Murata, Goshima et al. 2001) (Figure 9). An altered site mutagenesis kit (Promega) was used to construct a plasmid containing the two bp substitution, following the ssDNA protocol as outlined in the kit. Briefly, plasmid pM27, containing a 2.4 kb BamHI-SstI fragment of ICP27 from pBS27 inserted into the pSELECT vector at the BamHI-SstI sites, was used as the parental plasmid for site directed mutagenesis. An oligonucleotide was constructed to alter two bases

from the parental plasmid (5'-GTGGGGGTCTTCGGTGTCCTCGTCCG). The amino acid substitution was engineered on to a KOS 1.1 background. Briefly, plasmid and d27-1 DNA were transfected into Vero cells in combination using lipofectamine by a standard transfection protocol (GibcoBRL). Viral DNA was isolated from the resulting plaques. DNA sequencing and plaque reduction assay confirmed the mutation. M50T, unlike KOS 1.1 was resistant to 10ng/mL LMB as previously reported (Murata et al, 2001).

2.2 Infection of cells

Infections were carried out in serum-free DMEM at the multiplicities of infection (MOI) specified for each experiment, with or with the addition of LMB and phosphonoacetic acid (PAA) at 10ng/mL and 300μg/mL, respectively, or at the concentration indicated. After 1 hr. of adsorption at 37°C, the inoculum was aspirated and fresh medium was added. Cells were incubated at 37°C for the time indicated before they were harvested.

2.3 Transfection assays

Vero cells were seeded at a concentration of 1.4 x 10^6 cells/60mm dish as determined using hemacytometer counting. Cells were transfected with 1.2μg to 2μg DNA/well total plasmid concentration (puc19 DNA was utilized to ensure equal final concentration) using lipofectamine 2000. Six μL Lipofectamine2000 + 194μL Optimem (Gibco BRL) was added to Optimem + DNA to a volume of 400μL. Samples were mixed and incubated for 30 minutes at room temperature and transferred into wells containing standard media (1 to 2 mL/well), then incubated at 37° C overnight. Two plasmids were used, pCAT-RRE, and pFlagRev (Farjot, Buisson et al. 2000) (Figure 6). Transfections were done in the

presence of two different concentrations of LMB 6ng/mL (10mM) and 25ng/mL (42mM) as indicated.

Cells were harvested at 12 – 24 hr. post-transfection as indicated. Medium was aspirated and cells were washed three times with cold PBS (Phosphate Buffered Saline 10x -pH 7.5 – 0.2M phosphate, 1.5M NaCl). On the third wash, cells were scraped into the PBS and centrifuged for 10 minutes at 4°C, 2000 RPM. Medium was aspirated and 1 mL lysis buffer was added. Cells were resuspended and incubated for 30 minutes at room temperature. Samples were then centrifuged for 15 minutes at 14,000 RPM at 4°C.

2.4 CAT-ELISA

CAT-ELISA kit (Roche) was used to quantify CAT expression. CAT enzyme standards were prepared as per kit instructions and five concentrations were utilized to obtain a calibration curve: 0, 0.125, 0.25, 0.5, and 1ng/mL.

200μL of CAT standards or cell extracts were used per well and incubated, for one hour covered in foil at 37°C. The solution was removed and the wells were rinsed five times with 250μL of wash buffer (PBS-T 1xPBS, 0.05% Tween 20) before adding 200μL of anti-CAT-DIG (2mg/mL) per well and the microtitre plate was covered with foil and incubated for one hour at 37°C. The solution was removed and the wells were rinsed five times with 250μL of wash buffer (PBS-T). 200μL anti-DIG-POD was added to wells and the plate was again covered with foil and incubated at 37°C. The solution was removed and the wells were rinsed five times with 250μL of wash buffer (PBS-T); 200μL of POD substrate was added. The plate was placed on rocker for 10-40 minutes at room temperature until colour developed (green). Absorbance of samples was measured at 405nm using an ELISA plate reader. Results were normalized with respect to protein concentration using a BCA protein assay kit (Pierce).

2.5 Western Blotting

1 x 10^6 Vero cells were infected at an MOI of 10 pfu/cell, in the presence or absence of LMB and/or PAA (10ng/mL and 300 μg/mL, respectively), in a volume of 500μL/well SF DMEM (6 well plate). Samples were incubated for one hour at 37°C rocking plates every 15 minutes. The medium was aspirated and replaced with 2mL of 5% DMEM + supplemented drug. Cells were incubated for six to twelve hr. (as indicated) at 37°C. To harvest cells, medium was aspirated and 100μL of SDS-PAGE lysis buffer was added. DNA in the samples was sheared by drawing it up into a needle several times. Samples were heated for 5 minutes at 100°C before loading onto gel.

Several SDS-PAGE gel concentrations were used as indicated. The most commonly utilized concentration was 12%, with a 4% standard stacking gel. Samples were separated using SDS-PAGE at 50mA for approximately 3 hr. or until the dye front reached bottom of gel. Proteins were transferred onto PVDF transfer membrane (Hybond-F, Amersham Pharmacia Biosciences) using a wet protein transfer apparatus (Bio-Rad Trans-blot cell) at 1.8A for three hr. to ensure transfer of larger proteins. Membranes were blocked in 10% skim milk powder in TBST (TBS – Tris Buffered Saline (pH 7.6) –50mM Tris-HCl, 150mM NaCl, TBS-Tween – 0.05% Tween20 into TBS) for one hour at room temperature or overnight at 4ºC. Membranes were rinsed briefly with TBST. Primary antibody (Ab) was diluted in TBST with 5% skim milk powder to an appropriate concentration. Antibodies from the Goodwin Institute were used at a concentration of 1:1000 [1113(αICP27), 1114(αICP4), 1104(αVP16)]. αUL30 (polyclonal, 1:2000 (Haffey, Stevens et al. 1988)), αUS11 (1:2000, (Roller and Roizman 1992)), αUL42 (1:12,800, ZIF 11 (Murphy, Schenk et al. 1989), αVP16

(1:3000, LP1 (McLean, Buckmaster et al. 1982) and α-actin (1:5000 mAb AC-15, Anti-Bactin, Sigma Aldrich).

Following an overnight incubation at 4°C, membranes were washed three times for ten minutes each in TBST. Secondary antibodies (goat anti-mouse IgG-HRP1:3000, BioRad; goat anti-rabbit IgG-HRP, 1:3000, Molecular Probes Inc.) were diluted in TBST. Membranes were incubated for 30 minutes at room temperature in secondary Ab followed by three 10 minute washes with TBST. Proteins were detected using an enhanced chemiluminescence (ECL Plus) kit (Amersham Pharmacia Biotech) as per standard instructions and visualized using x-ray film (Fuji). Quantification of all protein bands was done using a phosphorimager (Storm 860) and Image Quant Software, as described in greater detail in Section 2.11.

2.6 Nuclear and Cytoplasmic RNA Fractionation

Vero cells were infected as described in six well plates and harvested at six hr. post-infection. Cells were washed with PBS and then trypsinized with 1mL trypsin/well. Following trypsinization, 2mL of DMEM + 5% serum was added and the cells were centrifuged at 2,000 RPM for five minutes at 4°C. Cells were resuspended in 175μL of cold RLT buffer (50mM Tris pH8.0, 1.5mM $MgCl_2$, 0.5% Nonidet P-40, 1,000U/mL Rnase-out, and 1mM DTT) and pipetted up and down gently to lyse the plasma membrane and not the nuclear membrane. Cells were incubated on ice for five minutes and centrifuged at 1,500 RPM for three minutes at 4°C. Supernatant was removed and transferred. 600μL of RLT buffer was added to the supernatant. Nuclear pellets were resuspended in 600μL RLT buffer, transferred to Quiashredders (Qiagen), and centrifuged for two minutes. To the nuclear samples, 600μL 70% EtOH was added and, to the cytoplasmic samples 430μL 95% EtOH was added. RNA was isolated using the RNeasy Kit

(Qiagen) as per standard protocol. RNA was separated on a 1% agarose gel (1g agarose, 88mL H_2O, 10mL 10X MOPS, 1.8mL formaldehyde, 5µL EtBr). 1X MOPS buffer (5X MOPS Buffer pH 7.0: 0.2M MOPS, 50mM sodium acetate, 5mM EDTA) was utilized for gel electrophoresis. RNA samples were prepared as follows: 3µL 10X MOPS, 5µL formaldehyde, 15µL Formamide, 10µL RNA total volume of 33 µL. Samples were incubated at 55°C for 15 minutes, separated at 100V for two hr., photographed under ultraviolet (UV) light to ensure equal loading, and transferred to nylon membranes (Gene Screen Plus, Perkin Elmer Life Sciences Inc.). Membrane was UV crosslinked in a stratalinker (Stratagene) (120mJ, 120 seconds).

2.7 ^{32}P-Labelling of probes

Probes for Northern and Southern blots were prepared by random priming labelling. 2µg DNA fragment was mixed with 4µL of random primers (500ng/mL), brought to a volume of 35µL with ddH_2O and boiled for five minutes. 7µL of oligo labelling buffer (315 mM Tris pH 8.0, 31.5 mM MgCl2, 7.2µL β-mercaptoethanol, 126µM dGTP, 126µM dATP, 126µM dTTP, 1.29M HEPES pH 6.6), 2µL BSA (10mg/mL), 50µCi α-^{32}P-dCTP and 1µL large fragment E.coli DNA polymerase 1 was added to this mix and incubated at 37°C for 30 minutes. Labelled fragments were purified using a NICK column (Amersham Pharmacia Biotech, Sephadex G-50) and eluted into 500µL TE. Probes were counted in a Scintillation counter and used at a concentration of 1 to 2 x10^6 cpm/mL hybridization buffer. Several fragments were utilized. pTK173 was restriction digested with SacI/SmaI to produce a 662bp fragment of TK. pRAM44 was restriction digested with XbaI/EcoRI to yield a 903bp UL44 fragment. pRAM42 was restriction digested

with PstI to produce a 740bp fragment of UL42. pC27 (pUHD10-3-2) was restriction digested with EcoRI to produce a 2004bp fragment of ICP27 coding sequence. The UL30 probe was amplified by PCR using primers HZ-7 and HZ-8 (5'-GCCATACGTACTATAGCGAA/5'- GATGGCCGGGCAGAAGTTGT) to produce a 680bp fragment. The VP16 fragment was amplified using primers VP16 5' and VP16 3' (5'- CGCCGTCGGGCGTCCCACAC/ 5'-CGGGGGATGCGGATCCGGTCGCGC) to produce a 1567bp fragment.

2.8 Hybridization

Blots were pre-hybridized in Church buffer (7% SDS, 7%BSA, 1mM EDTA, 250mM Na_2HPO_4, pH 7.2) for one hr. at 65°C. Probe was boiled for five minutes, added to the buffer, and hybridized overnight at 65°C. Blots were washed twice with Church wash 1 (2x SSC, 0.1% SDS) at 65°C and twice in Church wash 2 (0.1x SSC, 0.1% SDS) at 68°C.

2.9 PCR

Polymerase chain reaction was utilized to amplify the DNA probe fragments. Reactions were carried out in specialized PCR buffer. A specialized PCR buffer was utilized due to the high G/C content of the HSV-1 gene fragments (Bunnell and Rice 2000). Each DNA fragment was amplified over 30 cycles of denaturing, annealing, and extending. Specifically, each cycle consisted of: 94°C - 45s denature; 58°C - 45s anneal; and 72°C - 45s extend. Reactions were carried out in a 100μL volume: 10μL buffer, 10μL 2mM dNTPs, 5μL 5mM Primer 1, 5μL 5mM Primer 2, DNA (1ng (plasmid) or 20ng genomic), and ddH_2O to volume.

2.10 Southern Blotting

Cells were infected at an MOI of 10 as per standard protocol. 12 hr. post-infection, medium was aspirated from infected wells and 200μL (for a well of a 12 well plate) of 1X lysis buffer (0.6% SDS, 10mM EDTA, 10mM Tris pH 7.5, 100μg/mL) proteinase K was added to the well.

Lysates were incubated at 37°C for 5 to 6 hr. DNA was ethanol precipitated with an equal volume of 95% EtOH. The tube was shaken thoroughly and the white DNA precipitate was removed with a needle into 100μL TE (10mM Tris pH 7.0, 1mM EDTA) to be agitated on a nutator overnight at 4°C. Samples were centrifuged through a qiashredder (Qiagen) and phenol/chloroform extracted. A second ethanol precipitation was performed and DNA was resuspended in ddH_2O.

DNA for Southern blot analysis was restriction digested with BamHI and electrophoresed on a 1% agarose gel. DNA was denatured in 0.25M HCl for 20 minutes, depurinated in 0.5M NaOH for 20 minutes and neutralized in 1M Tris, 1.5M NaCl pH 7.0 for 20 minutes before it was transferred overnight onto nylon membrane (Gene Screen Plus, Life Sciences Inc.).

2.11 Quantification of phosphorimager data

The Northern and Southern blot ^{32}P signals were visualized by phosphorimager (STORM 860 model, Molecular Dynamics) and analysed using ImageQuant software from Amersham Biosciences. In ImageQuant, uniformly sized boxes encompassing the signals were selected, and the object average (signal) of each was calculated. The object average value (calculated signal) of the mock infected control sample was utilized as a background signal for each blot and was subtracted from the object averages. Values were transferred to Microsoft Excel for analysis.

2.12 Statistical analysis

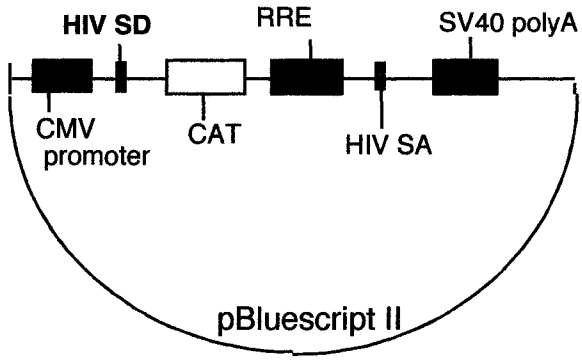
Data acquired by Northern and Western blot analysis was quantified as stated in section 2.11. I calculated the standard deviation between several trials of the same experiment using the standard equation. The determination of the average value and the standard deviation allowed us to compare levels of RNA or protein accumulation under several drug conditions for wild-type and mutant viruses. Values shown in Tables 1, 2, and 3 are the result of 3 or more experiments unless otherwise indicated and are reported relative to wild-type virus in the absence of any drug.

A comparison of RNA to protein data was done and summarized in Table 4. A ratio of RNA to protein was determined for VP16, UL42, UL30 and ICP27 gene products. The ratios were determined from the data found in Tables 1, 2, and 3. I divided the average % value of protein by the average % value of RNA. As this resulted in a very large range of error I arbitrarily chose to further analyse those values in which the ratio between RNA and protein was three-fold or higher. In order to test whether there was a significant difference between the means of these two groups (RNA and protein) we utilized a two-tailed student t-test. Many of our experiments did not have enough replicates to individually determine a normal distribution. However, when possible by virtue of a larger sample size, we determined that our data was unimodal, and that the mean, median, and mode were all located in the center of our data range. We therefore, assumed a random sampling and a normal distribution. By standard convention, $p < 0.05$ was considered significant. Values utilized in our t-tests are found in Tables 1, 2, and 3.

For example, UL42 RNA expression in d27-1 (no drugs) was determined to be 10.0 +/- 8.1% of KOS1.1. UL42 protein expression for d27-1 (no drugs) was determined to be 68.7 +/- 21.0% of KOS1.1. These values were analysed

with statistical analysis software (Free Web Based Software™ from www.software.com) to calculate a p value of 0.0107 and determined to be statistically significant.

A



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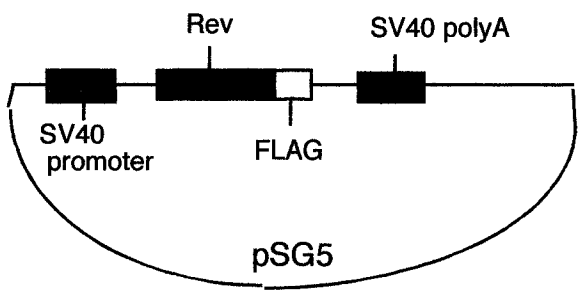

Figure 6
Plasmids utilized to measure LMB function.
A. Plasmid pCAT.RRE. CAT gene and RRE sequence are nestled between the splice donor and acceptor of HIV. CAT expression under the control of the CMV promoter.
B. Plasmid pFLAG.Rev. Rev is FLAG tagged and placed under the control of the SV40 promoter within the pSG5 expression vector.

Chapter 3: Results

At the time that the research described in this thesis was initiated, the favored model for the mRNA export function of ICP27 was that it acts in a fashion analogous to HIV Rev, i.e., by bridging unspliced viral mRNAs to the cellular export adaptor Crm-1, thus bypassing the splicing requirement for mRNA export. Apparently consistent with this hypothesis, Soliman and Silverstein (2000) had reported that leptomycin B (a potent anti-fungal antibiotic inhibitor of Crm-1), inhibited the nucleo-cytoplasmic shuttling of ICP27 and reduced the levels of expression of some, but not all, HSV-1 genes. In addition, Murata et al (2001) had just shown that a single amino acid change in ICP27 (M50->T) could render HSV-1 highly resistant to the inhibitory effects of LMB.

Dr. Peter Cheung (a former Ph.D. student in our lab) and Dr. Kimberly Ellison (a research associate in our lab) had previously shown that HSV-1 infection activates expression of the endogenous chromosomal α-globin gene in non-erythroid cells, leading to accumulation of readily detectable amounts of globin RNA. Both unspliced and fully spliced transcripts accumulated in the cytoplasm of cells infected with wild-type HSV-1, while only spliced mRNA was detected in cells infected with ICP27 null mutants (Cheung, Ellison et al. 2000). Thus, this system appeared to offer a particularly clear example of ICP27 ability to promote splicing-independent mRNA export. Dr. Ellison therefore reasoned that if ICP27 indeed functions through Crm-1 in the same fashion as rev, then the Crm-1 inhibitor LMB should block the export of unspliced (but not spliced) globin mRNA during infection with wild-type HSV-1. However, Dr. Ellison observed that LMB had essentially no effect on the export of either globin transcript. These

findings were not obviously consistent with the proposed role of Crm-1 in ICP27 mediated export of unspliced mRNAs. We therefore decided to reinvestigate the effects of LMB on HSV-1 replication. To this end, we first sought to confirm that the LMB preparation used in Dr. Ellison's experiments displayed the predicted inhibitory effect on the RNA export function of HIV Rev.

3.1 LMB is effective in inhibiting Rev Activity.

The HIV Rev protein provides one of the best-characterized examples of a viral RNA export protein. Rev shuttles between the nucleus and the cytoplasm (Meyer and Malim 1994) (Richard, Iacampo et al. 1994), and allows for the export of unspliced and partially spliced transcripts (Fischer, Meyer et al. 1994) by binding to a secondary RNA structure within the transcript, the rev response element (RRE) (Malim, Hauber et al. 1989). Rev contains a nuclear export signal (NES) consisting of a leucine rich stretch of amino acids (Fischer, Huber et al. 1995) that complexes with the cellular exportin Crm-1. Crm-1 (chromosome maintenance region 1) functions as an export receptor (Fornerod, Ohno et al. 1997) (Stade, Ford et al. 1997) and has been shown to bridge the interaction between Rev and the nuclear pore complex (Neville, Stutz et al. 1997).

In order to test the efficacy of our stock of LMB we utilized a transient transfection assay system based on the ability of Rev to export intron-containing transcripts in a splicing-independent manner. This system is comprised of three plasmid constructs kindly provided by Dr. Ivan Mikaelian (INSERM-Ecole Normale Superieure de Lyon, France). The first was a CAT reporter construct with a Rev Response element (RRE) flanked by HIV splice donor and acceptor sites, effectively placing it into an intron. The second plasmid was a derivative of the first with the RRE site removed to act as a control. The third construct contained the Rev transcript under the control of the SV40 promoter (Figure 6).

When the Rev protein binds the RRE of the transcript, the transcript is exported from the nucleus to the cytoplasm in a splicing-independent manner and is translated. This results in the expression of CAT protein from our pCAT-RRE plasmid even though the CAT sequence is effectively nestled within an intron. In the absence of Rev the transcript is spliced, the CAT gene removed and no CAT protein is expressed. If the function of Rev is inhibited by the covalent modification of Crm-1 by LMB, then there should be no expression of CAT protein. In this system, CAT expression is used as an indirect measure of Rev function.

Vero cells were transfected with the plasmid constructs as indicated in the presence and absence of LMB at a concentration of 10ng/mL shown previously to be adequate at inhibiting Rev export function. Cells were harvested at 21 and 26 hr. post-transfection, time points deemed suitable to allow for both Rev function and LMB inhibition. ELISA (Figure 7) measured CAT expression. The CAT protein concentration was normalized to total cellular protein as determined by the BCA protein assay. We observed only low-level background expression of CAT activity in the absence of Rev and a very low background in the absence of CAT. When the Rev expression vector and the CAT plasmid were transfected together, CAT protein expression increased 7-fold over background CAT expression, implying a functional Rev protein. Addition of LMB at 10ng/mL resulted in a 28-fold decrease in CAT expression as compared with Rev in the absence of LMB, thus indicating that our stock of LMB was effective at inhibiting Rev's function at this concentration. Experiments harvested at 21 or 26 hr. yielded similar results.

As the effects we observed on CAT expression in the presence of LMB could be due to an inhibition of function or an inhibition in the expression of Rev, Western blot analysis was used to distinguish between these two possibilities and confirmed a consistent expression of Rev in the presence and absence of LMB. Vero cells were transfected as indicated and protein was harvested at

21hr. post-transfection (Figure 8). Unless otherwise indicated LMB was added at a concentration of 10ng/mL. Proteins were separated by SDS-PAGE and anti-FLAG antibody was used to detect the tagged Rev protein. Two bands were visualized; the top (larger) band corresponds to the Rev protein (18kDa) and the smaller band is possibly a degradation product. We observed no alteration in the level of Rev protein in the presence of the drug LMB at 10ng/mL. Based on these observations we concluded that our stock of LMB was functioning as predicted and previously published and therefore Dr. Ellison's early observations involving α-globin export in the presence of LMB warranted further study.

3.2 ICP27 mutants used in this study

As we were able to confirm the functionality of our LMB preparation as per its inhibitory effect on the RNA export function of Rev, we concluded that ICP27's role in RNA export might not be as simple as that proposed by the favored model. We therefore initiated a detailed analysis of the effects of LMB on HSV-1 infection. For this study, we utilized the wild-type HSV-1 KOS1.1 and three ICP27 mutants (Figure 9). The rationale for using ICP27 mutants is as follows. Previous studies involving HSV-1 and LMB were built on the premise that all the effects of LMB on HSV-1 infection were due to LMB-mediated inhibition of ICP27 mediated nuclear export via Crm-1, the known target of LMB. ICP27 has been shown to shuttle between the nucleus and cytoplasm (Mears and Rice 1998) (Phelan and Clements 1997) (Sandri-Goldin 1998) (Hibbard and Sandri-Goldin 1995) and to bind viral intronless RNA (Sandri-Goldin 1998). It has also been proposed to affect the nuclear export of viral RNAs via Crm-1 dependent and independent pathways, as demonstrated by a decreased cytoplasmic accumulation of several but not all viral transcripts by LMB (Soliman and Silverstein 2000). Studies by Murata (2001) demonstrated that LMB inhibits viral

DNA replication of HSV-1 and that an HSV-1 mutant with a single amino acid substitution in the acidic region of ICP27 is resistant to the inhibitory effects of LMB. However, the authors never demonstrated a direct role for ICP27 in viral mRNA export. ICP27 contains several functional domains, including the arginine-rich RGG box that is required for RNA binding, a nuclear localization sequence (NLS), and a nuclear export sequence (NES). KOS 1.1 was used as the wild-type strain in our experiments. It is the background of d27-1 (Rice and Knipe 1990) that contains a deletion in ICP27 preventing the expression of ICP27. The ICP27 null mutant was utilized in these studies to understand the link between ICP27 and RNA export via Crm-1. If ICP27 shuttles via Crm-1 and exports viral RNA in a splicing independent manner then an ICP27 null virus should be defective in RNA export (at least to a degree, acknowledging that ICP27 may not be solely responsible) and should not be affected by LMB in any manner.

dLeu, (Lengyel, Guy et al. 2002) an HSV-1 mutant containing a deletion of ICP27 codons 6-19 was also investigated. dLeu contains a deletion of residues 6-19 of ICP27 that constitutes the NES of ICP27. If ICP27 exports RNA by interacting with Crm-1 via its NES (Soliman and Silverstein 2000), then dLeu infection should be defective in RNA export and be unaffected by LMB, as it lacks this site of interaction. If ICP27 exports RNA via Crm-1 by interacting with this exportin through its NES, then infection by KOS1.1 in the presence of LMB should mirror infection by dLeu in the presence or absence of LMB.

The final mutant in this panel is M50T. M50T is a viral mutant that we engineered to have a single amino acid substitution at residue 50, in the acidic region of ICP27 met –thr that renders the virus resistant to LMB (Murata, Goshima et al. 2001). An altered site mutagenesis kit (Promega) was used to construct a plasmid containing the two bp substitution, following the ssDNA protocol as outlined in the kit. The amino acid substitution was engineered on to

a KOS 1.1 background. DNA sequencing and a plaque reduction assay confirmed the mutation.

We confirmed the LMB-resistant phenotype of two independently isolated M50T mutants generated by the present author via a plaque reduction assay and found both M50T 1 and M50T 2 resistant to 25ng/mL LMB (Figure 10A/B) as previously reported. Two separate experiments are shown to illustrate that the concentration of LMB required for maximum inhibition of plaque formation by KOS 1.1 is somewhat variable, although the IC50 remains constant at 1ng/mL LMB.

3.3 Rationale for this study

Previous studies indicated that LMB altered viral gene expression during the course of infection (Soliman and Silverstein 2000). As discussed more comprehensively in the introduction, the observed changes in viral gene expression are reminiscent of observed changes in the absence of viral DNA replication. The RNA and protein expression of the IE genes surveyed by Soliman and Silverstein were not altered by LMB. Some E genes were affected by LMB while others were not. The RNA expression of leaky late gene Vp16 was partially inhibited, while expression of the true late gC was completely inhibited by LMB. Moreover, Murata (2001) reported (as data not shown) that LMB inhibits viral DNA replication. Therefore, it was conceivable that all of the previously observed effects of LMB on HSV-1 infection were the result of inhibition of viral DNA replication. To confirm this DNA replication defect, we examined viral DNA replication in Vero cells infected with wild-type KOS 1.1, the ICP27 null mutant d27-1, and dLeu or M50T. The rationale behind using these particular mutants of HSV-1 is addressed in detail in section 3.2. LMB and phosphonoacetic acid (PAA) were included in the culture media at 10ng/mL and 300μg/mL, respectively. PAA is an inhibitor of viral DNA replication. We used

PAA as a control to determine whether the inhibition of viral DNA replication was sufficient to mediate all the effects of LMB seen during HSV-1 infection. We used LMB and PAA in combination to determine if there were any effects of LMB beyond what can be attributed to inhibiting DNA replication. If the two drugs are used in combination, and the protein expression profile demonstrates a more severe defect than what is observed by either drug alone, then it would suggest that the two drugs were having an additive or perhaps synergistic effect on protein expression. This would suggest that they were affecting two distinct pathways or processes.

3.4 DNA replication

Given Murata et al's observation that LMB inhibits viral DNA replication of wild-type HSV-1 (cited as data not shown), (Murata, Goshima et al. 2001) we used Southern blot analysis to determine whether LMB inhibits the DNA replication of our ICP27 mutants. The suggestion that LMB inhibits viral DNA replication is consistent with the proposed model of ICP27 shuttling through Crm-1 to export viral RNA. If ICP27 shuttles RNA through Crm-1 and LMB inhibits this pathway, then viral RNA will accumulate in the nucleus and not be translated in the presence of LMB. If some of these RNAs encode required DNA replication machinery such as the polymerase (UL30), then DNA replication would be inhibited. If this hypothesis is correct, then dLeu, which lacks the ICP27-Crm-1 site of interaction, should be insensitive to inhibition of DNA replication by LMB. d27-1 is severely compromised for viral DNA replication due to its lack of ICP27 and exhibits only a small residual amount of DNA replication (McCarthy, McMahan et al. 1989)(Rice and Knipe 1990; Uprichard and Knipe 1996). This residual replication should be insensitive to LMB.

Vero cells were infected with an MOI of 10, in the presence or absence of LMB or PAA as indicated (Figure 11). Infected cells were harvested at 12 hr. p.i

unless otherwise indicated. Total cellular DNA was isolated, digested with BamHI and separated on a 1% agarose gel. Quantification via cybergold was utilized to ensure even loading. DNA was transferred to a nitrocellulose membrane and a $^{32}\alpha P$ labeled TK-gene fragment was used as a probe in Southern analysis (Figure 11). The top panel represents a 12 hr time course that follows KOS1.1 and d27-1 DNA replication during infection. In KOS1.1 infection (lanes 2-10) LMB inhibited viral DNA replication at both 8 and 12 hr. to less than 25% of wild-type levels (lanes 3 and 4 versus 6 and 7). This observation is consistent with what is reported in the literature (Murata, Goshima et al. 2001). However, LMB does not completely abolish DNA replication in KOS1.1, as PAA does (lanes 6 and 7 versus 9 and 10). Rather interestingly and unexpectedly, we observed a similar trend in d27-1 infection. LMB inhibited the residual replication of d27-1 at 12hr. p.i (lane 13 versus 16). The effects of PAA on d27-1 replication were approximately the same as those of LMB (lane 16 versus 19). We present a 24hr time course in the bottom panel to illustrate the effect of LMB on d27-1 replication when viral replication is allowed to progress for a longer period of time (lanes 8-16). At 24 hr. p.i, d27-1 DNA was at a more quantifiable level and was very obviously inhibited by LMB (lane 10 versus 13). ICP27 stimulates DNA replication by stimulating E gene expression and, as mentioned previously, this is consistent with the ICP27-Crm-1 model of export. However, the observed impairment of the residual replication of d27-1 (the ICP27 null) indicates that ICP27 cannot be the only target of LMB. It also raises the possibility that the replication defect observed with LMB may not involve ICP27 and, as such, that the M50T mutant may be resistant by a mechanism not directly involving ICP27 (i.e. not through a direct interaction).

We additionally examined the effects of LMB on viral DNA replication of dLeu, the viral mutant lacking the putative site of ICP27 – Crm-1 interaction, and M50T, the LMB resistant mutant (Figure 12). As the previous experiments indicated that LMB inhibition occurred at all time points during infection, this

experiment was done at only one time point, 12 hr. p.i. LMB inhibited DNA replication of dLeu (lane 5 versus 6) (40-50%), but not to the same level as PAA (lane 6 versus 7), nor as dramatically as it did inhibit KOS1.1 DNA replication. This result was unexpected given the d27-1 result and, although this experiment was repeated several times (data not shown), a more dramatic inhibition of dLeu DNA replication was not observed. Infection by M50T (lanes 8-13) was not altered by the addition of LMB. Lanes 8-12 show an increasing concentration of LMB added during infection; 25ng/mL is the highest concentration tested as it impedes plaque formation of M50T (Figures 10A/B). We observe no effect of LMB on viral DNA replication even at 25ng/mL. M50T DNA replication is completely resistant to LMB, but not to PAA, at all the concentrations we tested (lane 13).

We conclude from these experiments, that LMB likely has a target other than ICP27. Otherwise; LMB would have no effect on d27-1. In addition, it would appear that the ability of LMB to inhibit plaque formation of M50T at higher concentrations is not due to an inhibition of DNA replication.

3.5 RNA expression

Our DNA replication data demonstrate that LMB inhibits viral DNA replication strongly in KOS 1.1 and d27-1, slightly in dLeu, and not at all in M50T. As reviewed more thoroughly in the introduction, the previous model for ICP27 function implicated the protein in RNA export via Crm-1. As LMB is an inhibitor of Crm-1, and inhibition of viral RNA export would result in inhibition of viral DNA replication, we examined more closely the distribution and accumulation of several viral RNAs. We examined RNAs spanning all three temporal classes. We fractionated RNA into nuclear and cytoplasmic fractions to address whether ICP27 was required for viral RNA export. If ICP27 were required, then RNA should accumulate in the nucleus of cells infected with d27-1 as reported

previously by Sandri-Goldin (Sandri-Goldin, 1998). We also asked whether RNA export is blocked by LMB. If ICP27 exports mRNA through Crm-1, then LMB should also cause a nuclear accumulation of those viral RNAs that are dependent on ICP27 for export. Third, we asked whether the RNA expression profile for dLeu is identical to KOS1.1 in the presence of LMB. If RNA exports proceeds through Crm-1, then the profiles should be identical. PAA was included in these experiments to act as a control to determine which effects were attributable to inhibiting viral DNA replication alone. The two drugs were utilized in combination to determine whether there were some further effects of LMB on RNA accumulation or distribution beyond an inhibition of DNA replication.

3.5.1 ICP8 mRNA is ICP27 independent

Vero cells were infected with the indicated virus in the presence and absence of LMB and/or PAA as indicated (Figure 13). At 6 hr. post-infection, Nuclear (N) and cytoplasmic (C) RNA was isolated. RNA from equivalent numbers of cells was separated using gel electrophoresis and was analysed for expression of several transcripts by Northern blot. Quantification of all bands on Northern blots was carried out using a Storm 860 phosphorimager. We fractionated Vero cells and analyzed the fractions for ICP8, an mRNA previously described as being unaffected by the absence of ICP27 or viral DNA replication (Figure 13). ICP8 RNA levels remained constant in both nuclear and cytoplasmic fractions in cells infected with KOS1.1 or d27-1, regardless of the presence of PAA or LMB. Our results confirm that ICP8 RNA levels remain constant and are therefore ICP27 independent, as is their nuclear to cytoplasmic ratio. Ethidium bromide staining of ribosomal bands indicated even loading.

3.5.2 Northern Blot analysis of KOS1.1 and d27-1

RNA from equivalent numbers of cells was analyzed for ICP27 (IE gene), VP16 (γ1 gene), UL42 (E gene), UL30 (E gene), and UL44 (γ2 gene) RNA by Northern blot analysis (Figure 14). Triplicate experiments were performed and data is quantified and shown in Table 1. We quantified both the nuclear and cytoplasmic fractions of the RNA and added these values to find a value for total RNA. This total value was set to equal 100% and the percentage of the total RNA that is cytoplasmic is calculated from this value and shown in column B of Table 1.

In KOS 1.1 infected cells (lanes 1-8), 75-80% of the total RNA was cytoplasmic (Table 1A/B) for ICP27, UL42, UL44, and VP16. UL30 RNA had a unique distribution compared to the other RNAs; approximately 60-70% of its RNA was cytoplasmic. Although LMB or PAA significantly reduced the total amount of some of the mRNAs (discussed below), the drugs did not alter the nuclear/cytoplasmic distribution of any of these transcripts in wild-type infection. This observation is not obviously consistent with the hypothesis that LMB interferes with nuclear export of the transcripts examined. Our observation that the ratio of nuclear to cytoplasmic RNA remains constant, however, somewhat simplifies the examination of our data, as the amounts of RNA in the cytoplasmic fractions can be compared directly to one another.

When ICP27 mRNA levels were examined in KOS 1.1 (lanes 1-8 panel 1) in the presence of LMB or PAA, a slightly decreased expression was observed compared to KOS1.1 (Table 1). When the two drugs are used in combination, there does not appear to be any additional decrease in the ICP27 transcript. We next examined VP16 mRNA accumulation in KOS1.1 infection. In this experiment, VP16 RNA was not decreased by LMB, likely due to experimental error. The value for this experiment (lane 4) was 129% of KOS1.1 whereas, in six other experiments the values were 29%, 51%, 55%, 57%, 64%, and 72%, the overall mean of these was 66.7 +/- 29.3%. This was the only experiment in which LMB did not inhibit VP16 mRNA. If the experiment is not included in the

statistical analysis, the mean becomes 56.3 +/- 11.3%. This experiment was included in the statistical analysis due to a small sample set of experiments. An average experiment is shown in Figure 15 (lane 4). When PAA was added to the infection, VP16 RNA levels decreased to the levels observed when LMB was used in conjunction with PAA (Table 1). It appears that while PAA has a greater inhibitory effect on the accumulation of VP16 RNA than LMB, that there is no further effect when the drugs are used in combination. UL42, the E gene that encodes the polymerase processivity factor and UL30, the E gene that encodes the catalytic polymerase, demonstrated a similar profile to VP16 RNA (Table 1). UL44 RNA accumulation decreased more severely with the addition of PAA and LMB than the other transcripts. This was expected, as this transcript is a true late and, as such, more dependent on DNA replication for expression (Table 1).

For KOS1.1 it appears that LMB decreases the level of RNA accumulation. These observations with LMB are consistent with what was observed by Soliman and Silverstein (Soliman and Silverstein 2000), who observed that when cells were infected in the presence of LMB, and the RNA was fractionated, LMB decreased the cytoplasmic levels of certain mRNAs. They observed a reduction in VP16 mRNA levels to 42% of wild type, not unlike our 66%. They also observed a decrease in UL44 mRNA to 28% of wild type in the presence of LMB, similar to our observation of 31%. Soliman and Silverstein however, at odds with our findings, observed no nuclear RNA, suggesting an export defect. Our observations do not support a defect in mRNA export. However, upon closer examination, there may not actually be a discrepancy between our findings and those of Soliman and Silverstein as they did not detect any mRNA in their nuclear samples. Therefore, it is difficult to draw any conclusions about mRNA redistribution from their experiments. When one strictly compares the levels of RNA they observed in their cytoplasmic fractions to what we observed in our cytoplasmic fractions, there is no disagreement.

Our observation that LMB does not alter the nuclear/cytoplasmic ratio of the mRNAs examined and thus, does not inhibit RNA export is consistent with findings by Koffa (Koffa et al 2001) who observed that LMB treatment of HSV-1 infected cells did not inhibit ICP27 protein shuttling, an observation that was also confirmed by Chen et al (Chen, Sciabica et al. 2002). The inhibition of RNA accumulation that we observed was less severe than what was observed with the total inhibition of DNA replication (PAA), but this followed from the previous data showing LMB inhibiting viral DNA replication in the range of 75-90% of wild-type. There is no further additive effect of using LMB and PAA together. These data suggest that all of the effects observed on RNA accumulation by LMB were likely due to the ability of LMB to inhibit viral DNA replication.

Cells infected by d27-1 (Figure 14 panel 1 lanes 9-16) do not express the ICP27 transcript. Due to the lack of ICP27, this mutant has a very limited viral DNA replication and, as such, expression of UL44 (a true late) is abrogated. Expression of other mRNAs in d27-1 infected cells is reduced as a function of their dependence on viral DNA replication for expression. Although we observed a decrease in the level of RNA for the transcripts we examined we did not observe the nuclear retention of any of these transcripts. The ratio of nuclear to cytoplasmic RNA remained constant.

In d27-1 infection, the level of VP16 mRNA expression was lower than in KOS1.1 infection, and this level remained unchanged in the presence of LMB, PAA, or LMB/PAA (Table 1). As UL42 transcript expression is very dependent on ICP27 (Uprichard and Knipe 1996), there is very limited accumulation of this transcript in d27-1 infection (approximately 2-10% of KOS1.1). Due to the extremely limited expression of this transcript, it is difficult to determine whether it was altered by the addition of LMB or PAA (lanes 9-16, Table 1). As shown previously in KOS1.1 infection, UL30 mRNA was approximately 60-75% cytoplasmic, as opposed to 75-85% for the other transcripts. Infection by d27-1 exhibited a similar nuclear/cytoplasmic distribution as that which was observed

for KOS1.1 (approximately 60-75% cytoplasmic) (lanes 9-16). The level of transcript accumulation decreased relative to KOS1.1 and was not further altered by LMB, PAA, or the combination of the two (Table 1). These data indicate that LMB and PAA have no measurable effect on the levels of RNA accumulation for any of the transcripts examined for wild-type or an ICP27 null virus (with a very limited residual replication). Interestingly, we did not observe a nuclear accumulation of RNA in the absence of ICP27. This differs from previously published work. Sandri-Goldin 1998 observed a nuclear accumulation of several RNAs (TK, gD, gC, UL15, and UL41) in the absence of ICP27, an observation that greatly contributed to the current model of ICP27 in mRNA export. We did not examine the same RNAs as were examined in the study by Sandri-Goldin with the exception of gC or UL44. Sandri-Goldin observed a nuclear accumulation of UL44 RNA, while we were unable to quantify these Northern blots as the signals were not significantly above background. The most likely explanation for the observed differences between these two studies is the direct result of utilizing different cell lines. We used monkey cells while they used rabbit cells (RSF). RSF cells appear either to have a slower course of infection or to have a more stringent requirement for ICP27 for viral RNA export. There is evidence to support this conclusion. A later publication from this group (Chen, Sciabica et al. 2002) using RSF cells showed that at 6 hr. post infection, gB RNA exhibited nuclear accumulation in KOS infection that later disappeared at 8 hr. post infection. This would suggest that their cell system differs significantly from ours. It is possible that these cells may have a lower Aly/REF content and therefore transcripts may be more dependent on ICP27 for export than in Vero cells.

3.5.3 Northern Blot analysis of dLeu and M50T

We examined dLeu and M50T to determine the effects of LMB and/or PAA on their RNA localization and accumulation. One might assume that dLeu would be resistant to LMB, as this mutant lacks the putative site of interaction between ICP27 and Crm-1. Our DNA replication data (Figure 12), although not striking, suggest that this is not the case. dLeu DNA replication was less inhibited by LMB than KOS1.1 (65-90% inhibition versus 40-55% inhibition respectively). We examined by Northern blot analysis the level and distribution of several transcripts in dLeu infection (Figure 14 lanes 9-16).

We observed that the ICP27 transcript appeared to have a slight increase in accumulation in comparison to KOS1.1. However, when taking into account the error it is unlikely that this increase was significant. In dLeu infection, ICP27 transcript levels were not altered by the addition of LMB or PAA (Table 1). When the two drugs were used in combination, the levels of ICP27 RNA decreased (lane 16) although the differences were not statistically significant. VP16 and UL42 RNA expression decreased as compared to KOS1.1, but appeared to be unaffected by LMB, PAA or LMB/PAA (Table 1). As mentioned previously, UL30 was a slightly unusual transcript in KOS1.1 infection. The "normal" distribution of UL30 RNA in KOS1.1 infection was 60-75% cytoplasmic. With dLeu infection, this transcript becomes approximately 25-35% cytoplasmic. This distribution was not altered by the addition of either LMB or PAA. This was the only transcript tested whose localization was altered in this mutant. All other transcripts that we tested (ICP27, UL42, UL44 and VP16) had the usual 75-85% cytoplasmic distribution in dLeu infection. Due to the altered cellular distribution, we cannot compare these fractions directly to other mRNAs cytoplasmic distribution, which do not demonstrate this nuclear retention. They can, however, be compared to each other. LMB or PAA had no effect on the level of accumulation or the distribution of the UL30 transcript. The UL44 transcript was not expressed in dLeu infection due to the limited DNA replication of this mutant (approximately 20% of KOS1.1, Figure 11).

The data suggests that LMB has no effect on the distribution of these viral transcripts. There was also no measurable effect of LMB on the level of accumulation of these mRNAs. This would seem to imply two conclusions. First, that UL30 nuclear export is either directly or indirectly dependent on or inhibited by the lack of the ICP27 NES. Second, the resulting change in distribution that we observe is independent of Crm-1.

M50T was the final mutant in this panel examined for any effects of LMB on RNA localization or accumulation. The expression and localization of ICP27 mRNA in M50T infection was similar to what was observed in KOS1.1 infection (lanes 17-24). There was no change in the levels of expression or distribution of this RNA by LMB or PAA (Table 1A/B). VP16, UL44 and UL42 transcripts were expressed to a higher level in M50T infections as compared to KOS1.1 infections (lane 18). These transcripts were only slightly decreased in the presence of LMB, but were still higher than levels in KOS1.1 infection (Table 1). The level of these RNAs decreased in the presence of PAA but exhibited no further decrease when the two drugs were used in combination (Table 1). The nuclear/cytoplasmic distribution of either transcript was unaltered by the addition of either drug. UL30 expression by M50T appeared almost identical to KOS1.1 in both the level of accumulation and distribution. In the presence of LMB, both KOS1.1 and M50T levels of UL30 RNA decreased to 64.5 +/- 14.0% (lane 4) and 68.4 +/- 15.6% (lane 20), respectively. The addition of PAA further decreased UL30 expression in M50T to approximately 56.3% (lane 22) and to 52.1 +/-13.0% in KOS1.1 (lane 6). When both drugs were utilized in combination, no further decrease in RNA was observed (51.3 +/- 13.7%) for KOS1.1 (lane 8) or (53.3 +/- 17.5%) for M50T (lane 24). The nuclear/cytoplasmic distribution of this transcript was not altered in any way by the addition of LMB or PAA. This was a very interesting result, as it seemed to suggest that the regulation of UL30 expression was very dissimilar to UL42, UL44, and VP16. While there was an increased

accumulation of these transcripts in M50T infection, UL30 accumulation was not increased, and was almost identical to KOS1.1.

From these observations, it would appear that the mutation in M50T causes an increased accumulation of certain mRNAs in the absence of LMB as compared to the levels observed in KOS1.1. This raises the possibility that the mutation may in fact bypass the effects of LMB, rather than have its resistance conferred through a direct interaction of ICP27/Crm-1 and LMB. This could suggest a mechanism by which M50T could be resistant to LMB as measured by DNA replication and plaque assay, but still be affected by LMB at the level of transcript accumulation.

In summary, we can draw several conclusions. First, ICP27 does not appear to export viral RNAs to the cytoplasm via Crm-1. Second, the effects of LMB on viral RNA accumulation appear to be due to the effect of LMB on viral DNA replication. Third, infection with M50T results in the over-accumulation of several viral transcripts. Lastly, the UL30 transcript appears to be retained in the nucleus during dLeu infection.

3.5.4 UL30 RNA

As demonstrated previously (Figure 15) UL30 was the only mRNA for which we observed a nuclear accumulation. This unusual distribution was very clearly demonstrated in Figure 16 (lanes 11-18). When cells were infected with dLeu, we observed approximately 25-35% of the mRNA in the cytoplasmic fraction and 65-75% in the nuclear fraction. This was unlike KOS1.1 (lanes 3-10) where 60-70% of the RNA is in the cytoplasmic component. In the context of our other observations, this result is difficult to reconcile, as we observed no change in the mRNA distribution for any RNAs in the absence of ICP27 (Figure 14), nor do we see a change in the RNA localization in dLeu infection for any transcript that we examined. These results suggest inherent differences on UL30 mRNA in

dLeu infection. This observation suggests that an ICP27 protein that lacks a functional NES is more deleterious to UL30 RNA export than no ICP27 at all. Several scenarios might be envisioned of how this might happen. ICP27 may bind UL30 mRNA with a high affinity in the nucleus and, under normal circumstances, shuttles it to the cytoplasm. In the absence of ICP27, UL30 mRNA may use an alternate route to exit the nucleus. However, in the presence of an ICP27 that lacks a functional NES, UL30 may still bind ICP27 but become trapped in the nucleus with ICP27 and unable to use an alternate route as readily.

3.5.5 Two independent isolates of M50T exhibit a similar mRNA expression profile

As Northern blot analysis uncovered a surprising mRNA profile for M50T, which could perhaps be related to a mechanism of resistance to LMB, we sought to confirm that this phenotype was the result of our primary mutation and not a secondary mutation. In addition to sequencing the relevant segments (ICP27) of both isolates, we utilized Northern blot analysis to confirm that M50T-1 and M50T-2 gave rise to a similar RNA expression profile. We infected Vero cells with KOS1.1, M50T-1, or M50T-2 as indicated. Also where indicated, LMB and/or PAA were included at a concentration of 10ng/mL and 300µg/mL, respectively. At 6 hr. post-infection, nuclear and cytoplasmic RNA was isolated. RNA from equivalent numbers of cells was analyzed for ICP27 and UL44 RNA by Northern blots (Figure 17). As can be observed, M50T-1 and M50T-2 have similar profiles for both of these mRNAs. We could therefore reasonably confirm

that this phenotype of over accumulation of certain mRNAs was due to our primary mutation and not a secondary mutation. A rescue mutant was not engineered as this was the third independent isolate of this mutant with a similar phenotype as measured by resistance to LMB (Murata, Goshima et al. 2001) (Lengyel, Guy et al. 2002).

3.6 Analysis of the effects of LMB on protein expression

We initiated our study to determine whether ICP27 exports viral transcripts from the nucleus through Crm-1. At the time that this study was undertaken, Soliman and Silverstein (Soliman and Silverstein 2000) had observed changes in HSV-1 gene expression by LMB. We proceeded to characterize these changes in gene expression more carefully to determine if they were due to a block in nuclear export. We were able to confirm Murata et al.'s 2001 observation that LMB inhibits KOS1.1 viral DNA replication during infection. Additionally, we observed that LMB inhibits the residual replication of an ICP27 null virus (d27-1) and dLeu, the mutant lacking the putative NES of ICP27 (Figures 11 and 12). Our Northern blot data confirmed that the inhibition of DNA replication was not the result of a block in mRNA export (Figures 14 and 15). We did not observe nuclear accumulation for any but one mRNA examined and that only in dLeu infection. Northern blot analysis confirmed an unusual RNA profile for M50T, the LMB resistant mutant (Figure 15 and 17). Over-accumulation of several transcripts in M50T infection occurred even in the absence of LMB. If this mutant is bypassing the effects of LMB by over-expressing several transcripts, then the proteins encoded by these transcripts should also be over-expressed.

Recent data from Dr. Kimberly Ellison, a research associate in the Smiley lab, has suggested a possible role for ICP27 in the regulation of translation in addition to its role in mRNA processing. Dr. Ellison has observed a difference for mRNA associated with polyribosomes in the presence versus the absence of

ICP27 when ribosomes are separated on a gradient. When ICP27 is present, TK and VP16 transcripts are mostly clustered in fractions of ribosomes thought to be translationally active (polyribosomes). When ICP27 is absent, these same transcripts are found in fractions that are not thought to contain actively translating ribosomes (i.e. single ribosomal subunits). In relation to this study, this observation raises the possibility that LMB may affect viral protein accumulation during HSV-1 infection through effects on the translation function of ICP27. For these reasons, we undertook a detailed analysis of accumulation of several HSV-1 proteins with our four ICP27 mutants, and attempted to correlate mRNA accumulation to protein expression. We asked whether M50T over-accumulated proteins proportionally to its over-accumulation of mRNAs, and whether LMB had any effect on protein accumulation in HSV-1 infection, beyond the inhibitory effect we observed on mRNA accumulation.

We utilized western blot analysis to examine the expression of ICP27, UL30, UL42, VP16 and US11 (Figure 19). Vero cells were infected (MOI of 10 pfu/cell) in the presence of or absence of LMB or PAA as indicated. We harvested infected cells at six hr. post-infection and total cell lysates were resolved by SDS-PAGE. Western blotting was utilized as described previously and proteins were visualized using enhanced chemiluminescence. Blots were subsequently probed for the cellular protein actin as a loading control. Protein bands were quantified using a Storm 860 phosphorimager and normalized to the actin signal. All experiments were done in quadruplicate and data is summarized in Table 2.

3.6.1 The detection system utilized to visualize proteins is approximately quantitative

In order to determine if the detection system (ECL) was quantitative within the range of protein concentrations that we encountered in our assay, a

preliminary Western blot was done. Vero cells were infected with KOS1.1 and harvested at 6 hr. post infection. Samples were prepared as detailed above and separated by SDS-PAGE. One sample was serially diluted and loaded at 0%, 12.5% (1), 25% (2), 50% (3) and 100% (4) of a standard 20µl sample of protein. Western blot analysis was performed probing for the UL42 protein. We visualized our Western using ECL Plus and quantified bands on the phosphorimager. We observed that this detection system was linear for a small portion of the curve (Figure 18). All the samples were prepared in the same manner, and loading was controlled using the cellular protein actin however, protein concentrations that fall at the far ends of the spectrum will not be within a linear range and as such will have a greater range of error.

3.6.2 Protein accumulation at 6 hr p.i.

In KOS1.1 infection, ICP27 appears as two bands on the gel. Both bands were quantified for numerical analysis as both correspond to ICP27. The lower band is most likely a degradation product as it is commonly observed in samples that have been frozen and thawed previously. ICP27 is an IE protein whose expression is not dependent on viral DNA replication and our observations are consistent with previously published reports (Soliman and Silverstein 2000)(Phelan, Dunlop et al. 1996). ICP27 mRNA accumulation was not altered by the addition of LMB and/or PAA at 6 hr. and this was nicely mirrored by protein accumulation. For the UL42 protein in KOS1.1 infection, we observed equal decreases in protein levels in the presence of LMB, PAA, and LMB/PAA to approximately 60-70% of the control KOS1.1 sample containing no drugs (Table 2). At this time point, this does not mirror mRNA accumulation. At 6 hr. p.i. PAA decreased mRNA levels further than LMB (36.5 +/- 8.9% versus 60.5 +/- 21.0%). UL30 protein expression in KOS1.1 at 6 hr. (lanes 2-5) was minimal and therefore not likely to be in the linear range.

We examined VP16, a γ1 gene, for protein expression in wild-type infection. We observed no significant effect by LMB and/or PAA on VP16 protein expression. This was quite unlike what we observed at the level of mRNA between infection in the presence of LMB and infection in the presence of PAA (66.7 +/- 29.3% and 27.0 +/- 10.0% respectively). Six hr. may be too early for this gene product. We therefore examined protein expression of VP16 at 12 hr. p.i. Finally, we examined US11, a true late protein. US11 is completely dependent on DNA replication for expression and as such should not be made this early in infection (Roller and Roizman 1992). US11 is a tegument protein and it is part of the infective virion. As such, it is likely that the bands observed for US11 protein were the result of input virus and not US11 protein expression. This possibility is supported by our observation that LMB and/or PAA did not alter the level of protein. Infection in the presence of cyclohexamide (an inhibitor of protein synthesis) would resolve this issue.

In summary, we observed no significant effect on ICP27, VP16, or UL30 protein expression in KOS1.1 infection in the presence of the drugs we examined. We did observe a decrease in the protein accumulation of UL42 in the presence of LMB and/or PAA. To determine whether this lack of an effect on certain genes is due to the use of a time point too early in infection for good expression of γ1 and γ2 proteins, a 12 hr experiment was included in this study.

The processivity factor of the viral polymerase, UL42, is an E protein that is required for viral DNA replication. However, the expression of UL42 is also greatly boosted by the onset of viral DNA replication because of the increase in viral templates. Therefore, inhibiting viral DNA replication results in a decrease in the level of UL42 protein. In d27-1 infection, protein levels are decreased as compared to levels in KOS1.1. When LMB was added there was no significant change as compared to KOS1.1. With the addition of PAA or LMB/PAA, the protein levels decreased significantly (Table 2). This particular experiment shown here demonstrates the higher range of d27-1 expression of UL42. Other

experiments exhibited significantly lower amounts of UL42 protein. However, even in the lower range of UL42 expression in d27-1 infection, it was still higher than the mRNA accumulation observed. mRNA levels for UL42 were approximately 10% of KOS1.1, which was lower than the 68.7 +/- 28.9% protein observed even with such a large range of error.

We observed that the UL42 protein is slightly larger in d27-1 infection than in wild-type infection. This size difference is minute and perhaps the result of a differential post-transcriptional modification, such as phosphorylation, between the two proteins. UL42 has been shown to be phosphorylated in the carboxyl terminus during some stages of infection by cdc2 cyclin dependent kinase (Advani, Weichselbaum et al. 2001). Secondly, we observed that in d27-1 infection UL42 protein accumulation is significantly decreased by the presence of PAA. This is unlike what is observed in KOS1.1 infection. One possible explanation for this observation may be that when UL42 is in this unusual phosphorylation state the protein becomes less stable. UL42 is required to bind UL30, increase the DNA binding specificity of the polymerase, and decrease the rate of disassociation from the primer template without decreasing the elongation rate (Weisshart, Chow et al. 1999). One could possibly imagine that if the ability of UL42 to perform its function is compromised (by unusual phosphorylation), then perhaps the addition of PAA may further abrogate DNA replication and as such decrease UL42 stability.

UL30 protein expression in d27-1 infection was barely above background levels, and as such it is difficult to evaluate whether or not LMB or PAA had inhibitory effects on protein expression (Table 2). Interestingly, UL30 mRNA levels in d27-1 infection were observed ranging from 21-31% of KOS1.1. VP16 was also examined for protein expression in d27-1 infection. We observed that in d27-1 infection, VP16 protein expression was decreased as compared to KOS. However, it appears that the drugs we utilized have no further effect on the protein accumulation of VP16 in d27-1 infection. The mRNA data mirror this

observation. US11 protein expression was also examined in d27-1 infection. The protein bands that we observed were faint and probably the result of input virus protein. We believe variation between the drug conditions was the result of some variability in the MOI for this infection and not an inherent difference in susceptibility to the drugs (Table 2). An experiment to confirm that the US11 observed was input virus would be to infect cells in the presence of cyclohexamide with a range of MOI to determine whether this effect could be titred away.

In summary, in d27-1 infection we observed first a change in the mobility of the UL42 protein versus wild type. Second, a decrease in protein expressed. Lastly, LMB and/or PAA did not appear to have any effect on UL30, Vp16, and US11 protein expression. dLeu was the third mutant whose protein expression we characterized. This mutant is lacking residues 6-19 of ICP27, the putative region of the NES. ICP27 protein levels appear to be quite similar to what is observed in KOS1.1 infection and do not appear to be altered by either LMB or PAA (Table 2). The UL42 protein in dLeu infection was observed to be similar to what was observed in KOS1.1 infection (Table 2). In the presence of LMB, UL42 protein levels decreased. PAA had a further effect on protein accumulation, that was mirrored by the combination of LMB and PAA. When compared to the mRNA levels at this time point it is interesting to see that protein expression in dLeu was higher than mRNA levels as compared to wild-type (Table 2). However, this high ratio of protein expression to mRNA level was not observed when PAA and/or LMB were added. We observed a similar phenomenon in dLeu infection with UL42 protein as we observed in d27-1 infection, namely a small difference in the migration of the protein. The possible explanations for this are as discussed previously. UL30 protein expression in dLeu infection (lanes 10-13) appeared unaffected by LMB or PAA (Table 2). VP16 was also examined for protein expression in dLeu infection. We observed that in dLeu infection VP16 protein expression was decreased as compared to KOS1.1 (Table 2). The

drugs we utilized appear to have no effect on the protein accumulation of VP16 in dLeu infection. The mRNA levels observed for VP16 in dLeu infection were also not altered by LMB or PAA, consistent with the protein data. In dLeu infection, VP16 mRNA ranges from 10-18% of wild type, while protein ranges from 39-50% of wild type. When we evaluated US11 protein expression in dLeu infection, we observed very faint protein bands that once again we believe are probably the result of input virus protein (Table 2).

In summary, in dLeu infection LMB does not have an observable effect on protein accumulation for any of the proteins that we examined except for UL42. Additionally, PAA and LMB/PAA had a more significant effect on UL42 protein accumulation that LMB alone mirroring what was observed in d27-1 infection.

The final mutant in our panel was M50T. mRNA data demonstrated that M50T over-accumulated several transcripts raising the possibility that this mutant bypasses the inhibitory effects of LMB. If this is the case, then we believe that the proteins encoded by these transcripts should also be over-expressed. ICP27 expression in M50T infection is very similar to that in KOS1.1 (Table 2). Neither LMB nor PAA had any effect on protein accumulation, mirroring what we observed for mRNA. When the expression of UL42 was examined, we observed an overall increase in protein expression. UL42 protein levels in M50T infection were higher than in KOS1.1 infection (Table 2). This level of protein accumulation was unaffected by LMB and PAA. This mutant has been demonstrated to be resistant to LMB but has not been shown to be resistant to PAA for DNA replication and mRNA expression. It appears that the high levels of mRNA we observed for UL42 in M50T infection although inhibited by both LMB and PAA are sufficient for an over-accumulation of UL42 protein.

In M50T infection, protein levels of UL30 were increased as compared to its level in KOS1.1 infections. With the addition of LMB, PAA or LMB/PAA, the protein levels remained constant (Table 2). This observation for UL30 protein is unusual, as mRNA levels were not over-expressed in M50T infection. This

suggests that UL30 protein accumulation was upregulated in M50T infection. This may imply that the mutation in the acidic region of ICP27 may have the dual ability to up regulate both transcription and translation of viral gene products, or alter RNA and protein processing or stability. Alternatively, this may suggest that ICP27 is (in this altered form) acting on a viral or cellular protein which regulates the translation or degradation of UL30. Either possibility is equally likely. These observations also suggest that UL30 is an unusual gene, in that is regulated in a manner different from all other genes that we have examined.

VP16 was observed to over-accumulate as compared to its levels in KOS1.1 infections (Table 2). We observed that LMB, PAA, or LMB/PAA had some minor effect on the levels of this protein (Table 2). This does not mirror what we observe at the mRNA level. Interestingly, PAA decreases the level of RNA accumulation to below what is observed in KOS1.1 infection (Table 2). However, protein expression even in the presence of PAA is almost twice what is observed in wild-type infection, further supporting a role for ICP27 in the regulation of viral protein accumulation.

The final transcript that we examined was US11. At 6 hr., we did not observe any expression of this late protein in any of our mutants except M50T (lanes 14-17). As this is a very early time for this protein to be expressed, it raises the possibility that this mutation may uncouple US11 gene expression from DNA replication or conversely that this mutation increases the rate of progression of infection (ie replication occurs earlier than in wild-type virus). We observed nearly a four-fold increase in US11 expression over KOS1.1 (Table 2). Protein accumulation was unaffected by the addition of LMB, while the addition of PAA and LMB/PAA decreased the levels of this protein significantly (Table 2).

In summary, M50T exhibits an increased expression of several proteins that were examined, although this is not a direct result of increasing the amount of available transcripts in all cases. For example, UL30 transcript accumulation

was not increased in M50T infection, while UL30 protein accumulation nearly tripled.

3.6.3 Protein accumulation at 12 hr. p.i

As mentioned previously, we had examined VP16, a γ1 gene, for protein expression in wild-type infection at 6 hr. and observed that LMB and PAA did not decrease the levels significantly. We found this to be quite unlike what we observe at the level of mRNA between infection in the presence of LMB and infection in the presence of PAA (66.7 +/- 29.3% and 27.0 +/- 10.0% respectively). A possible explanation for this disparity was the early time point examined. A 12 hr. time point in infection was examined in this study for VP16 and US11, the two late genes that we surveyed (Figure 20, Table 3). As we had no 12 hr RNA data, the protein accumulation data at 12 hr cannot be compared to mRNA.

US11 protein expression was examined in KOS1.1 infection. At this later time point, LMB decreased levels of protein, as did PAA and PAA/LMB in combination (Table 3). VP16 was also examined for protein expression in KOS1.1 infection. We observed multiple bands on our Western blot due to the sensitivity of this protein to degradation. The progressive degradation of this protein can be observed after repeated freezing and thawing. In our quantification, all the bands were included in the analysis, as all are specific to VP16. In the presence of LMB, PAA, and LMB/PAA, the level of accumulation of VP16 protein is decreased equally as compared to KOS1.1 in the absence of drugs (Table 3). These drugs have a much more significant effect on protein accumulation of VP16 at 12 hr. p.i. than at 6 hr.

In d27-1 infection, protein levels of US11 decreased as compared to levels in KOS1.1. When LMB, PAA, or LMB/PAA was added, there was no significant change. When we examined VP16 expression in d27-1 infection, we observed a

decrease in protein expression as compared to wild type. In the presence of LMB and PAA, protein accumulation decreased. This was also observed when LMB and PAA were added together. However, there was no further decrease beyond what was observed with LMB or PAA (Table 3). This is a significant decrease over what is observed at 6 hr post infection.

We next examined US11 and VP16 protein expression in dLeu infection. US11 protein levels in dLeu infection (lane 9) were observed at 62.0 +/- 8.0% of KOS1.1. LMB decreased protein levels, as did PAA and PAA/LMB in combination (Table 3). We observed that in dLeu infection VP16 protein expression was decreased as compared to wild type (Table 3). In the presence of LMB, the levels of this protein further decreased (Table 3). PAA even further decreased the levels of VP16. However, the two drugs used in combination exhibited no difference from PAA alone. The protein expression that we observed for VP16 in dLeu infection at 12 hr. was similar to what we observed at 6 hr.

US11 expression in M50T infection was slightly decreased compared to KOS1.1 (Table 3). The addition of LMB had very little effect, while the addition of PAA and LMB/PAA resulted in a large decrease as compared to wild-type.

The final gene that we examined for protein expression was Vp16 in M50T infection. We observed that LMB had no effect on the levels of this protein (Table 3). The addition of PAA and LMB/PAA had a slightly larger effect on these levels (Table 3). It is interesting that the high level of over-expression that we observe for US11 and Vp16 in M50T-1 infection at 6 hr. p.i is essentially gone by 12 hr. This again raises the possibility that this mutant has a more rapid course of infection than KOS1.1. However, a time course of infection would have to be performed to determine whether this was in fact the case.

In summary, we observed a larger effect of LMB and PAA on Vp16 and US11 protein expression in KOS1.1 and dLeu infection at 12 hr. p.i. It appeared

that the over-expression of protein that we observed at six hr. by M50T was not obvious at 12 hr.

3.7 Ratio of protein to mRNA at 6 hr.

Due to an observed disparity between RNA accumulation and protein accumulation for several genes, we examined directly the ratio of RNA to protein at 6 hr. post infection. RNA and protein amounts were reported as a percent of KOS in the absence of any drugs. Therefore, we compare only relative changes in RNA to protein levels. The percent value we observed at 6 hr. for protein was divided by the percent value we observed for mRNA and these ratios are listed in Table 4. The ratio of protein to RNA that was observed for KOS was designated 1. A ratio greater than one indicated a high level of protein that did not correlate to a high level of RNA. If the ratio was less than one, then there was a low level of protein in a situation where RNA levels had not decreased correspondingly. We observed a significant range of error in both the mRNA and protein data. The range of error coupled with the indirect nature of this comparison understandably allows only for statistical analysis that is limited in its usefulness. However, we chose to further examine instances where the difference in the ratio between the mRNA and protein was three fold or higher. This was an arbitrary designation and these values were subjected to the t-test and a p value assigned. By standard convention, $p < 0.05$ was deemed significant. For a comprehensive explanation of the statistical analysis utilized, refer to section 2.12 in the materials and methods section. We collected this data at six hr. too early to get a true picture of late gene expression as indicated by the differences between the 6 hr. and 12 hr. protein expression data for VP16. Therefore, the data collected for VP16 was deemed inconclusive and was excluded from this comparison. Additionally, we had no US11 mRNA data to compare to protein

levels. Therefore, we examined the ratio of protein to RNA for ICP27, UL30, and UL42 in KOS1.1, dLeu, d27-1, and M50T-1 infection.

In KOS1.1 infection, we did not observe any transcript, under any drug conditions, where the difference between the percent of RNA and protein was 3 fold or higher (Table 4). In d27-1 infection, we did observe what we believe to be evidence for a translational regulation function for ICP27. For ICP27, the ratio was obviously not calculated, as d27-1 is an ICP27 null virus. In the absence of ICP27 (d27-1 infection), we observed significant changes in the ratio of protein to RNA for other genes. For UL42, the ratio was 6.87 ($p = 0.0107$), in the presence of LMB was 10.7 ($p = 0.0210$). In the presence of PAA, the ratio was 3.2, and in the presence of both LMB and PAA, it was 3.14. Our evidence, although indirect, seems to suggest a down-regulatory function for ICP27 in the translation of UL42 protein.

Statistical analysis for the UL30 gene in the absence of ICP27 was difficult as the mRNA levels bordered our detection limits. However, when no drugs were added the ratio was 0.6. When LMB was added, the ratio was 0.19. In the presence of PAA, the ratio could not be determined (mRNA was below the level of detection). When the two drugs were used in combination, the ratio was 0.27. This demonstrates a two- to four-fold decrease for protein to RNA. This observation does not strictly fit into our three-fold criterion but was believed to be significant as it was so diametrically opposed to the result for UL42 in the absence of ICP27. However, when the p values were assessed for UL30 in the absence of any drugs and in the presence of LMB they were found to be not significant.

In dLeu infection, the ratios of RNA to protein for ICP27 were not significant. For UL42, the ratio was 4.89 ($p = 0.0015$) in the absence of drugs, 3.98 ($p = 0.0298$) in the presence of LMB, 1.0 in the presence of PAA, and 1.02 in the presence of both LMB and PAA. For UL30, the ratio was 0.25 in the

absence of drugs, 0.13 in the presence of LMB, and not determined in the presence of PAA and both LMB and PAA. For UL30, the ratios demonstrated a four- to seven-fold decrease in protein. Statistical analysis, however, determined these values not to be significant.

In M50T infection, for ICP27 and UL42, we did not observe significant changes in the ratio of RNA to protein (Table 4). One final example of disparity for protein to RNA was in M50T-1 infection. The ratio of UL30 protein to RNA in M50T-1 infection was 3-4 fold higher than in KOS infection. For UL30 in M50T infection, the ratio was 2.87($p = 0.0434$) in the absence of drugs, 3.91($p = 0.0339$) in the presence of LMB, 4.46 ($p = 0.0583$) in the presence of PAA, and 4.07 ($p = 0.0160$) in the presence of both LMB and PAA. What is interesting about UL30 in M50T-1 infection is that it may allow us to separate a role for ICP27 in RNA accumulation and protein accumulation.

In summary, we observed several instances where it appeared that there was a disparity between the level of RNA vs. protein accumulated. This may point to a role for ICP27 in translation or protein accumulation.

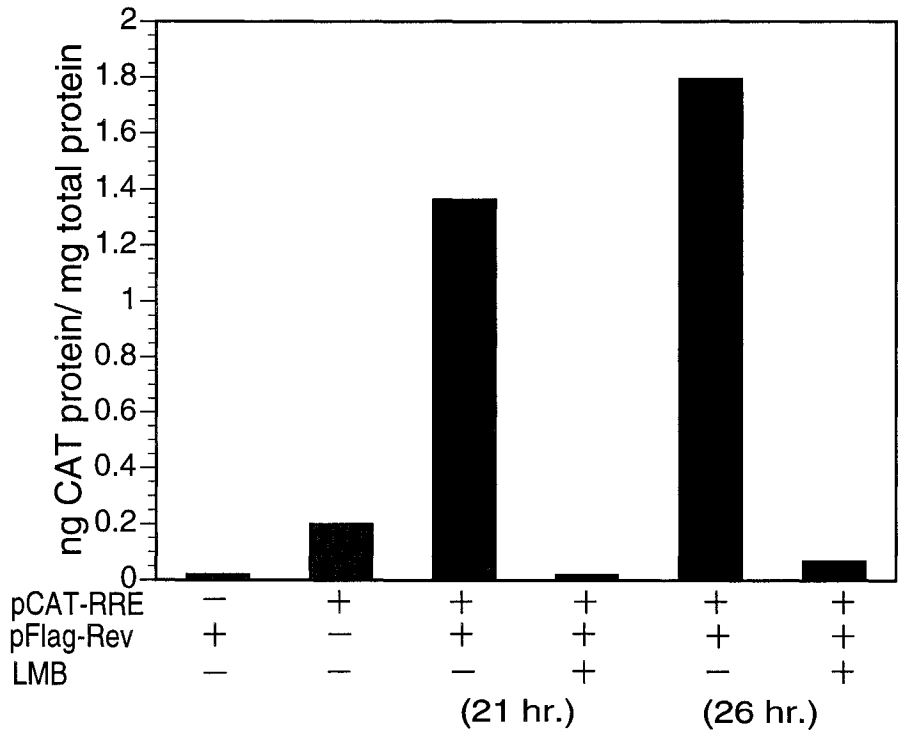

Figure 7
LMB inhibits Rev activity to less than 4% of wild-type levels. Transfection assay was performed to confirm efficacy of LMB. Vero cells were transfected with the plasmid constructs as indicated in the presence and absence of LMB (10ng/ml). At the time indicated post-transfectionn cells were harvested and a CAT-ELISA assay was performed. CAT protein concentration was normalized to total cellular protein as determined by the BCA protein assay and was utilized as an indirect measure of Rev activity. Our stock of LMB was effective at reducing Rev activity to less than 4% of wild-type levels and thus fully functional.

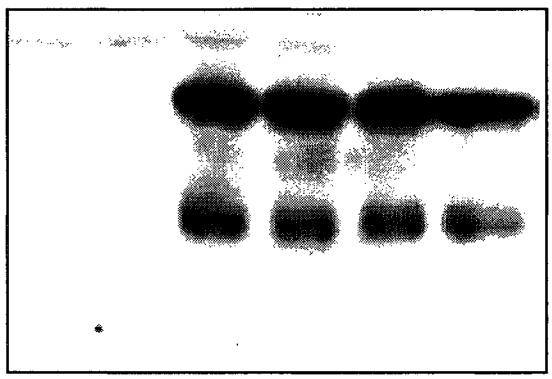

Figure 5
Expression of Rev protein is unaffected by LMB.
Vero cells were transfected with the plasmid constructs as indicated and protein was harvested at 21 hr. p.i. LMB was added at a concentration of 10ng/ml. Western blot analysis using the anti-FLAG antibody was utilized to detect the tagged Rev protein. The larger band corresponds to the Rev protein (18kDa). This confirmed that LMB was inhibiting Rev function,but not Rev expression.

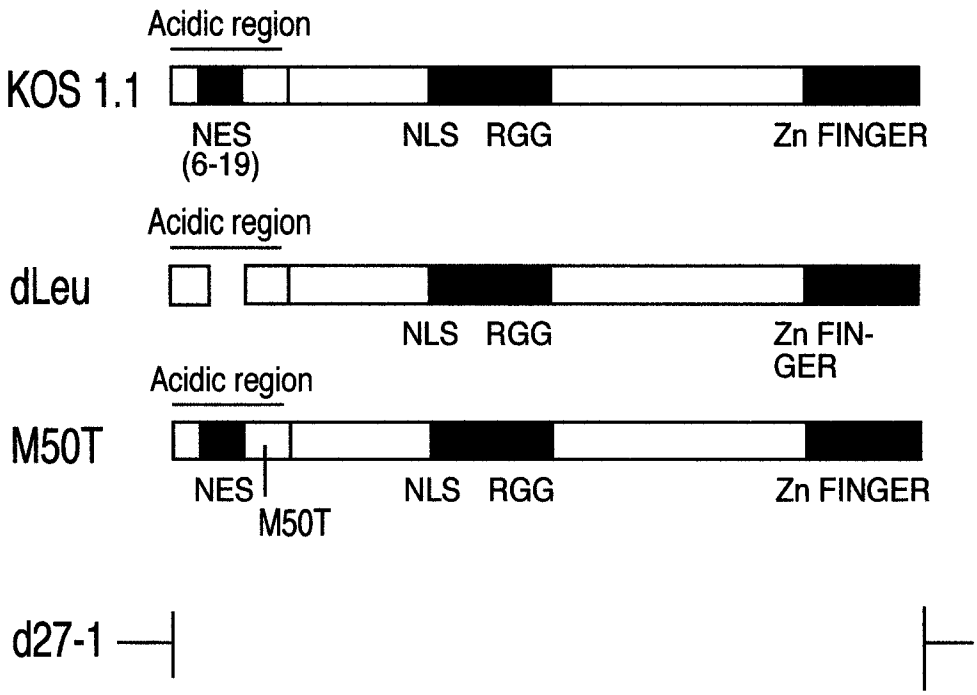

Figure 9
HSV-1 ICP27 mutants
KOS 1.1 was used as the wild-type strain of HSV-1 in these studies containing a full, non-altered ICP27 region. dLeu is a viral mutant lacking residues 6-19 resulting in the deletion of the Nuclear Export Sequence (NES). M50T is engineered to contain a two bp substitution (ACG-CCG) at residue 50 of ICP27 that converts a met to thr. d27-1 contains a deletion in ICP27 and was used as the ICP27 null virus. NES (Nuclear Export Sequence), NLS (Nuclear Localization Sequence), RGG (Arginine Rich Region).

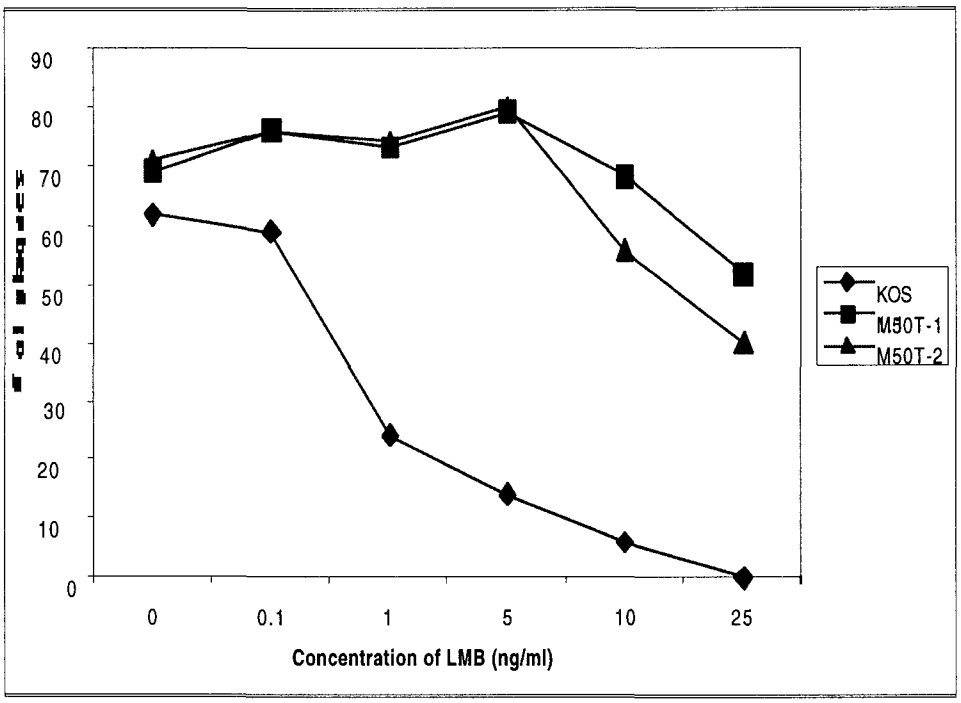

Figure 10A
Mutant M50T is resistant to LMB
A) The phenotype of two independent isolates of M50T was confirmed by plaque reduction assay. Each well of a 6 well dish was infected with 60 pfu of the virus indicated in the presence of varying concentrations of LMB as indicated. Plaques were counted at 48 hrs. post-infection. Both M50T-1 and M50T-2 were resistant to inhibition of plaque formation by LMB upto a concentration of 25 ng/mL, the highest concentration we utilized.

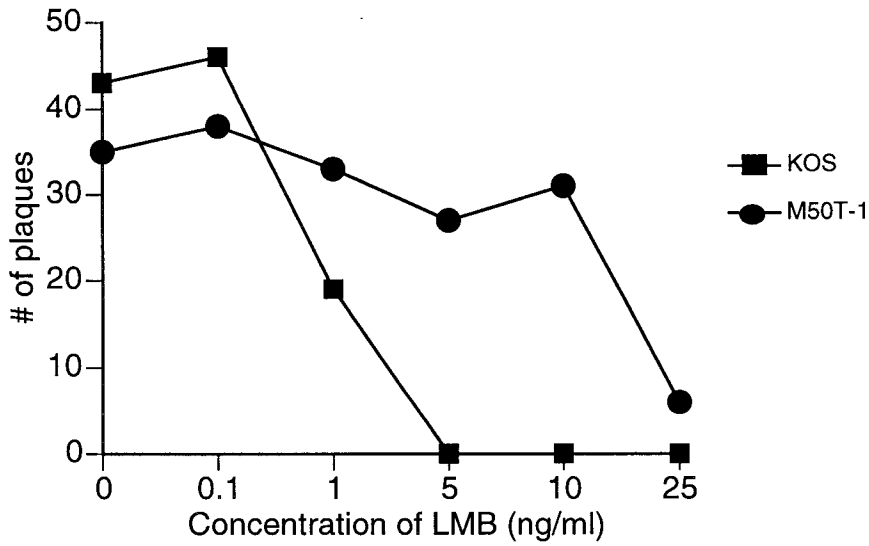

Figure 10B
Mutant M50T is resistant to LMB
B) Some variability exists between experiments. Each well of a 6 well dish was infected with 50 pfu of the virus indicated in the presence of varying concentrations of LMB as indicated. Plaques were counted at 48 hrs. post-infection. The concentration of LMB required to fully inhibit plaquing of KOS 1.1 and M50T is slightly variable although the IC50= 1ng/mL remains identical.

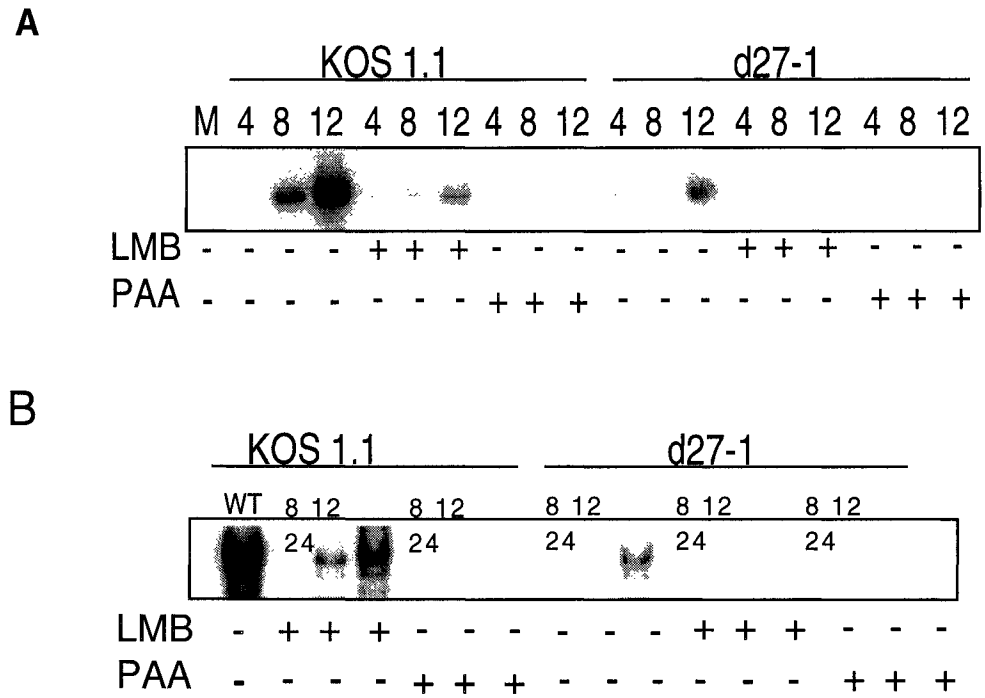

Figure 11
Viral DNA replication by KOS 1.1 and d27-1 is inhibited by LMB. Viral DNA replication is inhibited by LMB in the absence of ICP27 protein. A) Southern blot analysis of Vero cells infected at a multiplicity of infection of 10 with the indicated virus. Where indicated, LMB and/or PAA were included at a concentration of 10ng/ml and 300ug/ml respectively. At 4, 8, and 12 hr. post infection total cellular DNA was isolated, digested with BamHI and separated on a 1% agarose gel. DNA was visualized using a TK-gene fragment ^{32}P-labeled probe. B) Southern blot analysis done in a similar manner as panel A at 8, 12, and 24 hr. p.i.

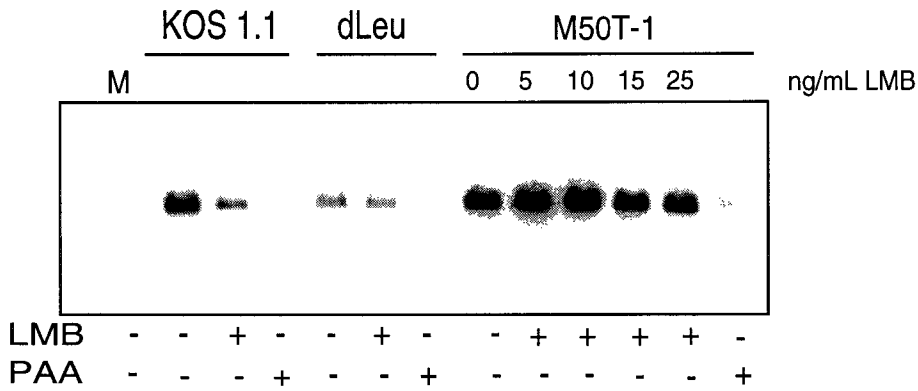

Figure 12
Viral DNA replication by dLeu is marginally affected by LMB while M50T is resistant to LMB.
Viral DNA replication is inhibited by LMB in the absence of ICP27 protein. Vero cells were infected at a multiplicity of infection of 10 with the indicated virus. Where indicated, LMB and/or PAA were included at a concentration of 10ng/ml and 300ug/ml respectively. At the times indicated post infection total cellular DNA was isolated, digested with BamHI and separated on a 1% agarose gel. Southern blotting was done using a TK-gene fragment 32P-labeled probe.
Viral DNA replication of dLeu is slightly inhibited by LMB to approximately 49% of dLeu replication in the absence of LMB.
Viral DNA replication of M50T-1 is unaffected by LMB.

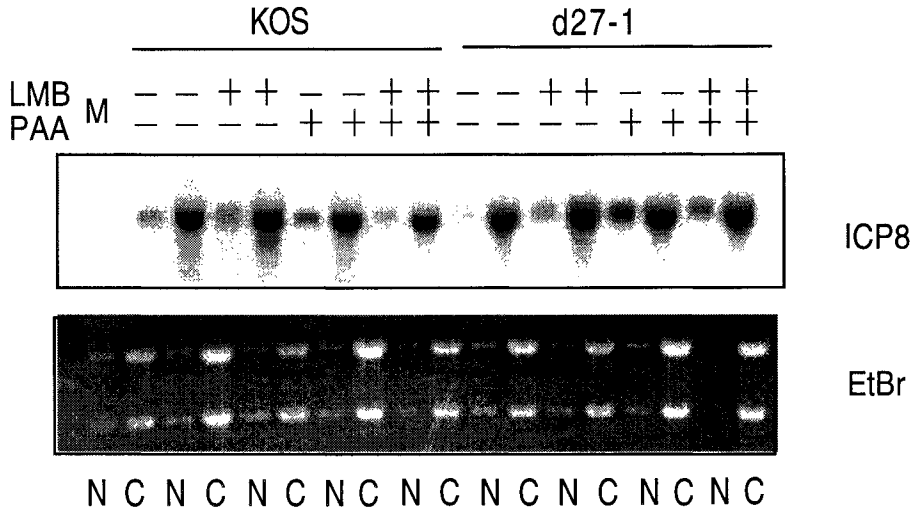

Figure 13
The expression and localization of ICP8 mRNA is ICP27 independent.
Vero cells were infected with KOS1.1 and d27-1 mRNA awas harvested and fractionated at 6 hrs. p.i. RNA bands were probed for ICP8 mRNA and were subsequently quantified on the phosphorimager. Ethidium bromide staining of ribosomal bands indicated even loading.

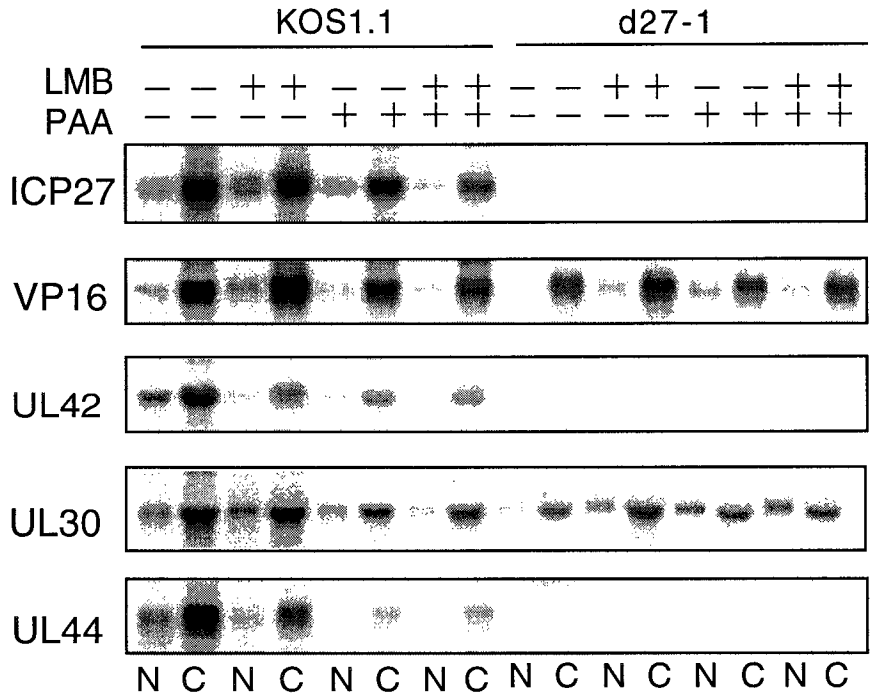

Figure 14
LMB alters viral RNA expression of KOS 1.1 and d27-1 but does not alter the nuclear/cytoplasmic distribution of these RNAs.
Northern analysis of viral RNA levels. Vero cells were infected with the indicate virus at an MOI of 10 in the presence or absence of LMB or PAA (10ng/ml and 300ug/ml respectively) as indicated. At 6 hours p.i nuclear (N) and cytoplasmic (C) RNA was isolated. RNA from equivalent numbers of cells was analyzed for ICP27, VP16, UL42, UL30, and UL44 RNA by Northern blot. Quantification of bands on all Northern blots was carried out using a Storm phosphorimager. Data is quantified and shown in Table 1.

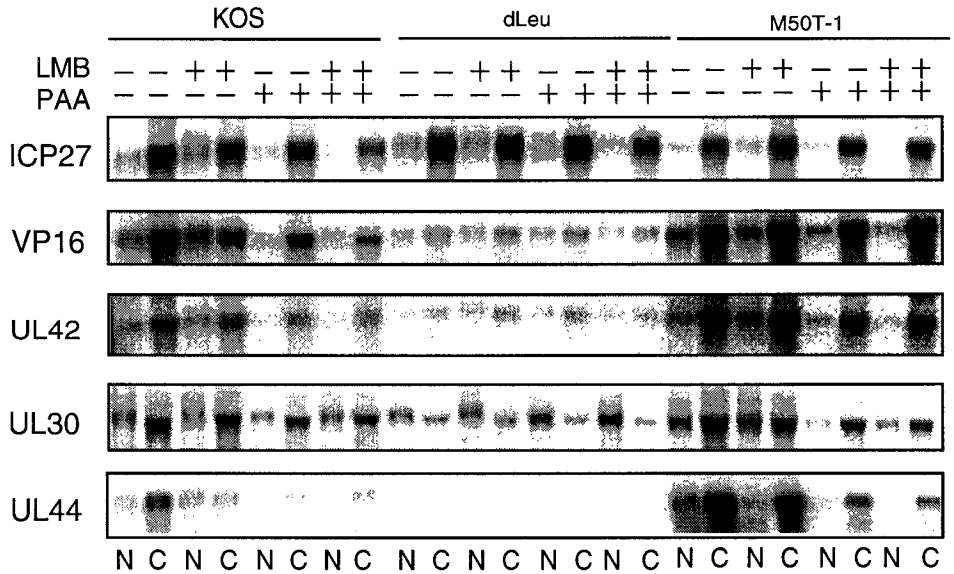

Figure 15
LMB alters viral RNA expression of dLeu and M50T but does not alter the nuclear/cytoplasmic distribution of these RNAs.
Northern analysis of viral RNA levels. Vero cells were infected with the indicate virus at an MOI of 10 in the presence or absence of LMB or PAA (10ng/ml and 300ug/ml respectively) as indicated. At 6 hours p.i nuclear (N) and cytoplasmic © RNA was isolated. RNA from equivalent numbers of cells was analyzed for ICP27, VP16, UL42, UL30, and UL44 RNA by Northern blot. Quantification of bands on all Northern blots was carried out using a Storm phosphorimager. Data is quantified and shown in Table 1.

Virus	Drug	VP16 rel. amount[a]	VP16 % of total[b]	UL42 rel. amount[a]	UL42 % of total[b]	UL44 rel. amount[a]	UL44 % of total[b]
WT (KOS1.1)	-	100	75.3 +/- 4.9	100	79.2 +/- 5.1	100	82.7 +/- 3.8
	LMB	66.7 +/- 29.3	71.7 +/- 2.9	60.5 +/- 21.0	71.2 +/- 6.4	31.3 +/- 6.3	75.0 +/- 3.0
	PAA	27.0 +/- 10.0	77.3 +/- 6.4	36.5 +/- 8.9	76.0 +/- 6.4	8.3 +/- 1.5	72.0 +/- 2.6
	LMB + PAA	21.3 +/- 14.6	83.0 +/- 1.0	25.1 +/- 8.6	76.2 +/- 5.0	8.7 +/- 2.1	77.0 +/- 4.0
d27-1	-	21.7 +/- 9.6	83.7 +/- 7.1	10.0 +/- 8.1	84.3 +/- 6.0	NQ	NQ
	LMB	30.3 +/- 9.5	83.7 +/- 4.0	6.4 +/- 4.5	80.3 +/- 7.3	NQ	NQ
	PAA	31.0[c]	80.0[c]	2.5[c]	75.2[c]	NQ	NQ
	LMB + PAA	29.3 +/- 7.0	79.3 +/- 4.1	3.5 +/- 1.3	77.0 +/- 1.0	NQ	NQ
dLeu	-	16.0+/- 14.1	75.3 +/- 7.6	17.7 +/- 6.0	66.1 +/- 1.3	NQ	NQ
	LMB	10.7 +/- 9.5	62.3 +/- 2.1	11.8+/- 5.2	54.3 +/- 8.9	NQ	NQ
	PAA	14.3 +/- 10.7	75.0 +/- 14.1	13.3 +/- 2.5	55.3 +/- 7.4	NQ	NQ
	LMB + PAA	18.0+/- 9.6	69.7 +/- 8.5	13.7 +/- 4.7	61.7 +/- 11.2	NQ	NQ
M50T (HB01)	-	187.1 +/- 11.9	77.3 +/- 2.9	185.0+/- 52.1	77.3 +/- 1.2	358.0 +/- 141.6	79.7 +/- 2.5
	LMB	147.0 +/- 53.2	74.3 +/- 4.5	140 .0+/- 39.1	76.0 +/- 7.5	153.7 +/- 16.1	68.7 +/- 7.1
	PAA	84.7 +/- 33.0	79.7 +/- 4.2	88.7+/- 26.0	75.3 +/- 5.1	42.3 +/- 20.5	66.7 +/- 7.6
	LMB + PAA	85.8 +/- 44.8	86.3 +/- 5.5	67.7 +/- 27.3	79.7 +/- 7.3	43.0 +/- 10.6	81.6+/- 3.8

Table 1A: Effects of LMB and PAA on the cytoplasmic accumulation of viral mRNAs.
Statistical analysis of Northern data for VP16, UL42, and UL44.
a - amount of mRNA in cytoplasm relative to WT virus. b - % of the total mRNA that is cytoplasmic
c - average of 2 experiments NQ - not quantifiable ND - not determined

		ICP27		UL30	
Virus	Drug	rel. amount[a]	% of total[b]	rel. amount[a]	% of total[b]
WT (KOS1.1)	-	100	86.0 +/- 3.1	100	72.0 +/- 4.4
	LMB	80.3+/- 11.2	78.3 +/- 5.2	64.5 +/- 14.0	61.1+/- 12.2
	PAA	77.0 +/- 16.8	82.7 +/- 3.5	52.1 +/- 13.0	72.0 +/- 11.0
	LMB + PAA	69.3 +/- 35.9	83.3 +/- 4.1	51.3 +/- 13.7	57.7+/- 4.9
d27-1	-	NQ	NQ	21.4 +/- 2.7	76.7 +/- 6.1
	LMB	NQ	NQ	31.4 +/- 5.2	69.3 +/- 5.5
	PAA	NQ	NQ	22.1	62.8
	LMB + PAA	NQ	NQ	25.9 +/- 6.3	64.2 +/- 3.7
dLeu	-	167.0 +/- 39.6	84.3 +/- 7.2	24.0 +/- 7.9	34.0 +/- 10.8
	LMB	134.0 +/- 61.3	77.7 +/- 7.1	27.3 +/- 7.6	33.3 +/- 8.5
	PAA	147.2 +/- 50.8	82.7 +/- 9.4	21.7 +/- 5.6	27.7 +/- 5.5
	LMB + PAA	77.0 +/- 22.5	78.0 +/- 12.1	17.3 +/- 4.0	26.3 +/- 8.0
M50T (HB01)	-	106.0 +/- 33.8	81.6 +/- 5.9	100.0 +/- 19.7	65.7 +/- 5.8
	LMB	92.3 +/- 26.5	79.3 +/- 7.2	68.4+/- 15.6	57.0 +/- 17.3
	PAA	85.3 +/- 36.2	84.0 +/- 2.7	56.3c	76.5[c]
	LMB + PAA	65.0 +/- 20.4	84.7 +/- 8.4	53.3 +/- 17.5	74.5c

Table 1B: Effects of LMB and PAA on the cytoplasmic accumulation of viral mRNAs.
Statistical analysis of Northern data for ICP27 and UL30.
a - amount of mRNA in cytoplasm relative to WT virus. b - % of the total mRNA that is cytoplasmic
c - average of 2 experiments NQ - not quantifiable ND - not determined

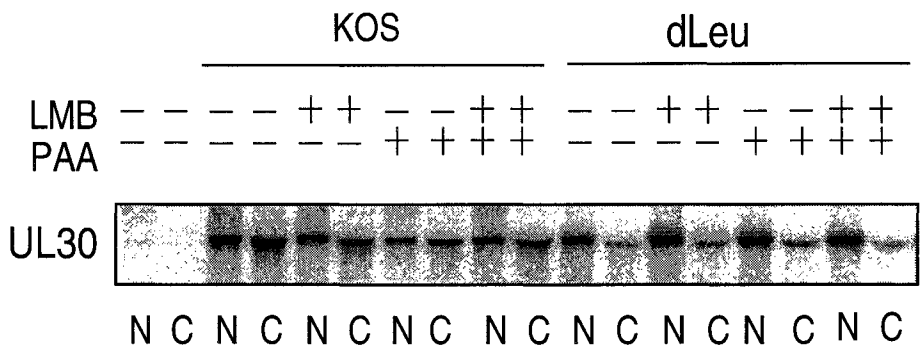

Figure 16
UL30mRNA demonstrates nuclear accumulation in dLeu infection.
Vero cells were infected with KOS1.1 and dLeu mRNA was harvested and fractionated as per protocol at 6 hrs. p.i. Northern blot analysis was performed and probed for UL30. Bands were visualized and quantified on the phosphor imager.

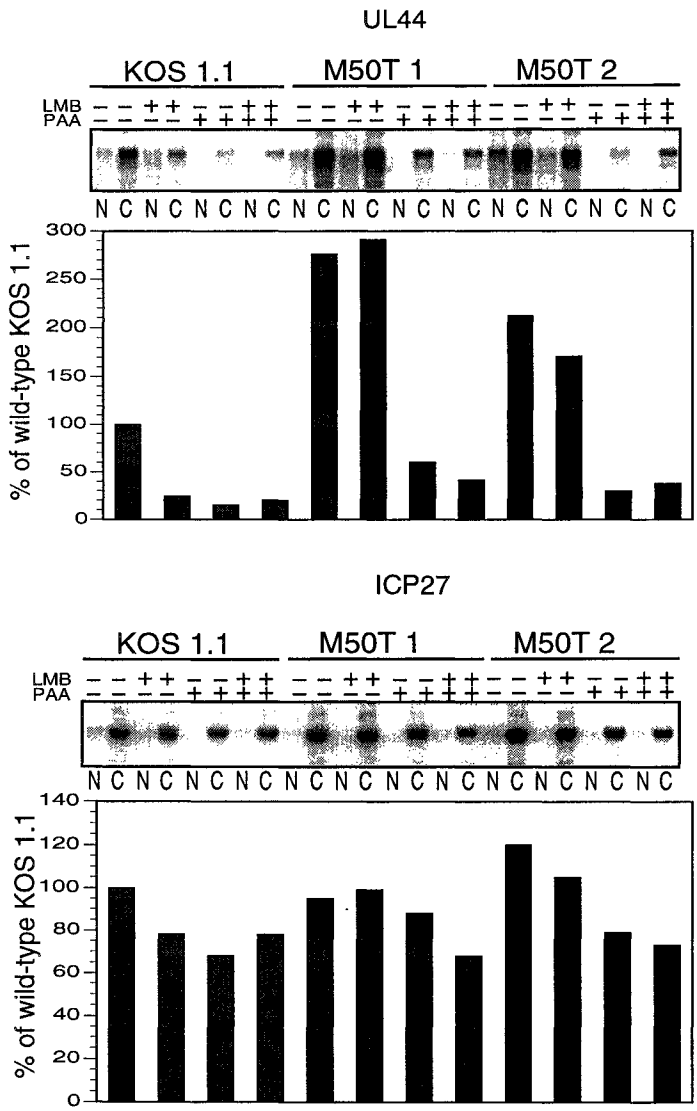

Figure 17
M50T exhibits an over-expression of several viral RNAs.
Northern blot analysis of two independently isolated M50T mutants confirms mutant phenotype. Vero cells were infected with KOS 1.1, M50T-1, or M50T-2 as indicated. At 6 hours p.i N/C RNA was isolated. RNA from equivalent numbers of cells was analyzed for ICP27 and UL44 RNA by Northern blot to confirm mutant phenotype.

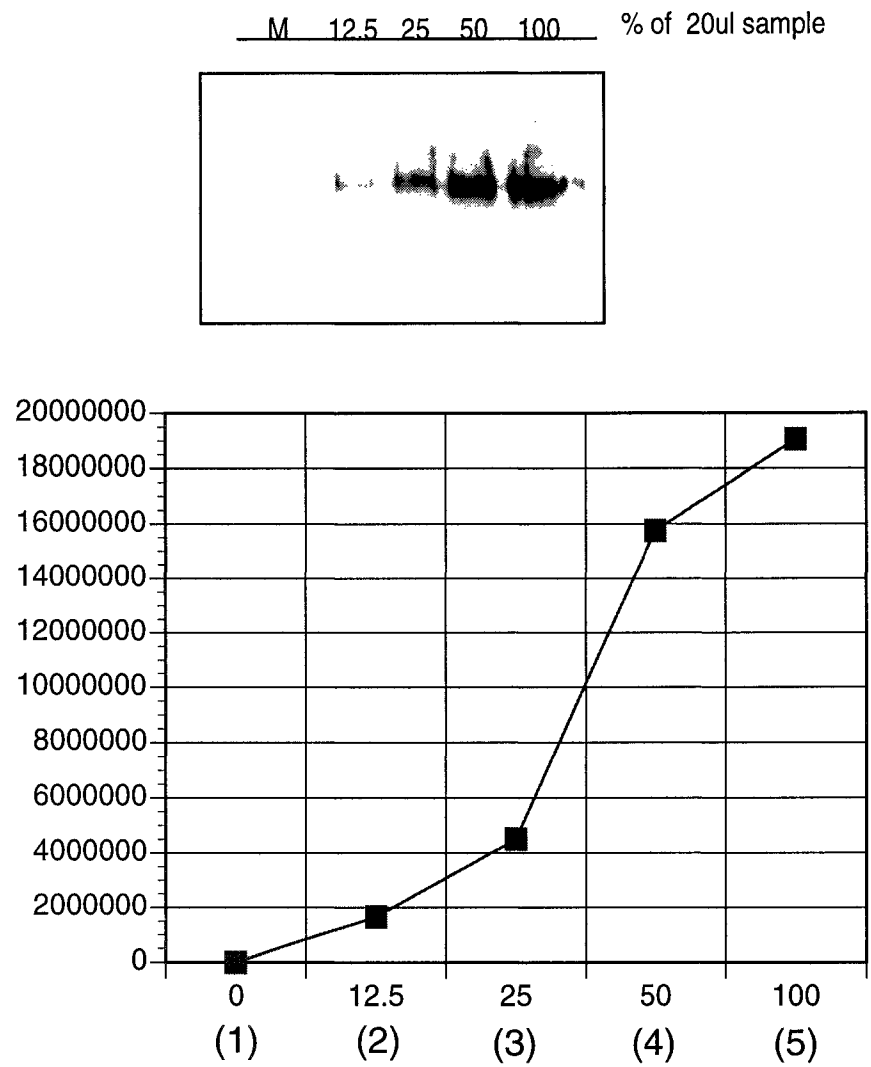

Figure 18
Detection system utilized to visualize proteins is quantiative within the linear range.
Vero cells were infected with KOS1.1 and harvested at 6 hrs. p.i. Samples were serially diluted and separated by SDS-PAGE. Western blot analysis was performed probing for the UL42 protein. Western was visualized using ECL Plus and the bands were quantified on the phosphorimager .

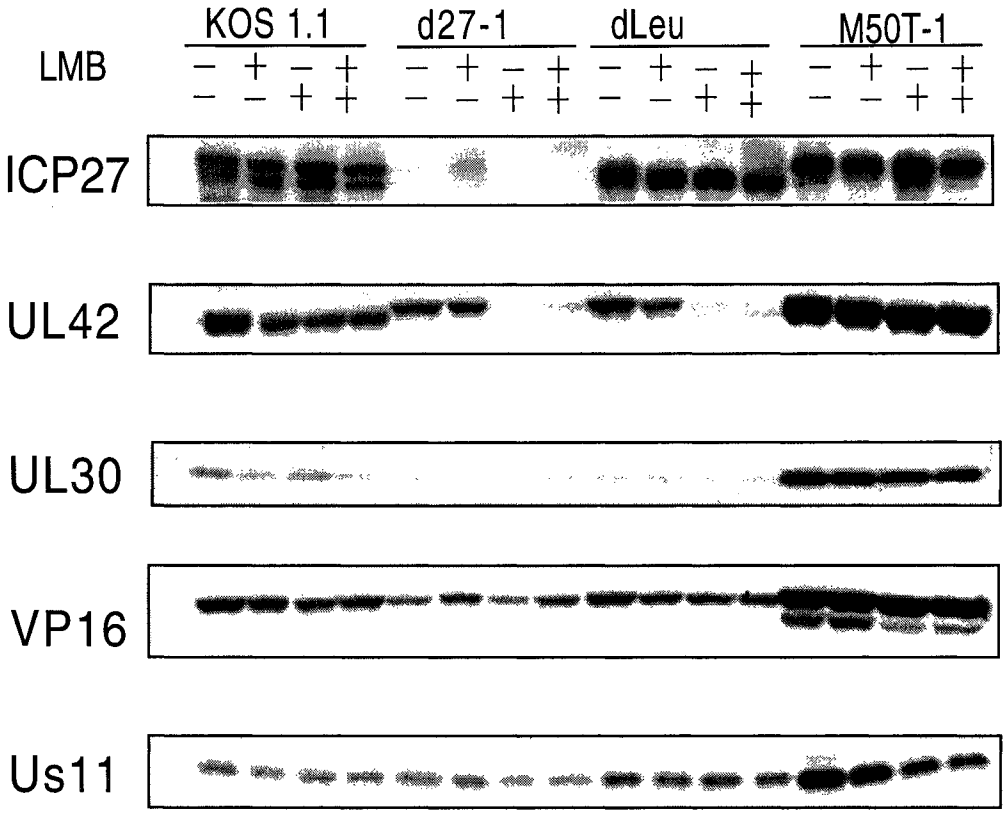

Figure 19
LMB alters viral protein expression at 6 hrs post-infection.
Vero cells were infected with wt HSV-1 or ICP27 mutants as indicated at an MOI of 10. Where indicated, LMB and/or PAA were included at a concentration of 10ng/ml and 300ug/ml respectively. Total cellular protein was isolated at 6 hours post-infection from infected cells and separated by SDS-PAGE. Antibodies to ICP27, UL42, UL44, UL30, us11 and VP16 were used to detect viral proteins. Actin was detected as a loading control and all protein quantification was normalized to the actin signal. Quantification on all western blots was carried out using a Storm 860 phosphorimager. Data obtained from these western blots is shown in Table 2.

Virus	Drug	VP16 rel. amount[a]	UL42 rel. amount[a]	us11 rel. amount[a]	ICP27 rel. amount[a]	UL30 rel. amount[a]
WT (KOS1.1)	-	100	100	100	100	100
	LMB	81.3 +/- 8.14	62.0 +/- 10.4	78.7 +/- 12.0	79.0 +/- 11.0	74.0 +/- 19.5
	PAA	74.3 +/- 16.6	71.3 +/- 14.5	98.0 +/- 18.0	82.7 +/- 13.6	83.7 +/- 15.5
	LMB + PAA	78.7 +/- 8.1	65.7 +/- 3.2	80.7 +/- 9.3	73.0 +/- 4.6	76.0 +/- 15.1
d27-1	-	32.7 +/- 7.1	68.7 +/- 21.0	98.0 +/- 5.0	0	12.7 +/- 11.2
	LMB	35.0 +/- 13.2	68.7 +/- 28.9	77.7 +/- 21.6	0	6.0 +/- 10.4
	PAA	19.0 +/- 10.0	8.0 +/- 11.4	54.7 +/- 10.6	0	NQ
	LMB + PAA	36.7 +/- 10.0	11.3 +/- 14.0	64.8 +/- 10.1	0	7.0 +/- 12.1
dLeu	-	50.3 +/- 25.8	86.7 +/- 14.2	115.0 +/- 25.5	95.3 +/- 20.5	6.0 +/- 10.4
	LMB	45.3 +/- 12.1	47.0 +/- 17.7	95.1 +/- 23.5	75.3 +/- 23.1	3.7 +/- 6.4
	PAA	39.7 +/- 10.8	13.3 +/- 15.4	116.0 +/- 16.9	73.3 +/- 13.8	NQ
	LMB + PAA	44.0 +/- 15.5	14.0 +/- 14.1	90.7 +/- 15.0	67.7 +/- 6.8	NQ
M50T (HB01)	-	195.0 +/- 44.9	188.0 +/- 51.5	377.0 +/- 86.9	90.7 +/- 34.1	287.0 +/- 109.3
	LMB	225.6 +/- 68.9	195.2 +/- 53.8	329.1 +/- 55.2	106.0 +/- 16.9	268.0 +/- 108.0
	PAA	183.0 +/- 34.7	169.0 +/- 34.1	197.0 +/- 45.0	100.1 +/- 19.6	250.2 +/- 87.1
	LMB + PAA	192.1 +/- 40.5	174.0 +/- 35.5	134.0 +/- 13.6	89.3 +/- 36.1	216.0 +/- 68.1

Table 2: Effects of LMB and PAA on viral protein expression at 6 hrs. p. i
Statistical analysis of Western data for VP16, UL42, us11, ICP27 and UL30.
a - amount of protein is relative to WT virus. NQ - Not Quantifiable.

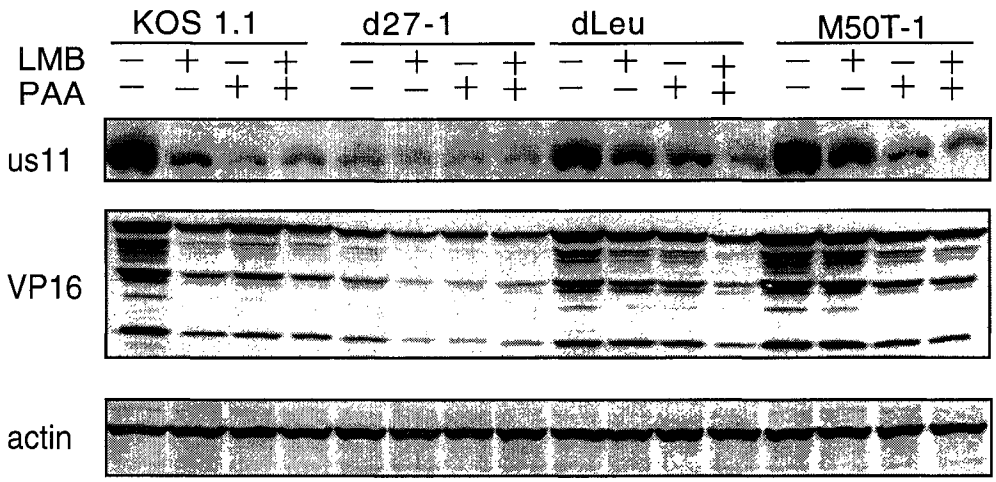

Figure 20
LMB alters viral protein expression at 12 hrs. post-infection in a manner similar to what is predicted in the absence of viral DNA replication.
Vero cells were infected with wt HSV-1 or ICP27 mutants as indicated at an MOI of 10. Where indicated, LMB and/or PAA were included at a concentration of 10ng/ml and 300ug/ml respectively. Total cellular protein was isolated at 12 hours post-infection from infected cells and separated by SDS-PAGE. Antibodies to us11 and VP16 were used to detect viral proteins. Actin was detected as a loading control and all protein quantification was normalized to the actin signal. Quantification on all western blots was carried out using a Storm 860 phosphorimager. Data obtained from these western blots is shown in Table 3.

Virus	Drug	VP16 rel. amount[a]	us11 rel. amount[a]
WT (KOS1.1)	-	100	100
	LMB	39.0 +/- 6.5	23.0 +/- 9.3
	PAA	44.8 +/- 5.0	13.2 +/- 5.0
	LMB + PAA	33.2 +/- 8.7	11.5 +/- 2.6
d27-1	-	17.2 +/- 6.0	5.0 +/- 2.5
	LMB	11.2 +/- 4.6	2.5 +/- 2.8
	PAA	12.8 +/- 4.0	5.8 +/- 3.8
	LMB + PAA	17.2 +/- 2.2	3.0 +/- 3.9
dLeu	-	78.5 +/- 7.9	62.0+/- 8.0
	LMB	53.2 +/- 8.0	29.8 +/- 9.1
	PAA	39.8 +/- 5.7	13.8 +/- 5.6
	LMB + PAA	31.8 +/- 8.8	9.0 +/- 5.0
M50T	-	96.0 +/- 10.7	76.5 +/- 5.2
	LMB	82.0 +/- 11.0	64.5 +/- 2.3
	PAA	49.1 +/- 6.8	10.8 +/- 3.0
	LMB + PAA	43.0 +/- 5.3	9.25 +/- 5.2

Table 3: Effects of LMB and PAA on viral protein expression at 12 hrs p. i
Statistical analysis of Western data for VP16and us11.
a - amount of protein relative to WT virus.

Virus	Drug	VP16	UL42	UL30	ICP27
WT (KOS1.1)	-	1	1	1	1
	LMB	1.21	1.02	1.15	1.01
	PAA	2.74	1.97	1.6	0.96
	LMB + PAA	3.7	2.61	1.48	1.13
d27-1	-	1.47	6.87p = 0.0107 10.7p = 0.0210	0.6p = 0.2610 0.19 p = 0.0194	ND
	LMB	1.15			ND
	PAA	0.61	3.2	ND	ND
	LMB + PAA	1.25	3.14	0.27	ND
dLeu	-	3.12	4.89 p = 0.0015 3.98p = 0.0298	0.25 p = 0.754 0.13 p = 0.0147	0.56
	LMB	4.23			0.56
	PAA	2.77	1.0	ND	0.49
	LMB + PAA	2.44	1.02	ND	0.87
M50T (HB01)	-	1.04	1.01	2.87 p = 0.0434 3.91 p = 0.339 4.46 p = 0.0583* 4.07 p = 0.0160	0.86
	LMB	1.53	1.39		1.14
	PAA	2.16	1.9		0.85
	LMB + PAA	2.23	2.57		1.3

Table 4: Ratio of RNA to protein at 6 hrs. p.i
Statistical analysis of Northern and Western data for VP16, UL42, and UL30 and ICP27.
Ratio of RNA to protein was compared and analyzed by the t-test. p values are given for to determine significance. By standard convention $p < 0.05$ is significant. Boxes are placed around values that are significant or borderline. * value fails t-test due to a lack of values (2 not 3). ND - Not determined.

Chapter 4: Discussion

It has become increasingly apparent that the role of ICP27 in HSV-1 infection is far more complex than originally thought. Recent data, along with that presented in this study, is inconsistent with the first model proposed for the mRNA export function of ICP27. The model proposed that ICP27 bypassed the splicing requirement for mRNA export in a fashion analogous to HIV Rev, by binding viral mRNAs and bridging an interaction with the cellular in Crm-1 (Soliman and Silverstein 2000)(Chen, Sciabica et al. 2002).

In this study, we attempted to reproduce and confirm previous observations, and to try to reconcile conflicting earlier studies. We propose and provide data to support an LMB induced inhibition of viral DNA replication that occurs even in the absence of ICP27. We observed no role for Crm-1 in mRNA export in our cell type and furthermore, observed no obvious role for ICP27 in nuclear mRNA export. Finally, we also confirm the resistant phenotype of M50T and observe a potential role in transcriptional and translational regulation for ICP27.

4.1 Neither LMB nor ICP27 alter the nuclear-cytoplasmic distribution of viral mRNAs

Our study revealed that neither LMB nor PAA had any affect on the nucleo-cytoplasmic ratio of the mRNAs we surveyed. As mentioned previously, our results seem to be at odds with the reported findings of Soliman and Silverstein (Soliman and Silverstein 2000). However, upon closer examination,

there are no major discrepancies. Soliman and Silverstein utilized Northern blot analysis to compare nuclear to cytoplasmic RNA. However, they were unable to detect any mRNA in any of the nuclear samples. Therefore, it is difficult to draw any conclusions about mRNA redistribution from their experiments. Interestingly, in our study we did not see a nuclear accumulation of RNA in the absence of ICP27. As mentioned previously in the result section this differs from the previously published work of Sandri-Goldin (Sandri-Goldin 1998). In that study, Sandri-Goldin observed a nuclear accumulation of several mRNAs (TK, gD, gC, UL15, and UL41) in the absence of ICP27. While it appears that these two sets of results cannot be reconciled, this may not necessarily be the case. There are some key differences between our methodology and that utilized by Sandri-Goldin. Briefly, in Sandri-Goldin's study, RSF (Rabbit Skin Fibroblasts) were infected with KOS or 27lacZ (an ICP27 null mutant) and UV cross-linked at 6 hr. post infection. Nuclear and cytoplasmic fractions were prepared and immuno-precipitation was done using a monoclonal ICP27 antibody bound to Protein A. The RNA bound to the beads was deemed the bound fraction and the RNA in the supernatant was the unbound fraction. RNA was separated using electrophoresis and visualized with radiolabeled probes. One potential source of discrepancy could be the use of UV cross-linking in their experiments. Another possibility for the observed differences between these two studies could be the direct result of utilizing different cell lines. RSF cells may have a more stringent requirement for ICP27 for viral RNA export. In Sandri-Goldin's study, they observed a nuclear retention of UL15 RNA, an intron containing transcript, which Sandri-Goldin claims is not dependent on ICP27 for export by virtue of its being spliced. This goes against the conclusions drawn in that paper.

Sandri-Goldin also observed a nuclear accumulation of ICP8 mRNA, which is in direct opposition to a study published by Knipe (Uprichard and Knipe 1996) that determined ICP8 mRNA to be unaffected by the absence of ICP27 or viral DNA replication. Our findings are consistent with this later study as we do

not observe a nuclear accumulation of ICP8 mRNA in the absence of ICP27. A recent study by Pearson (Pearson, Knipe et al. 2004), further provided evidence that ICP27 does not alter the nucleo-cytoplasmic localization of most mRNAs. They report, via Northern blot analysis, that VP16, UL42 and gC mRNAs are not dependent on ICP27 for nuclear export. These findings are again consistent with our own. The only transcript that they observed to exhibit a clear nuclear retention in the absence of ICP27 was the long UL24 transcript.

We observed the nuclear retention of only one transcript (UL30) and only in dLeu infection. We did not observe a change in UL30 mRNA distribution in the absence of ICP27. This observation seems to suggest that expression of an ICP27 protein lacking a functional NES is more deleterious to UL30 RNA export than expression of no ICP27. As previously mentioned in the Results section, one could imagine several possible scenarios to explain this observation. ICP27 may bind UL30 mRNA with a high affinity within the nucleus and, under normal circumstances, shuttle it to the cytoplasm. In the absence of ICP27, UL30 mRNA may use an alternate route to exit the nucleus. However, in the presence of an ICP27 lacking a functional NES, UL30 may still bind ICP27 but become trapped in the nucleus with ICP27 and unable to use an alternate route. Another possibility is that UL30 mRNA may be dependent on another protein for its export. This additionally required protein may be negatively affected by a build up of ICP27 in the nucleus, which is the phenotype observed for dLeu (Rice IWH 2003). This observation about UL30 localization leaves several unanswered questions. How is UL30 mRNA export regulated? Is it dependent on REF/Aly like other transcripts? Infecting cells with an ICP27 mutant that has the REF binding site abolished may be informative. Alternatively, the localization of ICP27 may be important. Would we observe a similar nuclear retention with other ICP27 mutants? Although ICP27 appears to bind mRNAs indiscriminately, there is some suggestion that it may bind specifically to cis-acting sequences within

certain viral transcripts (Sokolowski, Scott et al. 2003). UL30 may perhaps be an ideal transcript to examine to identify this putative recognition sequence.

4.1.1 ICP27 and transcriptional regulation

Our RNA expression data uncovered a surprising mRNA profile for M50T-1. In M50T infection, VP16, UL42, and UL44 mRNA accumulated 2-3 fold over wild type levels. This could suggest a role for ICP27 in transcriptional activation or in mRNA stabilization. ICP27 has been demonstrated to affect gene expression at a post-transcriptional level. It has been shown to be involved in the use of alternate poly (A) sites, mRNA export, and the inhibition of splicing. In addition to its post-transcriptional functions, there is also evidence that ICP27 can regulate transcription. Jean et al (Jean, LeVan et al. 2001) undertook a study to determine whether the expression of two viral late genes (gC and UL47) was up regulated by ICP27. Using nuclear run-on assays, to measure transcription of viral genes during WT infection and infection with a mutant virus lacking functional ICP27, they determined that ICP27 up regulated the transcription of these two transcripts. There is some contention in the literature, however, about the use of run-on assays in HSV-1 infection. It is unclear whether nuclear run-on assays can be utilized as a direct measure of the transcription rates of L genes in HSV-1 infection. This is due to the fact that later in infection most regions of the HSV-1 genome appear transcriptionally active by this assay (Godowski and Knipe 1986)(Smith, Hardwicke et al. 1992)(Weinheimer and McKnight 1987). This phenomenon or potential experimental artifact appears to be due to viral DNA replication, as it can be abrogated by the addition of PAA during infection. This may therefore not be a suitable assay to examine the transcription of L genes. To address this possibility, they also utilized in vivo RNA pulse labeling to measure the rate of

transcription. They reported that there was a 5-10 fold increase in the rate of transcription in the WT virus versus the ICP27 null. To determine whether this increase in mRNA accumulation could be due to an increase in mRNA stability (a valid concern as ICP27 has been shown to increase the stability of mRNA (Mosca, Pitha et al. 1992), they examined the stability of gC and UL47 mRNA. They determined that there was no difference in mRNA stability between infection with WT and the ICP27 null virus.

A recent study by Perkins et al (Perkins, Gregonis et al. 2003) possibly places these earlier observations into question. In this study, the authors reported that an increase in gC mRNA accumulation required both ICP27 and ICP0/ICP4, or ICP27 and a constitutively active promoter driving gC such as CMV. They determined that the effects of ICP27 on gC mRNA accumulation were post-transcriptional. The authors also utilized a nuclear run-on assay but with Vero cells stably transfected with gC. This allowed them to utilize PAA in their experiments and avoid the previously mentioned complication. In contrast to the findings of Jean (2001), they did not observe a significant increase in gC transcription by ICP27. It is, however, difficult to directly compare these two studies due to inherent differences between examining a virally encoded gene and the same gene transfected into a cell line.

While it remains unclear whether ICP27 directly stimulates transcription of HSV-1 genes, there is other evidence linking ICP27 to transcription. A study by Zhou (Zhou and Knipe 2002) provided evidence that ICP27 interacts with ICP8 and RNA pol II. The authors hypothesized that the interaction of ICP27 with RNA pol II may be related to its role in stimulating E and L gene expression. While our results with the M50T mutant could potentially point to a role for ICP27 in transcription regulation, there is also the possibility that ICP27 is playing a role in mRNA stability. Both scenarios are plausible, and unfortunately, our data does not distinguish between these two possibilities. At this junction, it would be important to examine the half-life or stability of the mRNAs in question in both the

nucleus and in the cytoplasm, to elucidate whether this effect on mRNA accumulation is transcriptional or post-transcriptional. In addition to nuclear run-on assays to measure the rate of transcription, mRNA stability should be examined. One could infect cells and allow mRNA to accumulate for a specific amount of time, for instance, 6 hr. Then an inhibitor of transcription such as actinomycin D could be added to stop new transcription. The rate of mRNA decay could be measured by taking samples every two hr. and quantifying the mRNA through Northern blot analysis.

4.2 LMB mediated inhibition of viral DNA replication

Murata (2001) reported that LMB inhibited wild type HSV-1 DNA replication, ostensibly through inhibiting RNA export. We observed that LMB did inhibit HSV-1 DNA replication as previously reported. Much more significantly, we also observed that LMB inhibited viral DNA replication of dLeu and the residual replication of d27-1 – the ICP27 null virus. This suggests either that the site of action for LMB is not ICP27, or that there are perhaps several sites of action and that at least one is not dependent on the presence of ICP27. M50T-1 was resistant to the LMB induced inhibition of viral DNA replication. We observed no inhibition of viral DNA replication in M50T-1 even at concentrations of LMB that prevented plaque formation. Murata et al commented in their study that it appeared that their mutant had egress defects at higher concentrations of LMB based upon a large titer difference between intracellular and extracellular virus. Experiments should be conducted to determine whether we also observed these effects.

When this study was initiated, the original goal was to determine whether ICP27 shuttled RNA via Crm-1 and whether the effects observed on HSV-1 infection by LMB were mediated by the inhibition of RNA export via Crm-1. Our observations do not support a model where ICP27 mediates RNA export through

Crm-1. We propose that the effects we observed on HSV-1 gene expression by LMB are due to an inhibition of viral DNA replication that appears to be independent of ICP27. If the forgoing is correct, then how does LMB inhibit DNA replication? There are several possibilities of how LMB may cause this inhibition. First, we must consider the function of the sole target of LMB – the cellular exportin Crm-1. Crm-1 is a known cellular protein exporter and has been demonstrated to export protein kinase inhibitor α and HIV rev (Fornerod, Ohno et al. 1997), IκBα (Sachdev, Bagchi et al. 2000), snurportin 1 (Paraskeva, Izaurralde et al. 1999), HTLV Rex (Hakata, Umemoto et al. 1998), cyclin B1 (Yang, Bardes et al. 1998), and the transcription factor NF-AT4 (Zhu and McKeon 1999). Therefore, it is likely that the effects of LMB are due to a block in the export of a cellular or viral protein. There is some evidence for both of these possibilities.

Recent studies conducted by the Rice lab (IHW 2003, Abstract) have shown that in KOS infection in the presence of LMB, ICP0, a protein normally found distributed in both the nucleus and the cytoplasm, becomes exclusively cytoplasmic. In infection with the LMB resistant mutant, M50T, ICP0 is almost exclusively nuclear. Additionally, in M50T infection, ICP4 appears predominantly nuclear unlike in KOS infection where it appears both nuclear and cytoplasmic.

LMB has been shown to affect the cell cycle and cause G1 cell cycle arrest in mammalian cells (Yoshida, Nishikawa et al. 1990). This is assumed to be due to a block in the export of a required protein for cell cycle progression. This observation clearly demonstrates that Crm-1, although a minor exporter, does function to export some important cellular proteins. This provides a potential area of investigation into how LMB affects HSV-1. Recent studies have indicated that HSV-1 infection blocks cell cycle progression into S-phase and arrests host cell growth in G1 phase (Song, Yeh et al. 2001)(Ehmann, McLean et al. 2000)(Song, Liu et al. 2000)(Lomonte and Everett 1999). However, it is

becoming increasingly clear that for HSV-1 the relationship between viral replication and cell cycle is a subtle one. HSV-1 in fact appears to require cellular cyclin dependent kinases (cdks) for replication. This is based on several pieces of evidence. First, pharmacological cdk inhibitors (PCIs) have been shown to inhibit the gene expression and replication of HSV-1 (Schang, Phillips et al. 1998)(Jordan, Schang et al. 1999)(Schang, Rosenberg et al. 2000). Second, HSV-1 establishes latency in resting neurons, which lack cdk1 and 2, and can be reactivated only in neurons expressing cdk2 (Schang, Bantly et al. 2002).

4.3 Protein expression does not always correlate with mRNA levels in infection

In this study, we observed an interesting protein expression profile for M50T-1. We observed that UL42, VP16, US11, and UL30 protein were highly over expressed as compared to KOS1.1. For VP16 and UL42, we also observed an increase in mRNA expression that may have directly led to the increase in protein expression. However, this was not the case for UL30. M50T-1 does not over express UL30 transcript but does highly over express UL30 protein. This would imply that for this gene product M50T-1 acts post-transcriptionally, possibly with a role in translation.

We examined our RNA and protein data to determine if we could identify any other instances of a possible role for ICP27 in the regulation of viral translation. Instances where the difference in RNA to protein levels was three-fold or higher were arbitrarily deemed potentially significant. We report RNA and protein amounts as a percent of KOS1.1 in the absence of any drugs (the ratio of RNA to protein in KOS1.1 was designated 1.0). Therefore, we compare only relative changes in RNA to protein levels. In the absence of ICP27 (d27-1 infection), we observed changes in the ratio of protein to RNA. For UL42, in d27-

1 infection the ratio ranged from a three- to ten-fold excess of protein to RNA when measured as a percent of what is observed in KOS1.1. This suggests a down-regulatory function for ICP27 in the translation or post-transcriptional processing of UL42 protein.

In dLeu infection, we see a similar trend however, although not as pronounced. For UL42, the ratio ranged from a one- to five-fold increase in protein to RNA. One final example of disparity for protein to RNA was in M50T-1 infection. We had observed that M50T-1 increased the amount of RNA accumulated for all transcripts except UL30. Interestingly, the amount of protein accumulated for all the gene products tested increased, including UL30. This could suggest that this mutation in ICP27 alters a translation function of ICP27. There are, however, several limitations to our data. The experimental system that we utilized to measure protein accumulation, western blotting followed by visualization by ECL and quantification, has several inherent problems. The first being, that ECL is linear in a very limited range of protein concentrations. Further complicating quantification was the sensitivity of some of our proteins to degradation. Therefore, "quantitative" analysis of the western blots should be utilized as an approximation of the amount of protein and not as an exact value. Second, our experimental methodology measures the amount of protein accumulated but does not distinguish an increase in the translational rate of synthesis from an increase in protein stability. A key next step would be to distinguish between these two possibilities. Pulse-metabolic labeling followed by immuno-precipitation could be one way to examine the rate of translation for a specific protein.

4.3.1 HSV-1 and translational regulation

In this study we examined the effects of LMB on HSV-1 protein synthesis. Our results support the conclusion that LMB does not directly affect protein

synthesis. The decreases in protein accumulation that we observed in the presence of LMB can most likely be attributed to LMB's effect on viral DNA replication. We do, however, observe what could potentially be a role for ICP27 in the regulation of protein synthesis.

In mammalian cells, cap-dependent translation initiation begins with eIF4F, the cap-binding complex (composed of eIF4E, eIF4A, and eIF4G), binding to the 5' cap structure on the mRNA. eIF4G interacts with the poly (A) binding protein (PABP) to circularize the mRNA and increase translational efficiency. The 40S ribosomal subunit is recruited and scans for an AUG codon. The 60S subunit is then bound and translation begins (Pestova, Kolupaeva et al. 2001).

The altering of the regulation of translation of cellular mRNAs by a virus is not a novel phenomenon. One example of this is the Picornavirus family of viruses, which selectively inhibit the translation of cellular transcripts but not viral mRNAs (reviewed in (Ehrenfeld and Semler 1995). Poliovirus (a member of the picorna family) proteinase 2A cleaves PABP and eIF4G. Cleavage of eIF4G results in the dissolution of the eIF4F complex (Etchison, Milburn et al. 1982) and the sequestering of eIF4E from eIF4G (Gingras, Svitkin et al. 1996). This results in an inhibition of cap-dependent translation. Picornavirus mRNAs are translated cap-independently utilizing internal ribosomal entry sites (IRES) on their mRNAs. Rotaviruses also disrupt cellular translation but in a slightly different way. Rotaviral mRNAs contain a 5' cap but not a 3' poly (A) tail. Nonstructural protein NSP3 binds to the 3' end of viral mRNAs and eIF4G, displacing PABP and preventing host mRNA translation (Piron, Vende et al. 1998).

Another mechanism by which viruses can affect host translation is through PKR activation. PKR kinase is a vital component of the host anti-viral response (reviewed in Barber 2001). It is activated in response to dsRNA and phosphorylates eIF2, preventing the GDP-GTP exchange factor eIF2B from recycling GTP on eIF2, which results in an inhibition of protein synthesis.

Hepatitis C virus (HCV) encodes two proteins, NS5A (Gale, Korth et al. 1998) and E2 (Taylor, Shi et al. 1999), that can bind to PKR and prevent its activation. Adenovirus does not inhibit PKR activation or the cessation of host protein synthesis (Burgert, Ruzsics et al. 2002). Adenovirus transcripts, unlike their cellular counterparts, are still translated as they encode a common 5' UTR, comprising a tripartite leader sequence that can direct cap-dependent translational initiation by a method called ribosomal shunting (Burgert, Ruzsics et al. 2002)(Yueh and Schneider 1996)(Yueh and Schneider 2000).

HSV-1 has been long known to cause a disaggregation of polyribosomes (Sydiskis and Roizman 1966) to reduce the level of host mRNA (Schek and Bachenheimer 1985)(Inglis 1982)(Mayman and Nishioka 1985) and to effect host protein synthesis (Roizman, Borman et al. 1965). However, it is unknown how this is all mechanistically linked.

Early shut-off of host protein synthesis by HSV-1 is the consequence of an indiscriminant degradation of all viral and cellular mRNAs by the virion host shut-off (vhs) protein. Secondary shut-off appears selective and under translational control (Roizman and Knipe 2001). It has been demonstrated that the synthesis of some proteins such as actin becomes less efficient, while other proteins like the ribosomal proteins are almost unaffected, regardless of the rate of degradation of their respective mRNAs by vhs (Simonin, Diaz et al. 1997).

It has long been suggested by multiple groups that viral gene expression is controlled to some degree by regulation at the level of translation (Schek and Bachenheimer 1985)(Weinheimer and McKnight 1987)(Honess and Roizman 1974). This hypothesis is based on several pieces of evidence. First, the rate of synthesis of some viral proteins does not correlate to the amount of mRNA accumulated during infection (Sandri-Goldin, Goldin et al. 1983)(Elshiekh, Harris-Hamilton et al. 1991)(Johnson and Spear 1984). A study conducted by Yager et al (1988) to investigate the relative discrepancy between the rate of UL30 transcription and the accumulation of UL30 protein demonstrated that UL30 is

regulated post-transcriptionally. Translation appears to be inefficient at the level of initiation. Second, infection by HSV-1 alters the translational machinery (Fenwick, Morse et al. 1979)(Kennedy, Stevely et al. 1981)(Masse, Garcin et al. 1990)(Simonin, Diaz et al. 1995). The ribosomal protein S6 is phosphorylated upon virus entry (Kennedy, Stevely et al. 1981) (Masse, Garcin et al. 1990). This is significant, as it has been demonstrated that an increase in S6 phosphorylation is correlated with a preferential translation of ribosomal mRNAs (Jefferies, Reinhard et al. 1994 2683). Four other ribosomal proteins, Sa, S3a, S2, and L30 are also phosphorylated upon HSV-1 infection (Masse, Garcin et al. 1990). Third, an HSV-1 late protein, γ34.5, has been shown to prevent the shut down of translation through PRK but not to act upon PKR directly. γ34.5 forms a complex with protein phosphotase 1 (PP1), which results in the dephosphorylation of eIF2α (He, Chou et al. 1997). This prevents the inhibition of translational initiation. Additionally, the viral US11 protein also appears to be involved in translational regulation of gene expression, as it has been shown to associate with the ribosomal fraction in polysome analysis (Roller and Roizman 1992)(Diaz, Simonin et al. 1993).

Recent work done by Dr. Kim Ellison in our lab with polyribosomal analysis has provided further evidence for translational regulation in HSV-1 infection and has demonstrated a role for ICP27 in this phenomenon. Dr. Ellison has observed that approximately 40% of VP16 mRNA is found in fractions containing actively translating polyribosomes in KOS1.1 and Δsma (vhs null) infection. In d27-1 infection, only a minor fraction of VP16 mRNA is associated with polyribosomes. A similar effect is observed with TK mRNA but not ICP8 mRNA (previously shown to be ICP27 independent for expression, Knipe 1996). Dr. Ellison has also examined two cellular transcripts, β-actin and GAPDH, and found that in mock infection, approximately 70% of transcripts are on translating polyribosomes. This is similar to what is observed in d27-1 infection. Infection

by Δsma alters the profile significantly with 20-25% of transcripts in these large fractions. Therefore, ICP27 appears to be required for this polyribosomal shift. Dr. Ellison also observed that the rate of synthesis of β-actin and GAPDH protein did not correlate with the mRNA levels observed in d27-1 infection. Infection with d27-1 exhibited a severe inhibitory effect on cellular mRNA levels and virtually no effect on their protein levels. These results strongly suggest that ICP27 plays a role in translational regulation in infection.

It is interesting to speculate how this translational regulation by ICP27 may occur. Several recent lines of study have raised the possibility that we may shed light on the role of ICP27 by examining its homologs in other herpesviruses. One such protein is the SM protein of Epstein Barr virus (EBV), which shares homology with ICP27 and can partially complement the growth of an ICP27 null virus (Boyer et al. 2002). The SM protein has recently been reported to contain a sequence that shows homology to the terminal domain of US11 (Poppers, Mulvey et al. 2003). As mentioned previously, the US11 protein of HSV-1 has been shown to associate with ribosomal fractions (Roller and Roizman 1992)(Diaz, Simonin et al. 1993) and to play a role in post-transcriptional regulation of gene expression (Diaz, Dodon et al. 1996)(Diaz-Latoud, Diaz et al. 1997). US11 has been shown to bind both DNA and RNA (Diaz, Dodon et al. 1996)(Roller, Monk et al. 1996), and to specifically bind UL34 mRNA, regulating its expression (Roller and Roizman 1991). This protein displays similarities to the retroviral regulatory proteins Rev and Rex (HIV-1 and HTLV-1, respectively). It has been demonstrated that US11 can bind the RRE and XRE sequences and substitute for Rex and Rev in trans-activating retroviral genes (Diaz, Dodon et al. 1996). US11 has also been shown to prevent PKR kinase activation (Roller, Monk et al. 1996) (Poppers, Mulvey et al. 2000). While none of these studies shed light on the role of ICP27 in translational regulation directly, they do appear to provide more circumstantial evidence linking ICP27 to translation.

We observed an increase in the ratios of protein accumulation versus RNA even in the absence of a cytoplasmically located ICP27 (dLeu) for the VP16 and UL42 transcripts. Nevertheless, this does not negate the possibility of a translational regulation function. It may in fact be suggestive of a mechanism of translational regulation that does not require ICP27 translocation into the cytoplasm. It has become apparent in recent studies that the nuclear history (splicing, polyadenylation, addition of the 5' CAP etc.) of a specific RNA plays a significant role in its ability to be translated when it reaches the cytoplasm. The process of splicing deposits protein complexes 20-24 nt upstream of exon-exon junctions in a splicing dependent and sequence independent manner. This exon junction complex (EJC) includes the spliceosome associated proteins DEK, SRM160, RNPS1, REF, Y14, and perhaps others (Zenklusen, Vinciguerra et al. 2001)(Le Hir, Gatfield et al. 2001)(Le Hir, Izaurralde et al. 2000)(Kim, Jong et al. 2001)(Kataoka, Yong et al. 2000). Whereas REF, known to promote export dissociates rapidly after nuclear exit, Y14 has been demonstrated to remain bound to mRNA until translation is completed. ICP27 interacts with at least one component of the EJC and may interact with others. It has been suggested that mRNAs not marked with the EJC, become targets for nonsense-mediated, decay. This degradation pathway is specific for mRNAs containing premature stop codons (Lykke-Anderson 2001).

4.4 Changes in the migration of the UL42 protein on SDS-PAGE in d27-1 and dLeu infection

We observed that in d27-1 and dLeu infection (two mutants that lack cytoplasmic ICP27) the UL42 protein migrates more slowly on an SDS-PAGE gel than in wild type infection (Figure 19). This difference may possibly be the result of a difference in the phosphorylation states of these two proteins. UL42 is phosphorylated in the carboxyl terminus during some stages of infection by cdc2

cycle dependent kinase (Advani, Weichselbaum et al. 2001). Activation of cdc2 kinase in HSV-1 infected cells appears to be required to enhance the expression of a subset of L genes. One possibility is that UL42 binds cdc2 kinase and facilitates an interaction between the kinase and several viral protein substrates to allow their phosphorylation by cdc2 kinase (Advani, Weichselbaum et al. 2001). We observed that in dLeu and d27-1 infection, UL42 protein accumulation is decreased by the presence of PAA. This is unlike what is observed in KOS or M50T-1 infection. One possible explanation for this observation may be that when UL42 is in this phosphorylation state, it may be less stable than under normal conditions in KOS infection. UL42 is required to bind UL30, increase the DNA binding specificity of the polymerase, and decrease the rate of disassociation from the primer template without decreasing the elongation rate (Weisshart, Chow et al. 1999). If the ability of UL42 to perform its function is compromised (by unusual phosphorylation that may cause a steric hindrance for interaction or by a decrease in the stability of the protein) then, perhaps, the addition of PAA may further abrogate DNA replication and, as such, decrease UL42 expression.

4.5 Model for M50T Function

The M50T mutation is a single base pair mutation that results in an amino acid substitution in the highly acidic region of the n-terminus adjacent to the NES. Soliman and Silverstein (2000) have suggested that a novel sequence they term "an export control sequence" (ECS), also found adjacent to the NES of ICP27, negatively regulates the export function of ICP27. While the borders of this putative ECS have not been defined, it is possible that this mutation M50 → T falls near this sequence. Murata et al (2001) suggested that this mutation may suppress the function of the ECS, thereby increasing the export of this protein. This was the original model for why M50T was resistant to LMB when ICP27 was

thought to function via Crm-1. Given new evidence that ICP27 functions via REF and TAP, and our recent observations on the effects of this mutation on gene expression, perhaps there is another explanation as to why this mutant is LMB resistant. In infection, this mutant up-regulates the accumulation of mRNA and protein of all but one gene that we tested. Given this possible indication that this mutation may also alter protein synthesis, polyribosomal analysis with M50T, similar to what Dr. Ellison has done may determine whether there is any effect on mRNA localization.

We also observed that these increased levels of RNA and protein are still susceptible to LMB and PAA, arguing against a mode of resistance involving a direct interaction between ICP27-Crm-1 and LMB. We observed that LMB does not inhibit the DNA replication of M50T at a concentration of 25ng/mL. We did observe a decrease in plaque formation of M50T at this concentration. Murata observed a similar phenomenon and concluded that LMB impairs the progress of -1 egress either directly or indirectly. These observations are consistent with the hypothesis that this mutation may bypass the effects of LMB, by upregulating HSV-1 gene expression. This could suggest a mechanism by which M50T would be resistant to LMB, as measured by DNA replication and plaque assay, but still be affected by LMB at the level of transcript accumulation. Potentially, any mutation that could cause a significant increase in mRNA expression could "appear" LMB resistant. It is possible that when this mutant was isolated originally, having been cultured in high concentrations of LMB, that it was merely coincidental that the mutation was in ICP27. Recent evidence from the Rice lab has indicated that making a deletion in the acidic region of ICP27 also confers an LMB resistant phenotype (personal communication), which supports the ECS model for LMB resistance but in no way clarifies the issue.

4.6 In Summary…

It is perhaps not surprising that this study brings up more questions than it answers. By what mechanism does LMB inhibit viral DNA replication? Is this mechanism mediated through ICP27? While ICP27 does not appear to be shuttling RNA via Crm-1, it does appear possible that ICP27 may shuttle a protein cargo such as another viral protein.

Assuming that the changes in protein accumulation that we observed for several gene products during infection with M50T, d27-1, and dLeu are due to regulation of translation by ICP27, it is interesting to speculate on the possible mechanisms of this translational function of ICP27. It is obviously gene specific, as there appears to be no consistency between genes of the same temporal class. How does the system differentiate between mRNAs? Is this alteration in translation the result of a nuclear event or is it due to something that occurs in the cytoplasm? dLeu, with its predominantly nuclear localization, and M50T, with its primarily cytoplasmic localization, may be useful in differentiating between these two possibilities. Additionally, is this effect of ICP27 on translation an up regulatory mechanism, or is it the removal of an inhibitory effect on translation by the host cell?

Many questions must be answered before we can truly understand this complex system.

Chapter 5: Bibliography

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