

FACTORS AFFECTING VIRION HOST SHUTOFF IN HSV-1 & HSV-2

CHARACTERIZATION OF FACTORS AFFECTING THE VIRION HOST SHUTOFF
FUNCTION OF HERPES SIMPLEX VIRUS

By

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ABSTRACT

Herpes Simplex virus (HSV) virions contain the protein vhs (virion host shutoff), which is known to trigger rapid shutoff of host protein synthesis and accelerated decay of viral and cellular mRNAs. HSV-1 strains generally cause weaker shutoff than HSV-2 strains. HSV viruses lacking the VP16 viral transactivator gene show uncontrolled shutoff late in infection (Lam *et al.*, 1996); vhs is known to bind to VP16 (Smibert *et al.*, 1994). In vitro experiments demonstrated that HSV-1 vhs protein (vhsT1) and an intertypic HSV-2 G vhs protein (vhsT2) did not differ in ability to effect mRNA degradation in a rabbit reticulocyte lysate (RRL) assay system, suggesting that virion factors might influence vhs shutoff phenotype. To investigate the possibility that VP16 influences early shutoff during HSV infection, a virus was constructed containing the HSV-2 strain G VP16 in place of HSV-1 VP16. This virus (8MA2R) grew in a noncomplementing cell line and was unaltered in shutoff phenotype compared to wt strain. Cotransfection assays demonstrated vhsT2 had greater shutoff ability than vhsT1, and this was confirmed in a viral system by constructing intertypic viruses containing portions of the HSV-2 G vhs ORF in a vhs-null HSV-1 tk locus. All intertypic constructs conferred increased shutoff ability relative to vhsT1, indicating that the strong shutoff ability of HSV-2 vhs is distributed along much of the vhs ORF. Also, to confirm the observation that UL13 null viruses show a lack of shutoff which is not due to lack of vhs in the virion (Overton *et al.*, 1994) a number of wild-type viruses and their UL13-null derivatives were tested for ability to shutoff host translation. All showed near-wt or wt levels of shutoff.

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LIST OF ABBREVIATIONS

M	molar
mM	millimolar
μ M	micromolar
nM	nanomolar
l	litre
ml	millilitre
μ l	microlitre
g	grams
mg	milligrams
μ g	micrograms
ng	nanograms
μ Ci	microcuries
w/v	weight/volume
w/w	weight/weight
v/v	volume/volume
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
DNA	deoxyribonucleic acid
rpm	revolutions per minute
k	1,000
min	minutes
h	hours
kb	kilobase
bp	base pair
HSV-1	herpes simplex virus type 1

HSV-2	herpes simplex virus type 2
VZV	Varicella-Zoster virus
EBV	Epstein-Barr virus
CMV	cytomegalovirus
BHV	bovine herpes virus
EHV	equine herpes virus
ORF	open reading frame
PFU	plaque forming unit
MOI	multiplicity of infection
UL	unique long
US	unique short
IE	immediate early
E	early
L	late
VP	virion protein
MEL	murine erythroleukemia
HEP	human epidermal carcinoma
HEL	human erythroleukemia
BHK	baby hamster kidney
ER	endoplasmic reticulum
kDa	kilodaltons
UV	ultraviolet
V	volts
aa	amino acid
g	gravities
G	guanine
A	adenine
T	thymidine

C	cytidine
U	uracil
vhs	virion host shutoff
ATP	adenosine triphosphate
CTP	cytidine triphosphate
UTP	uridine triphosphate
GTP	guanosine triphosphate
dATP	deoxyadenine triphosphate
dTTP	deoxythymidine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanine triphosphate
HIV	human immunodeficiency virus
LTR	long terminal repeat
ddH ₂ O	doubly distilled water
DMSO	dimethylsulfoxide
HMBA	hexamethylene bis-acetamide
CAT	chloramphenicol acyltransferase
β-gal	β-galactosidase

I. INTRODUCTION

1.1. Introduction to Herpesviruses.

The Herpes Simplex Viruses (HSV, singularly HSV-1 and HSV-2) are members of the alphavirinae subfamily of the herpesviridae family. Currently, there are 8 herpesviruses known to infect humans. Varicella-Zoster virus (VZV), also known as human herpesvirus-3 (HHV-3), causes the clinical syndromes chickenpox and shingles. Epstein-Barr virus (EBV) (human herpesvirus-4) is associated with Burkitt's lymphoma and nasopharyngeal carcinoma, and causes mononucleosis. Cytomegalovirus (CMV) (human herpesvirus-5) does not cause any overt diseases in immunocompetent adults, but infects children and fetuses asymptotically. Human herpesvirus-6 (HHV-6) was originally isolated from acquired immunodeficiency syndrome (AIDS) patients (Salahuddin *et al.*, 1986), but has only been shown to cause exanthem subitum in immunocompetent individuals. Human herpesvirus-7 (HHV-7) is closely related to HHV-6, and like HHV-6 has only been shown to cause exanthem subitum in immunocompetent populations. Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) was originally detected in the eponymous lesions in AIDS patients but has recently been implicated as a cause of multiple myeloma in immunocompetent populations (Rettig *et al.*, 1997). As three human herpesviruses (HHV-6,7,8) were discovered only within the last dozen years, there are likely more human herpesviruses which have yet to be identified.

1.1.1. General Epidemiology and Pathology of HSV.

Although new human herpesviruses are only now being discovered, HSV infections have been noted since ancient Greek times, most notably by Hippocrates. Both HSV-1 and HSV-2 cause lesions on skin or mucosal membranes, resulting in a blister which contains large quantities of virus as well as cell lysate.

To infect, the virus must come in contact with a mucosal surface or skin abrasions. The virus then replicates at the site of infection, and is transported to nearby dorsal root ganglia. In the ganglia, viral replication again occurs, after which latency is established. Although most work has focused on the HSV-1 in trigeminal ganglia or HSV-2 in the sacral ganglia as a reservoir of latent virus, recent work has shown that HSV is also detectable in the gastrointestinal tract, possibly through latent infection of gastrointestinal ganglia (Gesser and Koo, 1997). Latency allows the virus to recur at a later time even if the host has strong humoral immunity from the initial infection. Reactivation has been associated with damage to trigeminal ganglia, ultraviolet light, menstruation, immune suppression, and mental or physical stress (recently reviewed in Steiner, 1996).

It is commonly believed that HSV-1 is responsible for oral “cold sores” and HSV-2 is responsible for genital lesions. While HSV-1 can produce genital lesions, HSV-2 is not known to recur orally. Both viruses, although best characterized in epidermal, mucosal, or neural tissues, can also infect a wide range of organs and tissues in humans as

well as many other mammals (reviewed in Spear, 1993). Natural nonhuman reservoirs of HSV have not been demonstrated conclusively to date.

Serologic studies repeatedly have shown that almost 90% of adults are seropositive for HSV-1 but only approximately 33% of adults report that they suffer from cold sores once a year or more (reviewed in Nahmias *et al.*, 1990). HSV-2 prevalence varies widely with a number of factors, most significantly the number of sexual partners, but has been recently assessed at 23% in North America (Wald *et al.*, 1997). An important difference between HSV-1 and HSV-2 genital infections is that many HSV-2 infections shed virus asymptomatically, making detection difficult.

Although intertypic viruses are easily generated in cell culture infections (Amundsen and Parris, 1984), intertypic viruses have not been found in clinical studies (Kit *et al.*, 1983; Fife and Boggs, 1986). This may indicate that each HSV virus is ideally adapted to a specific pathological niche. The two HSV viruses are estimated to have diverged from a common ancestor 8-10 million years ago (Gentry *et al.*, 1988). 8-10 million years is an exceedingly long viral evolutionary period, certainly enough time to allow both viruses to adapt to their preferred sites of infection.

1.2. Structure of HSV.

HSV virions are comprised of four main structural elements. Proceeding from innermost to outermost virion components, these are the virion core, the capsid, the tegument, and the envelope (Fig. 1.1).

Figure 1.1. Schematic diagram of HSV components.

Where the common name of a protein differs from the gene name they are listed in the format: gene (common protein name). Common names are used for glycoproteins.

Core

Viral DNA, spermidine,
spermine

Nucleocapsid

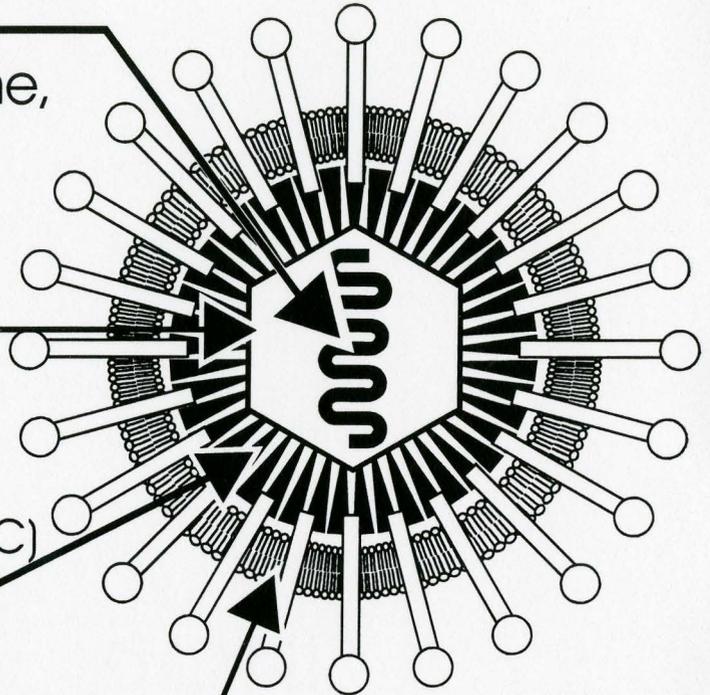
UL18 (VP23), UL19 (VP5),
UL26 (VP21, VP24),
UL26.5 (VP22a)
UL35 (VP26), UL38 (VP19C)

Tegument

UL13, UL11, UL37,
US9, US11
UL36 (VP1/2)
UL46 (VP11/12)
UL47 (VP13/14)
UL48 (VP16)
UL49 (VP22)
UL41 (vhs)

Envelope

gB, gC, gD, gE, gG,
gH, gI, gK, gL, gM
gJ(putative)
UL20, UL34



The virion core contains the HSV genome, approximately 150 kb of linear, double stranded DNA. It also contains two polyamines, spermidine and spermine, which partially neutralize the acidic DNA. A viral capsid protein (VP19C) has been shown to bind DNA (Braun *et al.*, 1984) but no other virion viral DNA-binding proteins have been identified.

Both HSV-1 and HSV-2 genomes contain approximately 70% G+C (HSV-1=68%, HSV-2=69%). There are two stretches of unique genes designated U_L (unique long) and U_S (unique short) each flanked by inverted repeats containing genes which are thus diploid (Fig. 1.2A). U_L contains 58 known viral genes and U_S contains 14 known genes. The inverted repeats flanking the U_L region contain three known genes, and the U_S flanking repeats contain a single gene (α 0). The genes in HSV are named according to their order within the U_L or U_S segments (e.g. U_L41, U_S6) while the gene products are the victims of a plethora of naming schemes. The L and S regions of the genome can invert relative to one another producing four isomers (Fig. 1.2B), the biological significance of which is unclear. The HSV genome does not seem to be spatially organized into gene groups, unlike other DNA viruses such as the adenoviruses.

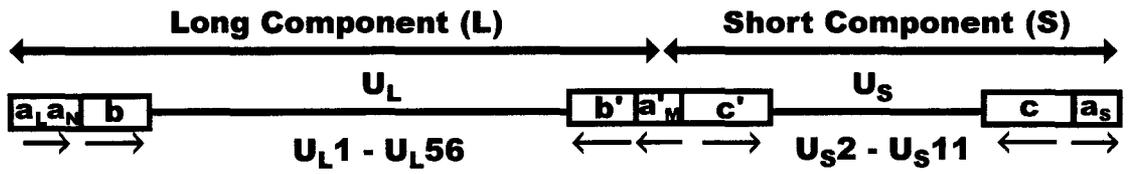
1.2.1. The HSV Capsid.

The most striking feature of herpesvirus virions is the architecture of the virion capsid. The capsid architecture is exceedingly well-conserved among widely divergent herpesviruses; it has recently been shown that channel catfish virus capsids are

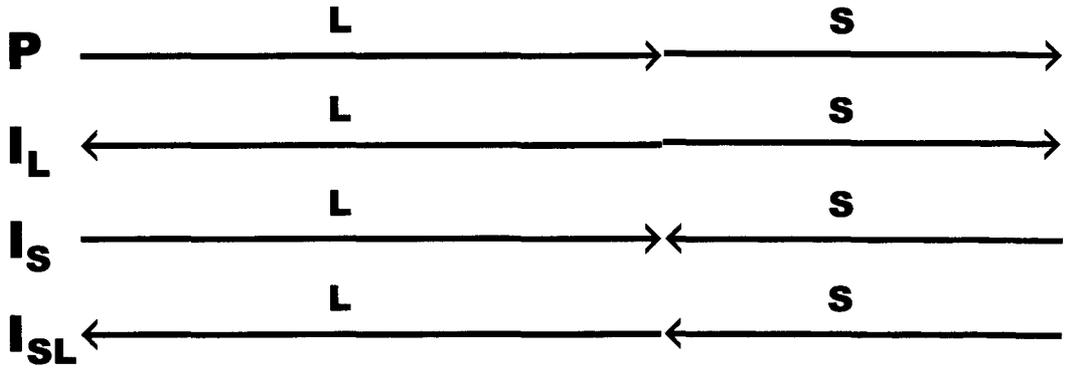
Figure 1.2. The structure of the HSV genome.

(A) The components of the HSV genome in prototype (P) orientation. U_L designates the unique long gene region, and U_S the unique short gene region. Arrows below a, b, and c regions designate segment orientation relative to each duplicate. A $a_L = >1$ terminal L region a sequence repeats; $a_N = >0$ a sequence repeats; $a_M = >1$ a sequence repeats; $a_S =$ terminal S region a sequence. (B) The four possible HSV genome isomers, in order, prototype (P), inversion of L component (I_L), inversion of S component (I_S), and inversion of S and L components (I_{SL}).

A.



B.



morphologically similar to HSV-1 capsids (Booy *et al.*, 1996). Other nonhuman herpesviruses appear indistinguishable from human herpesviruses by electron microscopy.

HSV capsid production requires six genes (UL18, UL19, UL26.5, UL35, and UL38) which produce seven proteins (VP5, VP19C, VP21, VP22a, VP23, VP24, and VP26) (reviewed in Tatman *et al.*, 1994). The UL26 gene encodes a proteinase that cleaves its own gene product into VP21 and VP24 as well as cleaving preVP22 (of UL26.5) into VP22a. Capsid formation occurs via the assembly of the outer capsid components (VP5, VP19C, VP23, and VP26) around the “scaffold” protein structure composed of VP21, VP24, and pre-VP22a (Preston *et al.*, 1994). Pre-VP22a is slowly converted by the UL26 proteinase into VP22a, which is absent from the mature virion.

1.2.2. The HSV Envelope

The envelope of mature HSV particles contains at least 10 known glycoproteins (gB, gC, gD, gE, gG, gH, gI, gK, gL, gM). At least three interact with immune system components. gC binds to complement protein C3 and prevents complement activity from destroying the HSV envelope (Friedman *et al.*, 1984). Glycoprotein E (gE) and I (gI) together form a complex that binds the Fc domain of monomeric IgG (Johnson and Feenstra, 1987; Johnson *et al.*, 1988).

The lipid component of the HSV envelope is cellular in origin, but it is not clear which cellular membranes are used to form the final virion envelope. One theory holds

that the capsid buds into the perinuclear space, acquiring a membrane containing immature glycoproteins, then moves into the endoplasmic reticulum (ER) where glycoprotein processing occurs (Johnson and Spear, 1982; Johnson and Spear, 1983). Another model proposes that capsids pass through the nuclear membrane, travel into the cytoplasm as unenveloped capsids, and enter transport vesicles containing mature glycoproteins (Jones and Grose, 1988). Recent studies argue that the HSV envelope is not acquired from the ER, as glycoprotein H which has been modified so as to “stick” in the ER membranes is not detectable in exiting virions (Browne *et al.*, 1996).

1.2.3. The HSV Tegument

The tegument of HSV is not well characterized. It contains a large number of virion proteins, including VP16, vhs, UL37, VP22, the kinase UL13, US9, UL36, VP11/12, VP13/14, VP1/2, US11, and likely others as yet unidentified. The only tegument protein thus far shown to be essential for virion formation is VP16 (Ace *et al.*, 1988; Weinheimer *et al.*, 1992). The failure of recent efforts to produce VP22 deletion mutants may indicate VP22 is needed for virion assembly as well (Elliott and O'Hare, 1997). Viable virus is still produced by mutants in the abundant proteins VP11/12 and VP13/14 (Zhang and McKnight, 1993) as well as less abundant components such as vhs (Fenwick and Everett, 1990a; Read *et al.*, 1993) or UL13 (Coulter *et al.*, 1993; Overton *et al.*, 1994).

As yet, formation of the tegument is a poorly understood process. It is unclear how the tegument associates with the capsid or the envelope of the virus. It is known that the tegument proteins VP16 and VP22, upon overexpression, form novel macromolecular assemblies dubbed “tegument bodies” (Elliott *et al.*, 1995). Other tegument proteins shown to interact with VP16 include vhs (Smibert *et al.*, 1994) and glycoprotein B (Zhu and Courtney, 1994). This suggests VP16 may couple tegument formation to envelope formation, as seems to occur in the formation of L-bodies. The tegument has been shown to retain its structural integrity after detergent-mediated removal of the envelope (McLauchlan and Rixon, 1992b), but tegument formation likely requires coupled envelope formation.

It is not known how proteins are packaged into the tegument. Overexpression of VP22 has been shown to increase the amount incorporated into the virion, indicating that VP22 is not under strict packing restrictions (Leslie *et al.*, 1996). However, similar experiments have shown that the amount of tegument UL37 is constant even if overexpressed (McLauchlan, 1997).

The tegument can form in the absence of the viral capsid and DNA, although the viral envelope may be required. L-particles (light particles) are noninfectious virion particles that lack the capsid and DNA which can be separated from complete particles on the basis of density (Szilagyi and Cunningham, 1991). L-particles lack any genome and thus do not act as defective interfering particles in replication of HSV. L-particle

formation can be separated from virion formation by using ts mutants in the UL26 gene (Rixon *et al.*, 1992). It has been shown that both vhs and VP16 from L-particles possess wt levels of activity when compared to whole virions (McLauchlan *et al.*, 1992a), thus demonstrating that L-particles can attach, enter, and allow the action of tegument components. L-particles also contain a number of proteins that are not found in virions (Szilagyi and Cunningham, 1991; McLauchlan and Rixon, 1992b), including the immediate early viral protein ICP4, which was previously identified as a component of the virion (Bibor-Hardy and Sakr, 1989; Yao and Courtney, 1989). These proteins are hypothesized to be present in inclusion vesicles present in many L-particles (Szilagyi and Berriman, 1994).

1.3. Overview of Early Stages in HSV Infection

1.3.1. Attachment to Susceptible Cells

HSV has been shown to be able to attach and enter to all cultured mammalian cells, although it naturally infects only humans and possibly chimpanzees (reviewed in Spear, 1993). Out of ten known glycoproteins, gC, gE, gG, gI, and gM are singly disposable for egress and entry of the virus.

gB and/or gC are involved in binding to cellular glycosaminoglycan (GAGs) chains. gD has recently been shown to interact directly with a cell surface protein dubbed HVEM (herpesvirus entry mediator) which is a new member of the tumor necrosis factor / nerve growth factor (TNF/NGF) receptor family (Montgomery *et al.*, 1996; Whitbeck

et al., 1997). Penetration of the envelope is pH-independent (Wittels and Spear, 1991). gB, gD, and gH-gL hetero-oligomers are required for fusion of the viral envelope with the cell membrane (Sarmiento *et al.*, 1979; Cai *et al.*, 1988; Ligas and Johnson, 1988; Forrester *et al.*, 1992; Roop *et al.*, 1993).

It appears that HSV glycoproteins are highly redundant with regard to entry. Cultured cells differ from natural epithelial cells in that there is no apical or basolateral membrane; receptors which normally would be expressed on one side alone are, in culture, expressed on all sides of the cells. HSV also infects neurons, each “side” of which must be distinguished, requiring four possibly distinct modes of entry. Thus, results obtained with cultured epithelial cells may not clearly indicate which glycoproteins are essential for replication in a living host. Cell surface heparin sulfate appears to be a cofactor for viral attachment, but is not essential for viral attachment or entry.

1.3.2. Tegument Dissociation

Upon entry, most tegument proteins dissociate from the capsid as seen by EM studies, with the varied tegument proteins moving to their sites of action. The phenomenon of tegument dissociation upon virion entry is well accepted but poorly understood. It is known that upon infection the individual tegument proteins seek out the cellular compartments in which they act; VP16 enters the nucleus to effect transactivation of IE genes; vhs diffuses throughout the cytoplasm to destroy cellular

mRNA; VP22 has been shown to spread to adjacent cells, using a novel trafficking pathway that involves the actin cytoskeleton (Elliott and O'Hare, 1997). There are no identified mutants that prevent tegument collapse upon entry, although the kinase UL13 has been speculated to act in this role (Pak *et al.*, 1995).

1.3.3. Tegument Protein Actions

1.3.3.1 VP16.

VP16 (also known as Vmw65, α TIF, ICP25) is encoded by the late gene UL48. It has been exhaustively studied and is one of the best understood HSV gene products. It is a 480 AA phosphoprotein that comprises a major amount of the virion tegument. The best studied aspect of VP16 is its ability to transactivate IE genes, which all contain a consensus TAATGARAT sequence which VP16 binds to (Triezenberg *et al.*, 1988b; Triezenberg *et al.*, 1988a). VP16 binds the TAATGARAT sequence in conjunction with the cellular proteins host cell factor (HCF, also known as VCAF, CFF, or C1 factor) and the ubiquitous cellular transcription factor Oct-1 (also known as NFIII, OBP100, OTF-1, POU2F1) (McKnight *et al.*, 1987a; Kristie and Roizman, 1988; O'Hare *et al.*, 1988b; O'Hare and Goding, 1988a; Gerster and Roeder, 1988; Goding and O'Hare, 1989; Kristie *et al.*, 1989; Xiao and Capone, 1990; Katan *et al.*, 1990; Werstuck and Capone, 1993). VP16 has been shown to bind DNA in the presence of low salt and very high concentrations of VP16 (Kristie and Sharp, 1990), but this weak binding does not seem to be relevant *in vivo*. Two distinct functional domains of VP16 are known: the C-terminal portion (AA 413-490) is called the acidic activation domain (AAD), and acts to recruit

transcription components to the vicinity of the TAATGARAT element, while the N-terminal portion (AA 1-412) interacts with the host cell factors Oct-1 and HCF, and appears to confer TAATGARAT specificity upon VP16 (Triezenberg *et al.*, 1988a).

The C-terminal portion of VP16 does not require the N-terminal portion of VP16 to transactivate, and can transactivate when fused to the DNA-binding domains of proteins such as yeast GAL4 or E. coli TetR (Sadowski *et al.*, 1988; Cousens *et al.*, 1989; Gossen *et al.*, 1995). The AAD is dispensable for Oct-1 / HCF / VP16 complex formation but is required for transactivation. This portion has been shown to directly interact with TBP (Stringer *et al.*, 1990), TFIIB (Hori *et al.*, 1995; Lin *et al.*, 1991), TFIIF (Xiao *et al.*, 1994), replication factor A (RPA) (He *et al.*, 1993; Li and Botchan, 1993), thus bringing elements of transcription preinitiation complexes to the TATA boxes of IE genes.

Cellular expression of the N-terminal portion of VP16 also inhibits VP16 transactivation of IE genes (Friedman *et al.*, 1988). The VP16 N-terminus appears to bind the GARAT sequence while Oct-1 binds the TAAT 5' sequence. This portion is inert as a transactivator in mammalian cells, but oddly has been shown to transactivate reporter sequences when fused to the GAL4 DNA-binding domain in yeast (Popova *et al.*, 1995). Bovine Herpesvirus (BHV), Equine Herpesvirus (EHV), and VZV all encode homologues of VP16 that do not contain well-conserved C-terminal acidic domains yet BHV, EHV and VZV transactivate TAATGARAT elements (Carpenter and Misra, 1992; Misra *et al.*, 1994; Misra *et al.*, 1996; Purewal *et al.*, 1994; Moriuchi *et al.*, 1993). BHV

VP16 seems to have at least two regions necessary for transactivation, in both the N and C termini (Misra *et al.*, 1995). Thus, it is possible that the N-terminal portion of VP16 may have a hidden transactivator function that is not obvious upon HSV infection of cultured mammalian cells. It has been suggested that the N-terminal portion of HSV-1 VP16 may bind a transactivating yeast protein with homology to vhs, since the VP16 portions required for yeast transactivation and vhs binding overlap (Popova *et al.*, 1995).

VP16 may have numerous functions in the HSV life cycle. There is considerable evidence that VP16 may be an essential structural protein (Ace *et al.*, 1988; Weinheimer *et al.*, 1992). VP16 has been shown to contact or bind a number of virion proteins in various studies, including gB (Zhu and Courtney, 1994), vhs (Smibert *et al.*, 1994), and VP22 (Elliott *et al.*, 1995). It has been shown that VP16 prevents vhs formed late in infection from shutting off viral transcription (Lam *et al.*, 1996) (discussed in more detail below).

The transactivation function of VP16 does not seem to be essential during early or late infection (Ace *et al.*, 1988; Lam *et al.*, 1996) but late synthesis of VP16 is required for production of virions (Ace *et al.*, 1989; Weinheimer *et al.*, 1992). VP16 could be needed due to a structural function or for late vhs downregulation. A VP16-/vhs- virus (8MA/ Δ Sma) requires VP16 in trans to produce infectious virions in the absence of virion host shutoff, arguing that the shutoff-dampening role of VP16 is not critical for virion formation (Lam *et al.*, 1996). A virion structural requirement seems likely for VP16 as it

forms a significant fraction of the tegument and contacts many other virion proteins, but this requirement has not yet been conclusively demonstrated.

Evidence that the transactivation function of VP16 is dispensable for virus replication emerged from studies of in1814, a viral mutant which contains a 12 bp insertion within the VP16 ORF. The insertion prevents IE transactivation and complex formation yet still allows virion formation (Ace *et al.*, 1989). However, plaquing efficiency and PFU/particle ratio are heavily reduced (Ace *et al.*, 1989). In the absence of IE transactivation, viral genomes lie “latent” within epithelial cells until rescued by viruses containing ICP0 (Preston *et al.*, 1994). The number of viral genomes entering “latency” can be increased by addition of interferon- α (IFN- α) to medium (Preston *et al.*, 1994). The poor infectivity of in1814 can also be countered by addition of hexamethylene bis-acetamide to the cellular medium (McFarlane *et al.*, 1992), which increases the plaquing efficiency ~ tenfold. It has recently been shown that the plaquing efficiency of V422, a VP16 mutant lacking the C-terminal AAD, can also be partially restored by addition of HMBA (Smiley and Duncan, 1997). The defect in plaque formation seems to be a consequence of decreased ICP0 synthesis in VP16 transactivation-deficient virus; U20S osteosarcoma cells, which have been shown to partially complement ICP0 null viruses (Yao and Schaffer, 1995), also act to complement the plaque formation defect in V422 and in1814 viruses, both of which are transactivation deficient (Smiley and Duncan, 1997). Thus, it seems that the requirement for VP16

transactivation reflects a need for ICP0 expression and activity, which allows a productive infection to ensue.

It has been suggested that IFN- α exerts a specific inhibitory effect on VP16 as IE gene expression in IFN- α treated cells is decreased while viral entry and delivery of viral DNA to the nucleus are unaffected (De Stasio and Taylor, 1990). IFN- α lowers plaquing efficiency of in1814, increasing the likelihood that the viral genome will enter a latent state in epithelial cells (Preston *et al.*, 1994). It seems that this effect is not VP16-specific, as cellular genes controlled by TAATGARAT elements can still be transactivated by virion VP16 in the presence of IFN- α (Nicholl and Preston, 1996). Thus, the effects of IFN- α are not specific to VP16, but are specific to expression of IE genes from the viral genome. It is interesting to note that IFN- α treatment of cells causes induction of proteins associated with POD bodies (also known as ND10 sites or Kr bodies) (Gongora *et al.*, 1997; Nason-Burchenal *et al.*, 1996; Doucas *et al.*, 1996; Lavau *et al.*, 1995) and overexpression of these proteins is correlated with antiviral activity (Doucas *et al.*, 1996). HSV ICP0 and the CMV ICP0 homologue both disrupt POD bodies upon expression (Everett and Maul, 1994; Koriath *et al.*, 1996), and the viral genome has been shown to localize to POD bodies before ICP0 expression (Maul *et al.*, 1996). It may be that IFN- α dampens IE gene expression and thus prevents ICP0 activity, causing viral genomes to be sequestered at POD bodies, rather than preventing VP16 function. The apparent latency of in1814 which can be rescued by ICP0-competent viruses may be due to a similar phenomenon.

The VP16 function of HSV-2 is not as well characterized as in HSV-1. VP16 amino acid similarity is 86% between HSV-1 KOS and HSV-2 333, although the acidic transactivation tail is less well conserved (70%) (Greaves and O'Hare, 1991). Despite these differences, both transactivate CAT reporter genes to a similar extent in transfection assays and form complexes of similar size on TAATGARAT elements (Greaves and O'Hare, 1991). A mutation in HSV-2 (ts13) that maps to the VP16 locus appears to render virions temperature sensitive (Moss, 1989), suggesting that VP16 plays an important structural role in HSV-2 as well.

The tegument proteins VP11/12 (coded by UL46) and VP13/14 (coded by UL47) have both been implicated in modulating VP16 activity (Zhang and McKnight, 1993; McKnight *et al.*, 1987b).

1.3.3.2. UL13.

The late UL13 gene (also known as VP18.8) has been predicted by sequence analysis to encode a serine-threonine protein kinase (Smith and Smith, 1989) and has recently been shown to phosphorylate or induce the phosphorylation of casein when expressed in a rabbit reticulocyte system (Prod'hon *et al.*, 1996). The UL13 gene is not essential for viral growth in cell culture (Purves and Roizman, 1992; Coulter *et al.*, 1993; Overton *et al.*, 1994) although UL13 null mutants create smaller plaques and suffer ~10-fold reduction in titer (Coulter *et al.*, 1993). UL13 is a ~57 kDa virion phosphoprotein (Cunningham *et al.*, 1992; Coulter *et al.*, 1993) produced late during infection, and is

present in low concentrations both during infection and within the virion tegument (Overton *et al.*, 1992; Coulter *et al.*, 1993). After nonionic detergent treatment in the presence of [γ ³²P]-ATP or GTP, virion extracts are able to phosphorylate endogenous UL13 (Cunningham *et al.*, 1992; Overton *et al.*, 1992). Because this kinase activity fractionates with UL13 on DNA-cellulose chromatography, UL13 has been suggested to autophosphorylate (Cunningham *et al.*, 1992). Supporting the role of UL13 as a nuclear protein kinase, Prod'hon *et al.* (1996) showed that UL13 transfection results in an ~6 fold increase of ³²P-labelled nuclear protein compared to empty vector, showing that UL13 can affect the phosphorylation of cellular proteins.

Oddly, cell lines expressing UL13 do not show phosphorylation of UL13 unless infected with UL13 null mutants (Coulter *et al.*, 1993). It has since been shown that the nuclear protein kinase activity does not occur in UL13 null mutants and that one of the other virion proteins underphosphorylated in the absence of UL13 is the abundant tegument phosphoprotein VP22 (Coulter *et al.*, 1993). More recent research has discounted the importance of this finding, showing that VP22 is phosphorylated predominantly by the cellular protein casein kinase II (CKII) (Elliott *et al.*, 1996).

Purves and Roizman (1992) have shown a role for UL13 in mediating processing of the IE gene product ICP22. UL13 null mutants produce only one minimally phosphorylated ICP22 protein during infection, while several hyperphosphorylated species exist in wt infection (Purves and Roizman, 1992). Further experiments have

shown that UL13 and ICP22 null mutants have some characteristics in common. Both viruses suffer impaired growth in baby hamster kidney (BHK) and HEL cells but are only mildly impaired in human epidermal carcinoma (Hep-2) or Vero cells, suggesting that the latter cell types may complement the mutants in some way (Purves *et al.*, 1993). Protein and mRNA levels of ICP0 and US11 are reduced in both UL13 and ICP22 null mutant BHK and rabbit skin cell infections, suggesting that an effect of UL13 on posttranslational modification of ICP22 is necessary for some aspect(s) of ICP22 function in these cell types.

Cycloheximide reversal experiments, in which translational inhibition is initially applied and then switched with transcriptional inhibition to prevent late gene expression, have been used to show that ICP22 produced early is not phosphorylated by virion UL13 (Purves *et al.*, 1993). However, the presence of UL13 was not verified and it is not obvious that virion UL13 would be intact at >10 h PI, leaving uncertainty as to whether virion or newly-synthesized UL13 effects the alteration of ICP22. Other results using transfection systems to assay UL13 activity have shown that transfection of UL13 alone results in a large inhibition of CAT production under the ICP4 promoter, and that at certain ratios of transfected DNA, ICP22 and UL13 together appear to reduce this inhibition (Prod'hon *et al.*, 1996).

The presence of ICP22 in complexes with RNA polymerase II, ICP4, the host nucleolar protein EAP (L22), and new viral DNA has been shown to be dependent upon

the presence of UL13, which phosphorylates ICP22 (Leopardi *et al.*, 1997). Substitution of the CMV gene UL97 for UL13 has been performed (Ng *et al.*, 1996). UL97+/UL13- viruses do not suffer a growth restriction in BHK cells. Phosphorylation of ICP22 is at best weakly stimulated by UL97, however, indicating host cell restriction and ICP22 phosphorylation may be unrelated. Interestingly, UL97 seems to complement a weakening of host shutoff in UL13- infection. Under conditions that prevented transcription, translation levels (expressed as a percentage relative to 100% actin production in mock infected) were 38% for wt and UL13 restorants, 28% for UL97+/UL13-, and 60% for UL13- virus (Ng *et al.*, 1996). This “dampening” of host shutoff in UL13- virus conflicts with the strong claim made by Overton *et al.* (1994) that virion host shutoff is completely dependent upon UL13.

1.3.3.3. Vhs Dependent and Independent Host Shutoff

It has been known for several decades that HSV infection causes the early shutoff of host protein synthesis, and reduced incorporation of tritiated uridine into RNA (Sydiskis and Roizman, 1966; Roizman *et al.*, 1965). These experiments showed two distinct phases of blocked RNA synthesis; (i) from 0-3 h. postinfection RNA synthesis dropped but rises afterwards until (ii) 6 h., at which time RNA synthesis begins declining and continues until cell death.

HSV infection was found to cause instability of mRNA along with dissociation of cellular polyribosomes (Nishioka and Silverstein, 1978). NaF treatment in MEL cells

dissociated polyribosomes but did not induce mRNA instability, indicating that dissociation was a secondary effect (Nishioka and Silverstein, 1978).

Nishioka and Silverstein (1978) suggested that HSV-induced mRNA instability was dependent on HSV gene expression, as UV irradiation and cycloheximide treatment prevented host shutoff. UV-irradiated HSV was shown to dissociate polyribosomes without degradation, suggesting that dissociation was a separate process from mRNA degradation. Other researchers have claimed (Hill *et al.*, 1983) that HSV-2 shutoff is caused by a virion component while HSV-1 shutoff is dependent upon gene expression, as determined by measuring HSV-induced shutoff in MEL cells using UV-inactivated virions, actinomycin D treatment, or cross-linked virus. Later studies in MEL cells (Smibert and Smiley, 1990) as well as Vero cells (Schek and Bachenheimer, 1985; Fenwick and Everett, 1990a) showed that virion UV inactivation or actinomycin D treatment did not impair early host shutoff. Conclusively, knockout of the UL41 gene (Fenwick and Everett, 1990a) showed that early host shutoff did not occur in the presence or absence of actinomycin D and polyribosomes did not dissociate. They also showed that the UL41 product was present within the virion in wt virus but absent from vhs null mutants in Vero cells. It is now generally accepted that early host shutoff does not require gene synthesis. The effect of the IE gene product ICP27 (described in more detail below) may be responsible for previously observed IE-dependent shutoff (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994).

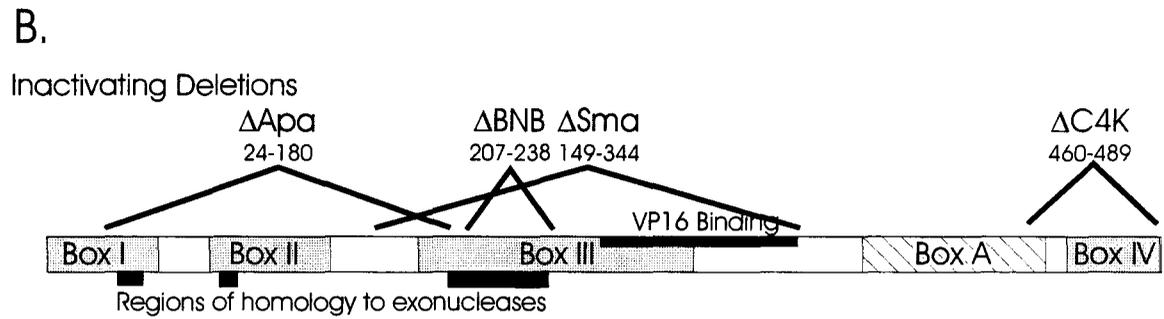
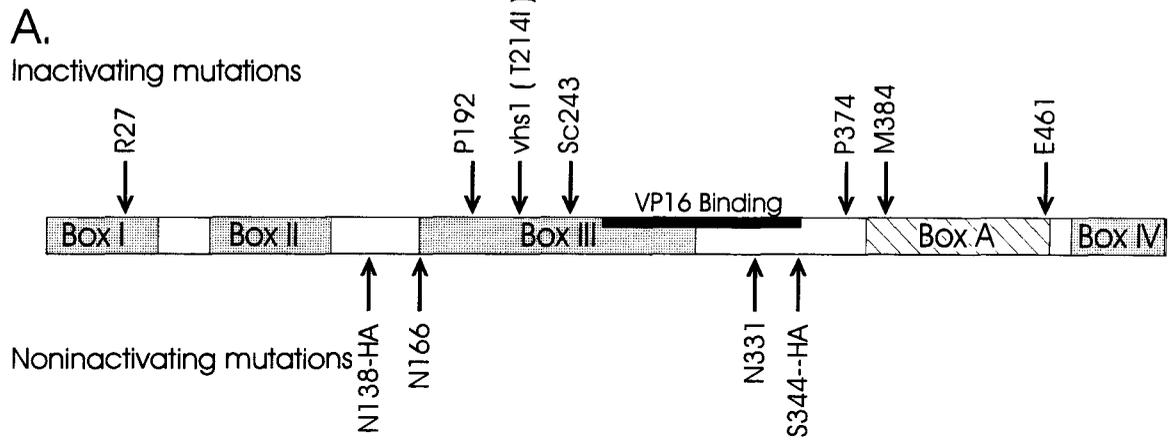
A possible complication with the use of differentiated MEL cells to test shutoff is that they are differentiated using either dimethylsulfoxide (DMSO) or hexamethylene bis-acetamide (HMBA). DMSO in combination with cycloheximide induces globin synthesis in both erythroid and non-erythroid cells (Cheung, P., personal communication). As well, HMBA or DMSO can be used to complement the loss of VP16 transactivation for viral replication, and have been shown to effect increased IE gene transcription (McFarlane *et al.*, 1992). The mechanism(s) by which either drug works to differentiate MEL cells is not fully known, but it is possible that the drug effects may remain after removal of the drug.

Mapping of the vhs1 mutation to the UL41 reading frame (Kwong *et al.*, 1988) allowed identification of the vhs gene product, with a predicted molecular weight of 55 kDa (McGeoch *et al.*, 1988). Vhs has been identified as a phosphoprotein of 58 kDa (Smibert *et al.*, 1992). Multiple forms of phosphorylated vhs have been found in infected cells, yet only the faster-migrating (less phosphorylated) form is present in virions (Read *et al.*, 1993). Properties of the vhs ORF are diagrammed in Fig. 1.3.

Vhs seems to be the only virion component needed for early viral shutoff independent of viral gene expression, although the mechanism by which vhs effects mRNA destabilization are as yet unknown (discussed below). Numerous experiments have verified that host cell shutoff still occurs if HSV virions are UV irradiated or translation of IE genes is halted by use of translation- or transcription-blocking drugs

Figure 1.3. Schematic diagram of the virion host shutoff (vhs) protein ORF.

The diagram is modified from Jones *et al.*, 1995, Fig. 8. (A.) The shaded regions (Box I - IV) and hatched region (Box A) indicate regions of >25% and 11% aa homology respectively between five different herpesviruses (Berthomme *et al.*, 1993). The solid bar indicates a region required for VP16 binding (Smibert *et al.* 1994). Linker insertion mutants that inactivate vhs in cotransfection assays are marked with arrows on the top of the ORF map, and those that did not inactivate are on the bottom (Jones *et al.*, 1994). (B.) Map of vhs inactivating deletions (Jones *et al.*, 1995; Strelow *et al.*, 1997). The dark bars at the bottom indicate regions of homology to exonucleases, as noted in the text (Doherty *et al.*, 1996).



such as cycloheximide or actinomycin D (Act D). In transfection assays using CAT as a reporter gene (Pak *et al.*, 1995) or β -galactosidase (β -gal) (Jones *et al.*, 1995), cotransfection of vhs alone blocks expression of the reporter genes, implying that vhs can destabilize mRNAs in the absence of other viral gene products. Some discrepancies between the two transfection systems were found; Pak *et al.* (1995) showed that transfection with vhs1 mutants increased reporter expression, but no such effect was found by Jones *et al.* (1995).

It is not clear what function of vhs is important for infection, although degradation of MHC (major histocompatibility complex) mRNA (Hill *et al.*, 1994) may serve to aid the function of the IE gene ICP47 (discussed later). Vhs null viruses consistently produce ~10-fold less virions in cell culture, implicating that vhs has a function that is independent of the host immune response. Vhs-induced shutoff does not discriminate between viral or cellular transcripts, which suggests that vhs may play a role in regulation of viral gene expression. The UL41 mutant vhs1 is hampered in shutoff and numerous studies (Oroskar and Read, 1987; Kwong and Frenkel, 1987; Oroskar and Read, 1989) have shown that this leads to abnormally long IE and E gene expression, increased stability of viral mRNAs, and overproduction of IE and E viral proteins. The disruption of ordered viral gene expression in shutoff-deficient virus may cause the reduction of viral titer observed in vhs mutants.

Vhs has been shown to bind VP16 both in vitro and in vivo (Smibert *et al.*, 1994). Vhs preincubated with protein A-VP16 blocks formation of the Oct1 / HCF / DNA complex but does not displace it if added afterwards (Smibert *et al.*, 1994). vhs-VP16 binding has been proposed to serve a number of possible functions (Smibert *et al.*, 1994): (i) VP16 could recruit vhs into the virion for packaging; (ii) vhs could block VP16 transactivation late in infection; (iii) VP16 may prevent the destruction of viral transcripts late during infection by large quantities of newly made vhs protein. The last function has been demonstrated by Lam *et al.* (1996). VP16 null viruses suffer a severe translational shutoff in noncomplementing cell types late in infection. This “late shutoff” is prevented in a dual VP16/vhs null mutant (8MA/ Δ Sma) and cell lines constitutively expressing VP16 are somewhat resistant to shutoff by incoming virions as well (Lam *et al.*, 1996). Thus, late shutoff in VP16 null viruses is due to the unrestrained activity of late-produced vhs.

Another type of late shutoff has been found in HSV γ 34.5 null mutants, in which late HSV protein translation ceases. This late shutoff is not caused by vhs as dual UL41-/ γ 34.5- mutants still suffer late protein shutoff (Poon and Roizman, 1997).

It is well established that HSV-2 strains generally have a stronger host shutoff function than HSV-1 strains, although the biological significance of strong host shutoff is unknown. Transfer of a HSV-2 G UL41 (vhs) gene into an HSV-1 vhs null mutant results in virions that cause strong shutoff (Fenwick and Everett, 1990b). In

cotransfections with ICP0, ICP4, and the CAT reporter gene, HSV-2 strain 333 vhs was slightly more effective than HSV-1 strain KOS vhs at shutting off CAT activity (Pak *et al.*, 1995). Recent workers attempting to use vhs as a suicide gene noted that HSV-2 strain 333 vhs was much more effective than HSV-1 vhs at inhibiting HIV replication in cotransfections of vhs and HIV proviral DNA (Hamouda *et al.*, 1997).

HSV-1 induced shutoff degrades ribosomal protein mRNA at the same rate as β -actin, but ribosomal protein translation continues at high efficiency late in infection (Simonin *et al.*, 1997). A previous report claimed that H3 histone mRNA does not degrade in MEL cells (Mayman and Nishioka, 1985) but this is not the case in Vero cells, where H3 mRNA is degraded (Schenk and Bachenheimer, 1985).

Vhs is necessary for productive infections in animal models and is involved in reactivation from latency. Trigeminal ganglia of mice infected with vhs null virus contains approximately 30-fold less viral DNA than wt; vhs null virus also reactivates less efficiently in explant cocultivation studies, at 4% versus 75% for wt virus (Strelow and Leib, 1995). The fact that vhs has active homologues (Berthomme *et al.*, 1993) in five neurotropic herpesviruses (HSV-1, HSV-2, VZV, pseudorabies virus (Berthomme *et al.*, 1993), and equine herpesvirus 1 (Feng *et al.*, 1996)) but not in the sequenced lymphotropic herpesviruses suggests that vhs may have an important role in neurotropism. Oddly, HSV infection of cultured peripheral neurons does not result in

host shutoff (Nichol *et al.*, 1994), implying that an enigmatic function of vhs is involved in neurotropism.

Many recent studies have been performed on the cytotoxicity of various HSV genes in an attempt to reduce cytotoxicity in proposed HSV-derived gene therapy vectors. Vhs seems to be an attractive candidate for causing cytotoxicity due to its aforementioned mRNA destabilizing effects. Contrary to expectations, in cell-survival assays, KOS strain and vhs1 did not differ in cellular toxicity (Johnson *et al.*, 1992), even if UV-irradiated to prevent gene expression (Johnson *et al.*, 1994). Also, disruption of UL41 did not affect cytopathicity in an ICP4 null background (Johnson *et al.*, 1994). These results indicate that vhs is not a major contributor to cytotoxic effects in the cell, and counterintuitively may reduce cytotoxicity by downregulating levels of cytotoxic IE genes (Kwong and Frenkel, 1987; Oroskar and Read, 1987).

The use of mixed infections to investigate the nature of the host shutoff response and vhs has yielded results that are difficult to interpret. An early report showed that HSV-1 strain KOS could interfere with the strong (early) shutoff of protein synthesis caused by HSV-2 strain 186 (Hill *et al.*, 1985). Mixed infections of wt HSV-1 and vhs1 virus result in blockage of translational shutoff (Kwong and Frenkel, 1989; Read *et al.*, 1993) but the deletion mutant vhs- Δ Sma does not inhibit shutoff (Read *et al.*, 1993). Fenwick and Everett (1990) showed that mixed infections of strains HSV-1(17+) and HSV-2 G resulted in weak shutoff. However, the virus 17G41, which contained both the

HSV-1 (17+) UL41 gene and HSV-2 G UL41 within the tk locus, showed a strong shutoff phenotype. The failure of the weak vhs function (type 1) to interfere with the strong response (type 2) when both were packaged within the same virion was surprising given that mixed infections result in dominant weak shutoff. A seemingly endless series of reports has claimed that inactivation of the shutoff defective (Matis and Szanto, 1985) HSV-1 strain HSZP virions by zinc (Matis and Krivjanská, 1994), antibody (Matis *et al.*, 1992), and heat (Matis and Krivjanská, 1988) prevents virion entry into the cell but allows HSZP to interfere with shutoff caused by a superinfecting strain of HSV-1 KOS.

Although extensive efforts have been made to investigate the mechanism by which vhs exerts its mRNA-destabilizing effects, its mechanism of action remains enigmatic. Although vhs has been shown to destabilize mRNA when translated in rabbit reticulocyte lysate without other viral genes (Elgadi *et al.*, 1996; Zelus *et al.*, 1996), it has not been shown conclusively that vhs is itself an RNase or whether it uses cellular factors to cause degradation. Zelus *et al.* (1996) show that virion extracts contain an mRNAse activity and that this activity is not present in vhs null mutants. A possible support for this claim is the observation that alphaherpesvirus virion host shutoff proteins contain sequences homologous to the 5' nuclease domain of pol I enzymes, and a family of endonucleases that includes mammalian FEN I (DNase IV), ERCC-5 (XPG), yeast RAD2, and T4 RNase H (Doherty *et al.*, 1996). The fact that a defective vhs mutant (vhs1) contains a Threonine → Isoleucine mutation in a conserved tripeptide supports the hypothesis that this domain is necessary for function. Interestingly, among the

homologous endonucleases, both vhs and T4 RNase H lack a proposed DNA-binding helix-hairpin-helix motif (Doherty *et al.*, 1996). Although vhs may have basal mRNAse activity, mixed infection data (discussed above) suggests that competition for host factors may limit shutoff, implying that vhs protein alone cannot cause the swift degradation observed.

1.3.4. Capsid Nuclear Transport.

The capsid, now freed of most (but likely not all) tegument proteins, moves through the cytosol via a microtubule mediated mechanism. Capsids have been shown to bind dynein, a microtubule-dependent minus end directed motor protein in the cytosol (Sodeik *et al.*, 1997). Via this interaction, capsids are transported to the microtubular minus end, near the nucleus. The release of the HSV genome into the nucleus requires a viral product, since tsB7 mutants accumulate DNA-filled capsids at the nuclear periphery (Batterson *et al.*, 1983).

1.3.5. HSV Immediate-early (IE) Genes

Upon nuclear entry, the IE (also known as α) genes are expressed with the aid of VP16. All but one IE genes have important roles in regulating gene expression, and likely perform several functions. The expression of the IE genes is essential expression of the early (E, β) and late (L, γ) genes. E gene expression is necessary for viral DNA replication, which amplifies expression of the L genes required for the production of progeny virions.

1.3.5.1. ICP0

ICP0 (Vmw110, IE110, IE1) is referred to as a “promiscuous” transactivator as it has the ability to increase activity of all classes of HSV viral gene promoters (Cai and Schaffer, 1992), as well as those of the SV40 early promoter, the HIV LTR (Mosca *et al.*, 1987), and the cellular α globin gene in transfection assays (Cheung *et al.*, 1997).

ICP0 is heavily conserved in the herpesviruses, including VZV and CMV. ICP0 is a 110 kDa protein which is encoded by the IE1 (or $\alpha 0$) genes in the long unique repeats of the viral genome and is therefore diploid. The 3' end of the latency-associated transcript (LAT) genes overlaps with that of the 3' end of ICP0. This led to the hypothesis that LAT controls ICP0 expression via antisense RNA. However, LAT gene expression is not necessary for establishment of latency (Sedarati *et al.*, 1989; Ho and Mocarski, 1989), casting doubt upon the importance of antisense regulation of ICP0. ICP0 is located primarily in the nucleus of infected cells but in some cells is localized to the cytoplasm as well (Knipe and Smith, 1986; Kawaguchi *et al.*, 1997).

ICP0 null viruses are severely impaired in viral growth and protein expression at low MOI. It seems that many of the ICP0 null virus genomes enter cells and reside in a quiescent state from which they can be reactivated as long as 7 days after initial infection. The tendency to enter this quiescent state is MOI dependent, and at high MOI these viruses enter the lytic cycle normally. This phenotype is very similar to those present in VP16 transactivation deficient mutants, as discussed previously. Latent genomes of

HSV-2 ts mutants can be recovered by superinfection with ICP0-competent HSV, adenovirus vectors expressing ICP0, wt CMV, or by transfection with ICP0 (Zhu *et al.*, 1990; Harris *et al.*, 1989). ICP0 null viruses exhibit marked reductions in E and L gene expression but IE gene expression is not hampered (Cai and Schaffer, 1992). ICP0 is involved with latency in ganglions as well. ICP0 null viruses do not reactivate efficiently from latency in mouse trigeminal ganglia (Clements and Stow, 1989; Leib *et al.*, 1989; Cai *et al.*, 1993).

The growth impairment of ICP0-null viruses can be partially complemented by an activity in osteosarcoma U2OS cells (Yao and Schaffer, 1995). A similar cell-cycle specific activity has been identified in Vero cells (Cai and Schaffer, 1991). It is not known whether treatment with HMBA can partially complement ICP0 null mutants, as it can with VP16-transactivation deficient mutants (Smiley and Duncan, 1997).

ICP0 has been shown to directly interact with ICP4 (Yao and Schaffer, 1994), as well as the translation factor elongation factor delta-1 (EF-1 δ) (Kawaguchi *et al.*, 1997). The C-terminal ICP0 fragments shown to bind EF-1 δ are also able to stop translation in reticulocyte lysate in a dose-dependent manner. EF-1 δ binding has been postulated to account for the presence of ICP0 in the cytoplasm (Kawaguchi *et al.*, 1997), as has ICP27 overexpression (Zhu *et al.*, 1996).

Recent work on the nuclear structures known variously as ND10 sites or POD bodies (Ascoli and Maul, 1991) has revealed that a number of DNA viruses disrupt these

punctate structures upon infection, including HCMV, adenovirus 5, and HSV (Maul *et al.*, 1993; Doucas *et al.*, 1996). HSV, SV40, and adenovirus DNA localize at these structures if viral-mediated disruption of ND10 sites is prevented (Ishov and Maul, 1996; Maul *et al.*, 1996). ICP0 colocalizes with these sites early in infection and effects dispersion of the punctate structures and their constituent proteins such as PML (Maul *et al.*, 1993; Maul and Everett, 1994). ND10 sites have been proposed as prereplicative sites for HSV (Lukonis *et al.*, 1997).

ND10 sites are also disrupted in promyelocytic leukemia, a rare condition in which a protein known as PML becomes fused to the retinoic acid receptor α (RAR α) as a result of a chromosomal translocation (Goddard *et al.*, 1991; Kakizuka *et al.*, 1991). PML:RAR α becomes localized to small cytoplasmic and nuclear speckles rather than the usual ~10 punctate foci seen in unaffected cells (Ascoli and Maul, 1991). Treatment with retinoic acid restores the foci to a normal staining pattern, accompanied by differentiation of leukocytic blasts (Dyck *et al.*, 1994; Weis *et al.*, 1994), leading to hypotheses that PML bodies influence cell growth and differentiation.

ICP0 has been found to possess a zinc-binding RING finger motif, as does PML. PML or the PML RING finger motif cannot functionally substitute for viral ICP0 or its RING finger motif (Everett *et al.*, 1995). Mutations in ICP0 which alter transactivation ability also alter interaction with ND10 sites, implying that the mechanism of ICP0 transactivation is related to dispersion of ND10 sites. ICP0 has also been recently found

to bind a 135 kDa nuclear protein termed HAUSP (herpesvirus associated ubiquitin-specific protease) that does not initially specifically localize to ND10 sites. HAUSP is recruited to ND10 sites in cells infected with ICP0 mutants that localize to but do not disrupt ND10 sites (Everett *et al.*, 1997).

There are at least two hypotheses available to explain the importance of ND10 sites in viral replication. It is possible that ND10 sites represent a cellular resource (possibly sequestered transcription factors) that these DNA viruses travel to in order to exploit. An alternate hypothesis is that these sites represent a cellular defense against incoming DNA viruses, and DNA viruses must disperse these sites to avoid the sequestration and shutoff of viral transcription and DNA replication. The fact that interferons have been shown to induce many of the identified ND10 proteins, including PML and Sp100, may suggest that ND10 sites play a role in intracellular defense against DNA virus infection (Doucas *et al.*, 1996; Lavau *et al.*, 1995; Nason-Burchenal *et al.*, 1996).

1.3.5.2. ICP4

ICP4 (Vmw175, α 4, IE3) is an essential nuclear phosphoprotein which is required for activation the expression of early and late viral genes, yet downregulates its own expression and that of ICP0 (Lium *et al.*, 1996; Michael and Roizman, 1993; Kuddus *et al.*, 1995; Leopardi *et al.*, 1995). The ICP4 gene is diploid within the HSV genome. ICP4 is a 175 kDa protein that is phosphorylated, ADP- and GDP-ribosylated. Although

ICP4 has been detected in L particles it appears to be a non-virion protein (Bibor-Hardy and Sakr, 1989; Yao and Courtney, 1989). A subform of ICP4 has been shown to be membrane associated (Xia and Courtney, 1995), which may play a role in ICP4 L particle association.

ICP4 contains domains associated with DNA-binding, nuclear localization, as well as two domains associated with transactivation. ICP4 binds DNA directly, and site specific DNA binding seems essential for repression (Koop *et al.*, 1993) but not transactivation (Smiley *et al.*, 1992; Gu and DeLuca, 1994). There seems to be two classes of binding sites; one class has a consensus site ATCGTC; another class of sites has no obvious consensus sites. The significance of DNA-binding in activation is unclear, as bound DNA sequences can be deleted in some cases without loss of ICP4 transactivation (Smiley *et al.*, 1992; Gu and DeLuca, 1994). DNA binding by ICP4 in the presence of TATA-binding protein (TBP) and TFIIB may serve to repress gene expression of ICP4 and ICP0, two sites for which strong binding of ICP4 has been established. These sites can confer repression on other genes (Koop *et al.*, 1993; Gu *et al.*, 1995; Kuddus *et al.*, 1995).

A C-terminal region of the protein required for transactivation has also been shown to interact with TBP-associated factor 250 (TAF250) (Carrozza and DeLuca, 1996), which is responsible for recruiting TBP into the TFIID complex. Interaction with TAF250 is the basis for interaction with holo-TFIID, of which TAF250 is a part. ICP4

requires the entire TFIID complex to enhance transcription; TBP alone is not sufficient (Gu and DeLuca, 1994; Carrozza and DeLuca, 1996). Oddly, the TATA boxes which display the lowest affinity for TFIID are transactivated to the greatest extent by ICP4 (Cook *et al.*, 1995), leading to the hypothesis that ICP4 acts to normalize TATA-box dependent gene expression. ICP4 has also been shown to bind TFIIB in a tripartite complex with TFIID (Smith *et al.*, 1993). TFIIB binding appears to be required for repression (Gu *et al.*, 1995) as well as transactivation (Gu and DeLuca, 1994).

ICP4 and ICP22 have recently been shown to localize in nuclear structures which also contain viral DNA, RNAP II, and host EBER-associated protein (EAP) (Leopardi *et al.*, 1997). EAP has been shown to relocalize from the nucleolus to ICP4 containing sites, and this process is dependent upon HSV DNA replication (Leopardi and Roizman, 1996). EAP has also been shown to bind Epstein-Barr associated small RNAs (Toczyski and Steitz, 1991) as well as disrupt ICP4 DNA-binding (Leopardi and Roizman, 1996).

The role of ICP4 in E and L gene expression is not yet fully understood, but seems to be related both to its localization in the nucleus and its association with TFIID. The late action of ICP4 may be to recruit transcription factors to viral replication sites, thus acting as an enhancer without necessarily binding DNA. It seems likely that spatial methods of transcriptional control are used by HSV. Cellular genes are transcriptionally activated when resident within the viral genome but are repressed in the cellular genome,

and this effect is dependent upon IE gene expression (Smiley and Duncan, 1992; Smibert and Smiley, 1990; Panning and Smiley, 1989; Smiley *et al.*, 1987).

1.3.5.3. ICP22

ICP22 ($\alpha 22$) is a 68 kDa protein not found in the virion. ICP22 null mutants can grow well in Vero but grow poorly in human embryonic lung (HEL), Rat-1, and BHK cells (Sears *et al.*, 1985). This phenotype has been correlated with a decrease in late gene expression (Rice *et al.*, 1995; Sears *et al.*, 1985). The transcription rates of cellular genes have been demonstrated to decrease after infection with HSV. How is cellular gene transcription repressed? It has been proposed that the ICP22 IE gene product acts to modify RNA polymerase II (RNAP II) such that it preferentially transcribes viral genes. The C-terminal domain (CTD) of the largest subunit of RNAP II is a repeating heptapeptide that is essential and conserved between yeast and humans. RNAP II in normal cells exists in two observed forms; I_{2a}, which is unphosphorylated and associates with preinitiation complexes, and I_{2o}, which is hyperphosphorylated and associated with elongating complexes (reviewed in Dahmus, 1995). HSV-infected cells contain novel intermediately phosphorylated forms of RNAP II dubbed I_{2i}. ICP22 is required for this modification but is not required for recruitment of RNAP II to viral replication compartments late in infection (Rice *et al.*, 1995). I_{2i} formation is not correlated with cell type restriction, as both infected HEL and Vero cells produce I_{2i} (Rice *et al.*, 1994). The significance of ICP22-dependent I_{2i} formation is still unknown, but depletion of the I_{2o}

form of RNAP II is correlated with repression of host cell transcription (Spencer *et al.*, 1997).

Transfection of SV40-controlled ICP22 decreases the expression of CAT under ICP0, ICP4, ICP22, or ICP27 promoters (Prod'hon *et al.*, 1996). Cotransfection of UL13 at certain ratios partially abolished the repression of IE-CAT expression. The proposed control of ICP22 function by UL13 is discussed under UL13 function. However, transfection results may not accurately model virus infection, as cells infected with virus lacking ICP22 do not show a greater accumulation of IE mRNA (Rice *et al.*, 1995), as one would predict if ICP22 is responsible for IE gene repression.

As previously mentioned, ICP22 has been shown to localize in nuclear structures which also contain viral DNA, RNAP II, and host EAP, and this localization requires modification by UL13 (Leopardi *et al.*, 1997). ICP22 has, like ICP4, been shown to interact with EAP when expressed as a fusion protein (Leopardi *et al.*, 1997). This data conflicts with the report that ICP22 is not localized to replication compartments created by transfection (Zhong and Hayward, 1997).

1.3.5.4. ICP27

ICP27 (Vmw63, IE63, α 27, IE2, UL54), an essential IE virus gene (Sacks *et al.*, 1985), has been proposed as one of the factors involved in non-vhs "late" host shutoff. ICP27 null mutants enhance IE gene expression but suppress late gene expression, leading to the theory that ICP27 has repressor and activator regions (Sekulovich *et al.*,

1988). There seem to be two distinct activation functions of ICP27 which mediate transition from E to L gene expression, including repression of IE and E gene expression (Rice and Knipe, 1990). A metal-binding domain is present in the C-terminus of the protein and binds zinc *in vitro*, although it is not clear whether this is functionally related to ICP27's activator and suppressor functions (Vaughan *et al.*, 1992). ICP27 has been demonstrated to bind the 3' ends of unstable mRNA, stabilizing the mRNA in cotransfection assays (Brown *et al.*, 1995). The RNA-binding ability of ICP27 is conferred by a RGG motif found in a number of cellular proteins (Mears and Rice, 1996). The significance of this RNA-binding during lytic infection is unclear, but may be related to ICP27 association with small nuclear ribonucleoprotein particles (snRNPs) (Sandri-Goldin *et al.*, 1995; Sandri-Goldin and Hibbard, 1996).

ICP27 has been shown to physically interact with ICP4 (Panagiotidis *et al.*, 1997), modulating the ability of ICP4 to form complexes on DNA. Overexpression of ICP27 inhibits the nuclear localization of ICP0 and ICP4, which suggests it may affect viral transcription indirectly (Zhu *et al.*, 1996). ICP27 does not seem to have promoter specific effects; rather, it downregulates expression or export of mRNAs containing introns and stimulates usage of certain polyadenylation signals (Sandri-Goldin and Mendoza, 1992; Phelan *et al.*, 1996). The downregulation function is the consequence of pre-mRNA splicing inhibition. ICP27 causes the redistribution of cellular snRNPs and splicing factors during infection, but redistribution is not sufficient for inhibition of splicing (Sandri-Goldin *et al.*, 1995). The inhibition of splicing is important in both viral

gene regulation and shutoff of cellular genes. Transcripts of intron-containing viral genes such as ICP0 or UL15 remain unspliced within the nucleus, downregulating their expression (Hardy and Sandri-Goldin, 1994; Phelan *et al.*, 1996). Cellular mRNAs containing introns are also not spliced and expression is inhibited. However, inhibition cannot be complete, as the DNA-packaging gene UL15 requires splicing and is expressed late in infection (Baines and Roizman, 1992; Baines *et al.*, 1994; Phelan *et al.*, 1996).

An early study (Sacks *et al.*, 1985) hinted that ICP27 could play a role in host shutoff. Hardwicke and Sandri-Goldin (1994) recently showed that although ICP27 ts mutants have wt vhs activity, functional ICP27 is required to effect a decrease in the amount of spliced cellular mRNAs. The observation that ICP27, which is not a virion component, is required for a “late” shutoff may explain previous reports that claimed HSV transcription is required for host shutoff (detailed under vhs). The defects in L gene expression in ICP27 null virus, coupled with the fact that cellular gene expression differences were noted at 5-13 h. postinfection without any transcriptional or translational blockage does not rule out the possibility that enhanced cellular gene expression in ts ICP27 virus relative to wt is due to decreased late gene expression and not due to splicing inhibition.

The influence of ICP27 on splicing and poly(A) site usage represents an interesting strategy by HSV to appropriate the transcriptional machinery of the cell. It may also explain the relative paucity of introns within the HSV genome; only five viral

genes (ICP0, ICP22, ICP46, UL15, and gC) are spliced during infection, and thus splicing inhibition may regulate the amount of these gene products translated late during infection.

1.3.5.5. ICP47

ICP47 is the only IE gene which does not have a role in gene regulation. It is also non-essential for HSV growth in tissue culture, unlike most other IE genes; however, ICP47 allows HSV-infected cells to escape anti-HSV CD8⁺ cytotoxic T-lymphocyte (CTL)-mediated death (York *et al.*, 1994). ICP47 has been shown to play a critical role in early establishment of infection by inhibiting the presentation of MHC complexes. ICP47 binds to the transporter associated with antigen processing (TAP) and prevents transport of viral peptides to the endoplasmic reticulum (Fruh *et al.*, 1995; Hill *et al.*, 1995). ICP47 binds stably to the peptide binding site of human but not mouse TAP (Tomazin *et al.*, 1996; Ahn *et al.*, 1996) which may account for the lack of HSV animal reservoirs.

1.4. Experimental Rationale.

The goal of this project was to determine the factors regulating vhs activity. As noted above, three factors are known to affect vhs activity: the absence of late-produced VP16 (Lam *et al.*, 1996), the viral strain of the UL41 gene (HSV-1 or HSV-2) (Fenwick and Everett, 1990b), and the absence of the gene UL13 (Overton *et al.*, 1994). Oddly, although transferring HSV-2 vhs genes into HSV-1 conferred a strong shutoff phenotype

(Fenwick and Everett, 1990b), HSV-1 and HSV-2 vhs seemed to show similar levels of mRNA destabilization in a RRL in vitro system (Elgadi, M., unpublished, this work).

Two hypotheses could explain these differences between HSV-1 and HSV-2 vhs activity: (i) HSV-2 vhs could be inherently more efficient at causing mRNA destabilization, and RRL does not accurately replicate the cellular environment in a way that determines shutoff strength, or (ii) vhs from HSV-1 and HSV-2 could have the same shutoff ability but other virion factors determine shutoff strength. One interesting point is that the virions produced by 8MA virus, which is provided with VP16 in trans from complementing cells, cause stronger shutoff than wt virus (Lam *et al.*, 1996). As cellular VP16 is produced in trans, it may not be properly modified before incorporation into the virus. VP16 is the only virion protein yet shown to bind to vhs (Smibert *et al.*, 1994), as such, it is a possible candidate for a virion regulator of vhs activity. VP16 has been shown to modulate the activity of vhs produced late in infection (Lam *et al.*, 1996). The mechanism by which the tegument dissociates is at present unclear, and since the HSV virion contains both vhs and VP16, it seemed possible that VP16 was also modulating vhs activity early during infection. To test this hypothesis, several methods were employed, including in vitro binding assays and the construction of a HSV-1 virus containing a HSV-2 VP16 gene.

A possible hypothesis to explain the apparent discrepancy between transfection and UL13(-) infection data is that a virion regulator of vhs is inactivated by UL13. Other

results from our lab using in vitro-translated vhs indicated that differences in mRNA-degradation activity were not greater than twofold between HSV-1 and HSV-2 vhs (Elgadi, M., personal communication). Together, these data indicate that other virion components may play a role in vhs activation and / or activity modulation.

Previous efforts had shown that an HSV-2 vhs gene inserted in the tk locus of an HSV-1 UL41 null virus could cause strong shutoff (Fenwick and Everett, 1990b), but it was not clear whether this was due to HSV-2 UL41 promoter effects on vhs abundance and packaging. It had not yet been demonstrated that the HSV-2 vhs protein composition was itself responsible for shutoff phenotype during viral infection. The virus vhsT2 had been constructed previously by Karen Koop in our lab, and contained a HSV-1 vhs gene containing a HSV-2 vhs ORF within the tk locus. Thus, the virus vhsT2 was tested for shutoff phenotype, and once it was determined that vhsT2 did possess a strong shutoff phenotype, several intertypics were created to further localize the differences in activity to a region of the vhs protein.

Overton *et al.*, (1994) claimed that UL13-null virus is deficient in host shutoff while virions still contained wt levels of vhs; the data supporting this claim was not conclusive for a number of reasons: (i) UL13(-) revertants were not completely restored to wild type host shutoff phenotype, indicating secondary mutations may have occurred during selection of mutants; (ii) The transcriptional blocker Act D was only used to measure virion shutoff capability in a one timepoint experiment; (iii) The UL13(-)

mutant used displays a weak but not absent shutoff phenotype upon infection in the presence of the transcriptional blocker Act D; (iv) The observed shutoff activity is very robust, almost completely knocking out protein synthesis at 1 h postinfection (MOI 10) without Act D, which has not been observed in other HSV strains; (v) Vhs null mutants were not used as shutoff-negative controls in the experiments and it is thus difficult to determine if UL13 mutants are deficient in shutoff to the same extent that vhs null mutants are. Thus, it was first important to replicate the results of Overton *et al.* (1994) before proceeding into further investigations.

The finding that vhs activity is dependent upon UL13 during infection (Overton *et al.*, 1994) raised several questions. It has been demonstrated several times that vhs does not require other host proteins to shut off cotransfected genes (Pak *et al.*, 1995; Jones *et al.*, 1995; Hamouda *et al.*, 1997), thus it is surprising that the presence of another viral protein is required for vhs activity per se in an infected cell. However, many other possibilities remain that could explain vhs inactivity. One hypothesis is that UL13 is involved in tegument dissociation (Pak *et al.*, 1995)

II. MATERIALS AND METHODS

2.1 Production and manipulation of plasmids

2.1.1 Bacterial culture growth

Recombinant plasmids were maintained in the *Escherichia coli* (*E. coli*) strain DH5 α (Woodcock *et al.*, 1989; Hanahan, 1983), or JM109 (Yanisch-Perron *et al.*, 1985) for production of dcm-site unmethylated plasmid. Bacteria were grown in Luria-Bertani medium (LB) (1.0% bacto-tryptone, 0.5% w/v yeast extract, 1.0% w/v NaCl, pH 7.0) and plated on LB agar (LB containing 1.5% w/v Bacto agar) for isolation of individual colonies. Ampicillin was used for plasmid selection at a concentration of 100 μ g/ml in LB and LB agar.

2.1.2 Preparation of competent bacteria

A single colony of bacterial strains DH5 α or JM109 was used to inoculate an overnight 2.5 ml LB culture and incubated with shaking at 37°C. This culture was subcultured 1:100 in LB + 20 mM MgSO₄, and grown to an OD₆₀₀ of 0.6. This culture was then centrifuged at 5000 rpm for 5 min at 4°C. The supernatant was decanted and the bacterial pellet resuspended in 0.4 volumes ice-cold TFBI (30 mM KOAc, 100 mM RbCl, 10 mM CaCl₂, 50 mM MnCl₂, 15% glycerol, pH 5.8), and incubated on ice for 5 min. The mixture was centrifuged at 5000 rpm for 5 min, the supernatant decanted, and the pellet resuspended in 1/25 volume ice-cold TFBII (10 mM MOPS, 75 mM CaCl₂, 10

mM RbCl, 15% glycerol, pH 6.5). The mixture was incubated on ice for 1 h, aliquoted into 100 μ l aliquots in eppendorf tubes, and quick-frozen on liquid N₂.

2.1.3 Transformation of competent bacteria

5-10 μ l of plasmid DNA or ligation mixture was added to 100 μ l of competent bacteria in a 1.5 ml eppendorf tube. This mixture was kept on ice for 15-30 min, transferred to a 37°C water bath for 1 min, and placed on ice again for 5 min. 1 ml of LB was added to the tube and the mixture incubated with shaking at 37°C for 1 hour. Bacteria were then pelleted by brief centrifugation and the supernatant decanted. Bacteria were resuspended in the remaining liquid and spread onto LB agar plates containing the appropriate antibiotic. Plates were incubated overnight at 37°C and viewed the next day to check for resistant colonies.

2.1.4. Small-scale DNA preparation

Isolated resistant bacterial colonies from LB agar plates were picked using a sterile loop and incubated in 2 ml LB with the appropriate selection drug overnight at 37°C with shaking. Saturated bacterial cultures were harvested by decanting into a 1.5 ml eppendorf tube, briefly centrifuging to pellet bacteria, and the remaining LB aspirated. The bacterial pellet was then resuspended in 100 μ l P1 (50 mM Tris pH 7.5, 10 mM EDTA) by vortexing. 200 μ l P2 (200 mM NaOH, 1% SDS) was added to obtain lysis, and tubes were inverted several times. 150 μ l P3 (3.0 M KOAc, pH 5.5) was added to precipitate bacterial chromosomal DNA and cell debris, and the tubes were mixed and

incubated for 5-15 min at 4°C. The tubes were then spun at 14000 rpm in an eppendorf centrifuge for 5 min at 4°C. The supernatant was taken off to a new eppendorf tube and 1 ml 95% EtOH at -20°C was added to precipitate plasmid DNA. The tube was then incubated at -70°C for 5-15 min and spun for 10 min at 4°C in an eppendorf centrifuge. The supernatant was decanted and 500 µl of 70% EtOH at -20°C was added, and centrifuged again for 5 min. The supernatant was decanted again, and the remaining liquid was eliminated by drying in a vacuum dessicator (SpeedVac) for 5 min. Nucleic acid pellets were then resuspended in 50 µl of TE (10 mM Tris pH 7.4, 1 mM EDTA) containing 1.0 µg/ml RNase A. 5-10 µl of the resulting plasmid preparation was used in restriction digest analysis.

2.1.5. Cesium chloride gradient DNA preparation

Overnight cultures of plasmid-bearing bacteria were spun out at 8000 rpm for 5 min. Pellets were resuspended in 10 ml of P1 buffer supplemented with 1 mg/ml lysozyme. 10 ml P2 buffer was added to lyse the bacteria and incubated at room temperature for 5 min. 10 ml P3 was added, the mixture left on ice for 15 min, and centrifuged in an SS-34 rotor for 15 min at 4°C. The supernatant (cleared bacterial lysate) was transferred to a tube, mixed with 0.6 X isopropanol, and incubated at rt for 15 min, and spun at 8000 rpm for 10 min to pellet nucleic acids. The supernatant was drained off and the pellet resuspended in 9.5 ml TE. 10 g of CsCl was added and dissolved by incubation at 37°C. 1.0 ml of 10 mg/ml EtBr was added, and the mixture spun at 10000 for 10 min to pellet protein and RNA. The supernatant was placed in a 13

ml Beckman polyallomer seal cap tube and centrifuged in a Ti60.5 rotor for 14-20 h at 65000 rpm. The visible band of plasmid DNA was extracted by puncturing the tube with a 20 gauge needle next to the band and drawing the band into a 3 ml syringe. EtBr was removed by addition of 3 ml of CsCl saturated isopropanol followed by vortexing and removal of the top isopropanol band, which was repeated until no EtBr was visible (3-4 extractions). The remaining liquid was made up to 12.5 ml with ddH₂O, 32.5 ml of 95% ethanol added, cooled at -20°C for 15 min, and spun in a Sorvall RT6000B at 3200 rpm for 45 min at 4°C. The supernatant was discarded, the pellet washed with ice-cold 70% ethanol, and the DNA pellet was air-dried and resuspended in sterile ddH₂O.

2.1.6. Qiagen large scale DNA preparation

Qiagen DNA preparations were performed by use of a Qiagen kit (Qiagen) according to the manufacturers' instructions. Briefly, a Qiagen-tip 500 DNA isolation column was equilibrated by addition of 10 ml of QBT (50 mM MOPS, pH 7.0, 750 mM NaCl, 15% EtOH, 0.15% Triton X-100). Cleared bacterial lysate (as described below but preincubated for 15 min with 33 µg/ml RNase A) was added to the pre-equilibrated column and the flow-through was discarded. The column was then washed with 30 ml of QC (50 mM MOPS, pH 7.0, 1.0 M NaCl, 15% EtOH). Plasmid DNA was eluted via addition of 15 ml QF (50 mM Tris, pH 8.5, 1.25M NaCl, 15% EtOH). The eluted DNA was precipitated by addition of 12 ml of isopropanol, vortexing, and centrifugation at 10000 rpm for 15 min at 4°C. The pellet was washed with 70% EtOH, centrifuged at 10000 rpm for 5 min, the supernatant decanted, and the pellet dried in a SpeedVac for 5

min. The pellet was then resuspended in TE or sterile ddH₂O in an eppendorf, and plasmid DNA quantified and verified by restriction analysis.

2.1.7. Quantitation of DNA

Plasmid preps used for cloning were quantitated by measuring absorbance at 260 nm and 280 nm (1 OD₂₆₀ = 50 mg/ml double stranded DNA). Plasmids used in transfection trials were quantitated by both UV spectrophotometry and fluorimetry using Hoechst 33258 dye (Labarca and Paigen, 1980) and a Hoechst DNA fluorimeter according to the manufacturer's instructions. Briefly, DNA samples were suspended at an appropriate dilution in 1 X TNE to which Hoechst 33258 dye was added to a final concentration of 1.0 µg/ml. Readings were standardized to known quantities of DNA before each reading. Quantitation was redone unless UV and fluorimetry results agreed within a 10% margin of error.

2.1.8. Restriction enzyme digestion and modification of DNA

Restriction and modifying enzymes were obtained from New England Biolabs unless otherwise noted. Digests were performed in the manufacturer's recommended buffer and at the recommended temperature for 1-4 hours. If double digests were required, the buffer giving maximal activity to both enzymes was used. If different temperature digests were required, digestion proceeded at the lower temperature before switching to the higher temperature.

2.1.9. DNA agarose gel electrophoresis

10X DNA loading dye (50% glycerol, 0.4% xylene cyanol, 0.4% bromophenol blue, 1 mM EDTA) was added to DNA to a final concentration of 1X and loaded onto 1% or 0.7% agarose TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA) gels. Gels were run at variable voltages in 1X TBE buffer. Upon completion of the gel run, gels were visualized by staining in a solution of 1 µg/ml EtBr for 20 min with shaking, placed on a UV transilluminator (Stratagene), and photographed.

2.1.10. Ligation

Vectors for ligation were, unless otherwise noted, treated with Calf Intestinal Phosphatase (CIP) (New England Biolabs) to decrease the frequency of self-ligation. Vector DNA was then purified by running on a TBE 1% agarose gel, visualization by EtBr staining, and extraction with a Quiex kit (Qiagen) as per manufacturers instructions. Purified vector and insert were added at a 1:3 ratio and ligated in the recommended buffer for 4 h at room temp or placed in a room temperature water in an insulated container left at 4°C to equilibrate overnight.

2.1.11. Random labeling of DNA probes

Plasmid DNA was labeled for use as probe in Southern or Northern blots using the following method. Approx. 200 ng DNA was mixed with 5 mM random hexamer DNA primers (Pharmacia) in a 38 µl volume, boiled for 5 min, and snap cooled on ice for 5 min. The reaction was made up to a total volume of 60 µl 50 mM dATP, 50 mM

dGTP, 50 mM dTTP, and 800 $\mu\text{Ci/ml}$ $\alpha^{32}\text{P}$ -dCTP, and 0.3 U/ μl of Klenow polymerase (NEB). The mix was incubated at room temperature for 30 min. Afterwards, 200 μl ddH₂O was added, the total mix added to the top of a 0.9 ml bed of G-50 Sephadex (Pharmacia) spin column packed with silanized glass wool, and spun down through the column to remove unincorporated nucleotides. The remaining liquid was transferred to an eppendorf tube and boiled for 5 min to denature the probe, and added to 10 ml Church buffer (250 mM Na_xPO₄ pH 7.2, 7% SDS, 1% BSA, 1 mM EDTA) in a hybridization cylinder (Hybaid).

2.1.12. Southern blotting

DNA agarose gels were prepared as described above, with the exception of EtBr staining. Gels were placed in 0.25 M HCl for 15 min, followed by 0.4 M NaOH, 1 M NaCl for 15 min. Gel contents were then transferred in 20X SSC (3M NaCl, 0.3M NaCitrate pH 6.35) to uncharged Nytran membrane (S&S) with a PosiBlot pressure blotter (Stratagene).

After blotting, DNA was cross-linked to the membrane using a Stratalink 2000 UV crosslinker. The membrane was then prehybridized at 65°C in Church buffer for 2 X 5 min with changes of buffer. random labeled DNA probe was added and hybridized to the membrane overnight at 65°C. The membrane was then washed with 0.2 X SSC, 0.1% SDS once for 5 min, followed by washing with 0.02 X SSC, 0.1% SDS for 5 min. The

blot was then checked using a Geiger counter to check background and exposed to X-ray film (Kodak) at -70°C for variable periods.

2.3. Cell culture and viral procedures

2.3.1. Maintenance of mammalian cell culture

Unless otherwise noted, the adherent cell lines Vero (African green monkey kidney cells), 16-8 (Weinheimer *et al.*, 1992), and Cell 10 (Lam *et al.*, 1996) were grown as monolayers in Corning tissue culture flasks or plates in a humidified incubator at 37°C and 5.0% CO₂. Upon confluency, cell monolayers were removed from the plate or flask surface by aspiration of growth media, washing with phosphate-buffered saline (PBS) (0.88 mM KH₂PO₄, 6.41 mM Na₂HPO₄ pH 7.3, 137 mM NaCl, 2.68 mM KCl), followed by addition of trypsin-EDTA (Gibco) at 37°C for 2-6 min. Cultures were then tapped to dislodge remaining cells from the growth surface, and the cells resuspended in the appropriate growth medium supplemented with 10% fetal calf serum (Promega), 1.0% L-glutamine (Gibco), and 1.0% penicillin streptomycin (Gibco).

The suspension cell line MEL (mouse erythroleukemia cells) were grown in the same conditions as above. MEL cell cultures were split by gentle agitation to disperse cell clumps followed by addition of suspended cells to new media at a ratio of 1:20. Differentiated MEL cells were obtained by addition of hexamethylene bisacetamide (HMBA) two days after splitting to a final concentration of 5 mM and incubated for a further 3 days before use in experiments.

The respective media and selection conditions for each cell line areas follows:
Vero cells were grown in α MEM (Gibco) without selection; 16-8 cells were grown in α MEM and selected for using 440 mg/ml active Geneticin G418 (Gibco/BRL); Cell-10 cells were grown in DUL-His (Gibco) and selected for using 0.35 mM histidinol. MEL cells were grown in α MEM and differentiated via addition of 5 mM HMBA Cell lines were grown in the presence of selective agents unless the cells were to be infected or transfected, in which case the cells were split and grown without selection previous to use.

Cell numbers per plate were obtained by washing cells once with PBS, trypsinizing for 2-5 min followed by agitation to dislodge cells, addition of α MEM without FCS, and 2X diluted in 0.4% Trypan Blue (Gibco). Live cells were counted on a hemocytometer under a light microscope.

2.3.2. Preparation of infectious viral DNA

10 150cm² plates of Vero cells were infected with HSV strains at an MOI of 10 PFU/cell. After 24 hours, cells were harvested into media with a sterile scraper. The media was collected and the cells pelleted by centrifugation for 10 min at 1100 rpm at 4°C. The supernatant was discarded, and the cells washed once with 1X PBS and recentrifuged. Cells were resuspended in 10 ml of proteinase K buffer (200 mM EDTA, pH 8.0, 0.5% SDS, 100 μ g/ml proteinase K) and incubated overnight at 4°C. Phenol:chloroform:isoamyl alcohol was added in equal volumes, mixed via gentle

inversion for 5 min, and centrifuged at 2700 rpm in a Sorvall benchtop centrifuge to separate the phases. The top aqueous layer was extracted, and phenol extraction repeated until no proteinaceous crust was visible on the interface. The supernatant was then placed in boiled dialysis tubing, clamped, and dialyzed against 4 L of 0.1X SSC overnight at 4°C with several changes of 0.1X SSC. The resulting viral DNA was collected using sterile technique from the dialysis tubing and stored at 4°C, to be used in transfection and generation of recombinant virus.

2.3.3. Co-transfection and generation of recombinant virus

Infectious viral DNA (as prepared above) and plasmid DNA containing viral sequences for marker rescue were co-transfected as described previously (Smiley, 1980; Smiley *et al.*, 1981). Co-transfection was performed using a modification (Wigler *et al.*, 1977) of the calcium phosphate method (Graham and van der Eb, 1973). Vero cells were split as described above one day prior to transfection into 35 mm plates and allowed to grow to 50%-70% confluency. Media was replaced with 5 ml of new media shortly before transfection.

A previously determined optimal concentration of infectious viral DNA (20-70µl) was added to an eppendorf tube (tube #1) along with 2-4 µg of linearized plasmid DNA containing viral sequences for recombination, and filter sterilized ddH₂O to a final volume of 225 µl. 25 µl of 2.5 M CaCl₂ was then added and the contents mixed by inversion. To tube #2, 250 µl of 2X HEPES (62 mM HEPES, 225 mM NaCl, 2 mM

Na_2HPO_4 , pH 7.05 - 7.10) was added. A Pasteur pipette attached to a mechanical pipettor was used to gently expel air into the liquid in tube #2 while the contents of tube #1 were added slowly dropwise into the bubbling mixture. The mixture was then allowed to incubate at room temperature for exactly 20 min, and then added dropwise with shaking to the media in the 35 mm cell culture dish to distribute the mixture evenly and to avoid cell death from pH extremes. Cell-DNA mixtures were incubated in a cell incubator for 16 hours at 37°C and precipitates were washed off the cells with α MEM 3 times. 5 ml of 10% α MEM was added to each cell dish and cultures were incubated until viral infection was evident and allowed to proceed until 100% of the cells were infected. Cells were harvested via scraping, broken by repeated freeze-thawing with liquid N_2 , and stored at -70°C. Before infection of cells for plaque isolation (as described below), a portion of the lysate was filtered through a 0.45 micron sterile filter to ensure that plaques would result from single virions.

2.3.4. Isolation of recombinant virus

0.45 micron filtered viral transfection progeny were used to infect susceptible monolayers of cells at varying 10-fold concentration ranges (usually from $10^1 \mu\text{l}$ - $10^5 \mu\text{l}$ virus in 500 ml α MEM). Cells were incubated with virus dilutions for 1 hour, the media removed by aspiration, and media containing 0.1% HGG (human gamma-globulin, Immunostat) was added to prevent virion infection of nonadjacent cells by neutralizing virions secreted into the medium. BrDC (5-bromodeoxycytidine) was added to 100 $\mu\text{g/ml}$ if selecting for tk(-) recombinant virus. Selection for β -gal (+) virus was done by

covering MOI dilutions of infected cells with agarose-media overlays (0.5% agarose, 300 $\mu\text{g/ml}$ X-gal, 0.003% DMSO, 1 X α MEM complete media, 5% FCS), and waiting for the appearance of blue plaques. Blue plaques were then picked with a glass pipette and amplified as below.

Well-separated plaques were selected from the appropriate dilution plate and isolated by aspiration of media, picking of the plaque with a sterile applicator, and mixing of the applicator into a 10 mm cell dish containing 1 ml media and freshly split cells in the absence of selective agents. Cells were then incubated until all cells showed signs of infection, scraped, and harvested into two aliquots. One aliquot was stored at -70°C for future infection, and one aliquot was used for viral DNA isolation, diagnostic restriction enzyme digest, and Southern blot to verify recombinant DNA structure.

2.3.5. Growth and titration of virus

150 cm^2 70-100% confluent plates of cells were infected with 10^6 PFU (0.1 MOI) of virus and incubated until all cells showed cytopathic effects (CPE). Cells were then scraped from the plate, the media and cells transferred to 50 ml Corning tubes, and the cells spun down at 1,200 rpm for 5 min at 4°C in an IEC tabletop centrifuge. The supernatant was poured off, and 1 ml of α MEM without FCS per plate harvested was added to the pelleted cells. Virus to be used as stock was prepared by freeze-thawing the cells 5 X in liquid N_2 , centrifuged at 2000 rpm for 5 min to spin out cellular debris, the supernatant taken off and distributed into Nunc cryovials which were frozen at -70°C for

storage. Virus to be used in experiments was prepared by sonicating the mixture 3X, cooling on ice between 10 second sonication bursts. After sonication, the cellular debris was spun down at 2000 rpm for 5 min and the supernatant distributed into Nunc cryovials and stored as above.

Determination of virus titer was performed by serially diluting virus stocks tenfold from a 10^{-3} to 10^{-8} range in α MEM without FCS. 500 μ l of dilution was added to 35 mm plates of confluent cells and incubated at 37°C for 1.5 h with occasionally tilting to prevent drying. Afterwards, the medium was aspirated and 2 ml of the appropriate media containing 10% FCS and 0.1% HGG was added and the cells incubated until viral plaques became visible using a light microscope. Media was then aspirated and the cells stained with crystal violet stain (1% crystal violet, 61% EtOH, 8.7% formalin, 4.3% acetic acid). Wells containing between 30-300 plaques were used for calculation of viral titer.

2.3.6. Viral DNA isolation for Southern Blot

Viral plaque picks were isolated and amplified as described above. 500 μ l of scraped infected cells were spun down briefly at 14K rpm in an eppendorf centrifuge, and the supernatant decanted. 500 μ l of viral lysis buffer (7M urea, 350 mM NaCl, 10 mM Tris-HCl pH 7.5, 20 mM EDTA, 1% SDS) was added, and each sample was vortexed. 500 μ l of phenol:chloroform:isoamyl alcohol (25:24:1) was added, samples were vortexed for 1 min, and spun at 14k rpm at 4°C for 15 min. The supernatant was

transferred to a new tube and 500 μ l chloroform:isoamyl alcohol (24:1) added. Samples were vortexed, spun at 14k rpm at 4°C for 5 min, the supernatant taken off to new tubes, 1 ml 95% EtOH added, and the samples spun at 14k rpm for 15 min. The supernatant was decanted and the pellet washed with 70% EtOH, respun for 5 min, and dried in a SpeedVac.

2.4. Formaldehyde-agarose gel electrophoresis

Dry RNA samples were dissolved in 1 X MOPS (pH 7, 20 mM 3-n-morpholinopropanesulfonic acid, 5 mM NaAc, 0.5 mM EDTA) 50% deionized formamide, 6% formaldehyde), and heated at 75°C for 10 min. RNA loading dye (50% glycerol, 1% (w/v) xylene cyanol, 1.5% (w/v) bromophenol blue, 5 mM EDTA) was added, and samples were vortexed briefly then centrifuged to pellet the sample and loading dye. The samples were loaded in a formaldehyde-agarose gel (1% Seakem S&S agarose, 6% formaldehyde, 1 X MOPS) and run at 70-110V depending on gel size in 1X MOPS, 6% formaldehyde running buffer.

2.5. Northern blotting

After RNA gel electrophoresis, gels were soaked in 1 L of ddH₂O for 20 min; 500 ml of 50 mM NaOH, 10 mM NaCl for 15 min; and 500 ml 100 mM Tris-HCl pH 7.5 for 15 min. Gel contents were then transferred in 20X SSC (3M NaCl, 0.3M NaCitrate pH 6.35) to positively charged Nytran membrane (S&S) with a PosiBlot pressure blotter (Stratagene).

2.6. Cotransfection shutoff assay

Duplicate cultures of Vero cells in 6 well (35 mM) culture dishes (Corning) in 5 ml of α MEM were transfected with 15 μ g pUC18 carrier DNA, 15 μ g of pRSV β -gal reporter plasmid, and variable amounts of pCMVvhsT1, pCMVvhsT2, or pCMVvhs1. The amount of HCMV promoter transfected was kept constant by addition of pCMV vector. Cells were transfected as described above, incubated with DNA precipitate for 16 h, washed several times to remove the precipitate, and incubated for 24 hours in 5 ml of fresh α MEM, after which time β -gal activity was assayed.

To assay β -gal activity in transfected cells, cells were washed once with PBS, once with TEN (40 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl), lysed with ice-cold assay buffer (250 mM Tris pH 8.0, 0.5% Nonidet P-40, 1mM phenylmethylsulfonyl fluoride (PMSF)), transferred to tubes and placed on ice. 40 μ l of lysate was added to a microtiter plate containing 275 μ l of reaction mix (100 mM Na_2PO_4 pH 7.2, 10 mM KCl, 1 mM MgCl_2 , 50 mM 2-mercaptoethanol, 10 mM o-nitrophenyl- β -D-galactopyranoside (Sigma)) on ice, transferred to 37°C, and incubated for 10 - 60 min. β -gal activity was determined by measuring the A_{410} of each well with a Precision Microplate Reader (Molecular Devices).

2.7. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

An equal volume of 2X sample buffer (2XSB, 125 mM Tris pH 6.8, 4% SDS, 600 mM 2- β -mercaptoethanol, 5% glycerol, 0.001% bromophenol blue) was added to

samples and boiled for 5 min before loading. The denatured protein samples were electrophoresed through a 5% acrylamide stacking gel (4.9% acrylamide, 0.17% bis-acrylamide, 125 mM Tris-HCl pH 6.8, 0.1% SDS, 0.1% ammonium persulfate (APS), 0.001% TEMED) followed by a 12% separating gel (12% acrylamide, 0.42% bis-acrylamide, 375 mM Tris-HCl pH 8.8, 0.1% SDS, 0.07% ammonium persulfate (APS), 0.0006% TEMED) in running buffer (RB, 25 mM Tris, 192 mM glycine, 1% SDS). Gels were run in Hoefer gel rigs or Biorad minigels at respectively 60 V or 150 V (Laemmli, 1970).

Upon completion of the gel run, SDS-PAGE gel used for autoradiography were fixed in 50% methanol:10% acetic acid for 30 min, washed with ddH₂O, soaked in the autoradiographic signal enhancer Enlightning (Dupont) for 15 min, dried on a Biorad gel drier, and exposed to X-ray film or blanked PhosphorImager cassettes.

2.8. Western blots

SDS-PAGE gels were run as above but were rinsed after completion of the run in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Nitrocellulose membranes were also wet with transfer buffer before use. Gel proteins were transferred to nitrocellulose at 100 V for 1 h in a Biorad gel transfer apparatus in ice-cold transfer buffer according to the manufacturer's instructions. Prestained molecular weight markers (Promega) were used to determine if proteins had fully transferred to nitrocellulose. The nitrocellulose was then blocked for >1 h in TBS-T (20 mM Tris-HCl pH 7.5, 150 mM

NaCl, 0.05% Tween-20) with 1% instant skim milk (Carnation). The membrane was then washed 2 X 5 min in TBS-T, and the primary antibody applied at a recommended dilution for 1 h in TBS-T. The membrane was then rinsed 5 X 5 min in TBS-T, and the secondary antibody applied in TBS-T for 1 h, washed 5 X 5 min with TBS-T, and visualized with chemiluminescence reagent (Dupont NEN). The membrane was soaked in chemiluminescence mixture for 1 min, placed on Whatman paper to dry briefly, and placed in acetate sheets for exposure to X-ray film.

2.9. Translational shutoff assays

All shutoff assays were performed in 35 mm 6 well cell culture dishes (Corning). At 20 min before infection, cell media was removed and replaced with 10% FCS α MEM supplemented with 10 μ g/ml Act D to halt transcription. At time 0, cells were infected with varying MOIs of virus in α MEM without FCS and incubated for 1 h. 1 h postinfection (PI), infectious media was aspirated off and replaced with 10% FCS α MEM + Act D. Cells were then incubated until 1 h before harvest, the media aspirated, and washed 2 X with 199 – methionine media (Gibco) + Act D. The cells were then aspirated, 0.5 ml labeling media (0.9 parts media 199 - methionine, 0.1 parts α MEM, 50 μ Ci 35 S-methionine/ml, 10 μ g/ml Act D) added, and further incubated for 1 h. To harvest, cells were removed from the incubator and placed on ice. Labeling media was drawn off with a pipettor, the plates washed 2 X with ice-cold PBS, the PBS completely removed, and 200 μ l ice cold RIPA++ (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM PMSF) applied so as to cover the

plate. The lysate was then removed into eppendorf tubes, spun for 10 min at 14krpm in an eppendorf centrifuge at 4°C, the supernatant removed, and stored at -20°C.

25 µl of cell lysate was mixed with an equal volume of 2X SB, boiled for 5 min, and loaded on a 12% SDS-PAGE gel (Hoefer). Upon completion of the gel run, the stacking gel was removed, the gel fixed in 50% methanol, 10% acetic acid for 1 h, soaked in Enlightening (Dupont) for 15 min, dried on a Bio-Rad gel dryer at 80°C for 45 min, and either exposed to X-ray film or quantified using a PhosphorImager cassette.

2.10. VP16 in vitro binding assay

Plasmids pMBP-VP16₄₁₁ and pMBP have been described previously (Popova *et al.*, 1995) and are based on the pMAL-2 vector (New England Biolabs). Plasmids were transformed into E. Coli strain TB1 (New England Biolabs). Cultures were grown in 500 ml LB/5% glucose and induced with 0.3 mM IPTG at an OD₆₀₀ of ~ 0.6 and allowed to grow for 2 h before harvesting. Cells were centrifuged at 4000 X g at 4°C for 20 min, and the supernatant discarded. The bacterial pellet was resuspended in 25 ml column buffer (CB; 20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA, 0.5 mM PMSF, 1 mM dithiothreitol (DTT)) and frozen at -20°C for several hours. The frozen culture was thawed in cool water, placed on ice, and sonicated for 3 X for 30 seconds and cooled between sonications. The lysate was then centrifuged for 20 min at 14,000 rpm at 4°C and the supernatant frozen for storage at -20°C.

1 ml of MBP-VP16 or MBP protein lysate was incubated with 100 μ l of amylose-agarose beads (NEB) overnight at 4°C with gentle inversion. The beads and proteins were then spun down at 1000rpm for 30 sec at 4°C, and washed with: 1 ml CB; 1 ml CB plus 0.2 mg/ml BSA; and 1 ml of CB plus 0.2 mg/ml BSA, 0.05% NP40.

In vitro translated 35 S-met labeled proteins were diluted 100-fold in CB and 200 μ l of the diluted protein added to the washed beads, and incubated for 1 h at 4°C with gentle inversion. Following the incubation, the samples were spun down at 1000 rpm, the supernatant decanted, and the beads washed 1 X with 1 ml CB, 3 X with 1 ml CB plus BSA, and 3 X with CB + BSA + NP40. 20 μ l of 2X SDS loading buffer was added to the beads and boiled for 10 min. The beads were spun out at 14 krpm and 10 μ l of supernatant loaded and run on an SDS-PAGE gel. SDS-PAGE gels were fixed in 50% methanol 10 % acetic acid for 30 min, followed by 15 min incubation with Enlightning (Dupont), drying, and exposure to a blanked PhosphoImager cassette. Protein amounts were quantitated by PhosphoImager and ImageQuant 3.3 software.

2.11. In vitro degradation assay

2.11.1. In vitro transcription of RNA

In vitro degradation assay substrate RNA was generated from the plasmid construct pCite (Novagen). 5 μ g of circular plasmid DNA was incubated in a 50 μ l reaction containing 1X transcription optimized buffer (Promega, 40 mM Tris-HCl pH 7.5, 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl), 10 mM DTT, 1 U/ μ l RNAsin

(Promega), 0.5 mM ATP, 0.5 mM UTP, 0.5 mM CTP, 0.05 mM GTP, 0.5 mM m⁷GpppG, 1 μ Ci α^{32} GTP, 20 U T7 DNA polymerase) for 30 min at 30°C.

Unincorporated nucleotides were removed by running the products through a Sepharose G-50 column plugged with silanized glasswool. The eluent was transferred to an eppendorf tube, made up to 200 μ l with DEPC-treated ddH₂O, and phenol:chloroform:isoamyl alcohol extracted followed by chloroform:isoamyl alcohol extraction. 100 μ l of 7.5 M NaAc and 750 μ l of 95% EtOH were added and the mixture frozen at -20°C for 30 min. The samples were centrifuged at 14 krpm at 4°C for 15 min, the supernatant poured off, washed with 1 ml of 70% EtOH, and dried in a SpeedVac. The amount of incorporated radioactivity was then measured via Cherenkov counting in a Beckman scintillation counter.

2.11.2. In vitro transcription / translation of vhs protein

The Single Tube Protein System 2 kit (Novagen) was used to transcribe and translate vhsT1 and vhsT2. 0.5 μ g of circular plasmid DNA (pSP6vhsT1 or pSP6vhsT2) was added to 8 μ l of STP2 SP6 transcription mix (Novagen) and made up to 10 μ l with ddH₂O. The transcription mix was then incubated at 30°C for 15 min. To the transcription mix 40 μ Ci of ³⁵S-methionine, 30 μ l STP2 translation mix (Novagen) were added and the total volume made up to 50 μ l. This mixture was then incubated at 30°C for 60 min, a portion taken off for autoradiographic quantitation via SDS-PAGE, quickly frozen in liquid N₂ then stored at -70°C. Blank reticulocyte lysate was obtained by omitting plasmid DNA from the transcription mixture.

2.11.3. In vitro degradation assay for vhsT1 and vhsT2

Equal amounts of vhsT1 and vhsT2 protein were dispensed into eppendorf tubes and the volumes equalized with blank reticulocyte lysate. A range of concentrations of vhs was obtained by serially diluting vhs into equal volumes of blank reticulocyte. 2 μ l of the final dilution volume was removed for analysis and quantitation on an SDS-PAGE gel. ~6000 counts of capped pCITE transcript were added to each tube while on ice, and then incubated for 10 min at 30°C. The reaction was then stopped by addition of 200 μ l of Trizol, 40 μ l chloroform, and 5 μ g tRNA. The samples were vortexed for 1 min each, centrifuged at 14 krpm at 4°C for 15 min, the supernatant removed and transferred to a new tube. 2 volumes of isopropanol were added and samples cooled at -20°C for 1 h, re-centrifuged, the supernatant removed, and the samples washed with 70% EtOH before drying in a SpeedVac. Samples were then resuspended in DEPC-treated ddH₂O and frozen at -70°C.

2.12. Viruses

The viruses Δ UL13 (referred to as R7356) and parental HSV-1 strain F were obtained from Bernard Roizman (Purves and Roizman, 1992). The viruses D13lacZ and parental strain HSV-1 KOS1.1 were obtained from Stephen Rice (unpublished data).

2.13. Plasmid and virus creation

To create pUL13(-), an ICP6:lacZ cassette from the plasmid pD6P (Goldstein and Weller, 1988) was excised using BamHI, blunted, and inserted into a 490 bp Sma I-Sma I

deletion in pUL13 such that the lacZ ORF is antisense relative to the UL13 ORF. This plasmid was used in cotransfections with KOS viral DNA and selected via blue-white screening to produce a virus which contains an insertion in the UL13 gene, UL13(-).

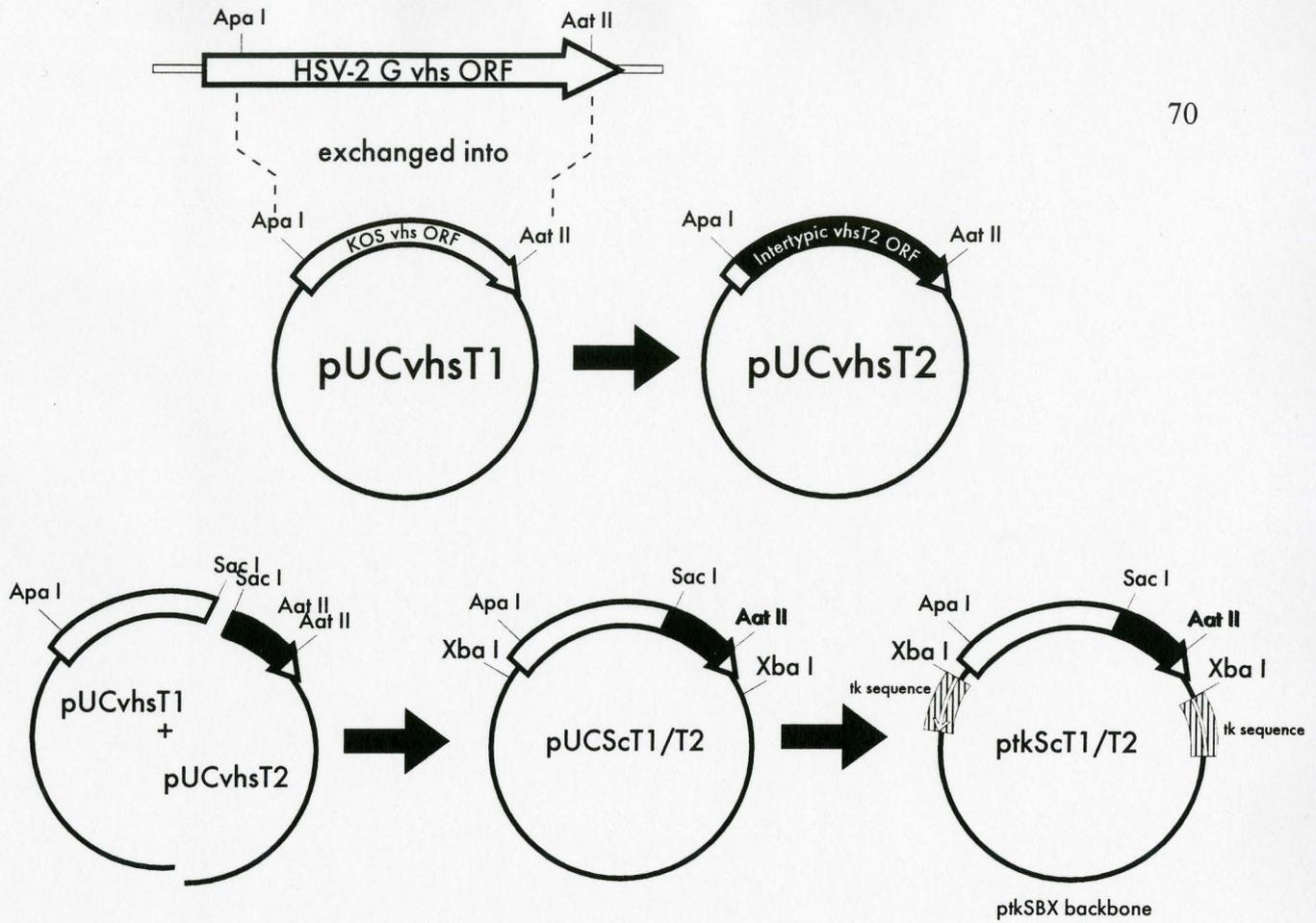
To create pUC/CMVvhsT2, an Apa I - Msc I fragment of the vhs ORF from the plasmid pSPvhsT2 was inserted into the complimentary fragment of an Apa I - Msc I cut pUC/CMVvhsT1 (Jones *et al.*, 1995). This plasmid contains a vhs ORF that is identical to those in pUCvhsT2 and ptkvhsT2 but which is under the control the same promoter sequence as that of pUC/CMVvhsT1.

To create ptkVP16-2, a Sac I 3.2 kb fragment was cloned out of the HSV-2 strain G genome which contained the HSV-2 UL48 (VP16) ORF and promoter (Genbank accession number: M57289). This fragment was cloned into a Sac I site on the plasmid ptk173 such that the VP16 ORF is antisense to the thymidine kinase ORF. This plasmid was then used in cotransfections with 8MA viral DNA followed by BrDC selection of progeny virus on Vero cells to produce a virus which contains the HSV-2 UL48 gene in the tk locus, named 8MA2R.

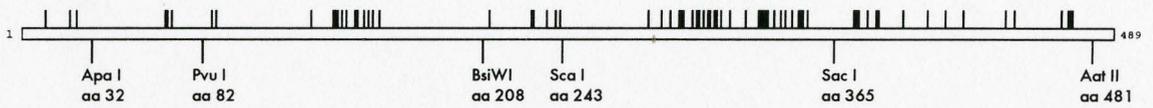
The plasmid pUCvhsT1 contains an Xba I - Xba I fragment of HSV-1 strain KOS vhs DNA in a pUC19 vector (detailed in Jones *et al.*, 1995). An Apa I - Aat II fragment of the HSV-2 G strain vhs ORF was exchanged into this plasmid by Frank Jones in our lab, creating pUCvhsT2 (Fig. 2.1). pUCvhsT2 contains a vhs ORF which is type 1 from N-terminal 1-31 aa, type 2 from 32 – 481 aa, and type 1 at the C-terminal 482-489 aa.

Figure 2.1. Intertypic plasmid creation and composition.

A schematic diagram of intertypic plasmid creation. Top: pUCvhsT2 was created by exchanging a Apa I – Aat II portion of the HSV-2 G vhs ORF into pUCvhsT1. Middle: Example of intertypic plasmid creation. Subsequent intertypics were created by exchanging portions of pUCvhsT1 and pUCvhsT2 cut at restriction sites within the vhs ORF and plasmid backbone to create chimeric vhs ORFs. These pUC-based constructs were then excised as Xba I fragments and ligated into Xba I – cut ptkSBX such that the vhs ORF is in the opposite orientation relative to the tk ORF. The ptk-based intertypics were then inserted into the vhs(-) genome via transfection and selection for tk- mutants as described in Materials and Methods. Bottom: Diagram of the intertypic vhs ORFs constructed. Differences between the HSV-2 G and HSV-1 KOS vhs ORF are noted as vertical lines on the top of the first bar. Relevant restriction enzyme sites used to create intertypics are marked on the bottom of the first bar, along with their amino acid location. Open boxes indicate HSV-1 KOS vhs sequence while black boxes indicate HSV-2 G sequence.



Substitutions & Insertions



Virus



Three amino acid differences exist between KOS and G sequence in aa 1-31, and none exist in the terminal 7 aa. Thus, three aa in the pUCvhsT2 vhs ORF differ from the HSV-2 G vhs ORF sequence: aa 11 (Q→H), aa 22 (R→G), and aa 25 (E→A).

All subsequent intertypic vhs constructs were constructed from pUCvhsT1 and pUCvhsT2 by digesting both plasmids with an appropriate enzyme to cut within the vhs ORF, digesting at a site in the non-vhs plasmid sequence, and exchanging the resultant fragments to produce an intertypic vhs ORF. Afterwards, Xba I - Xba I vhs fragments were transferred into Xba I -cut ptkSBX such that the vhs ORF runs antisense to the tk ORF. The ptkvhs plasmids were used to transfer intertypic constructs into the tk locus of vhs(-) virus, to create several intertypic viruses (PvT2/T1, BsT2/T1, ScT2/T1, SaT2/T1, and SaT1/T2, constructed by Karen Koop). Several second generation intertypics were created by the procedures described above, except that the first generation of pUC-based intertypics were digested to create a second generation of intertypic vhs plasmids (ptkBsaT1vhs, ptkScsaT1vhs, ptkBsaT2vhs, ptkScsaT2vhs) and viruses.

III. RESULTS

3.1. The Effect of VP16 on Vhs Shutoff Phenotype

VP16 has been shown to control vhs activity late in infection (Lam *et al.*, 1996). It was thus of interest to determine if VP16 controlled activity of vhs early during infection as well. As preliminary results from M. Elgadi suggested that vhsT1 and vhsT2 possess similar levels of mRNA degradative ability in an in vitro assay, it seemed possible that the early host shutoff phenotype of HSV could be influenced by VP16.

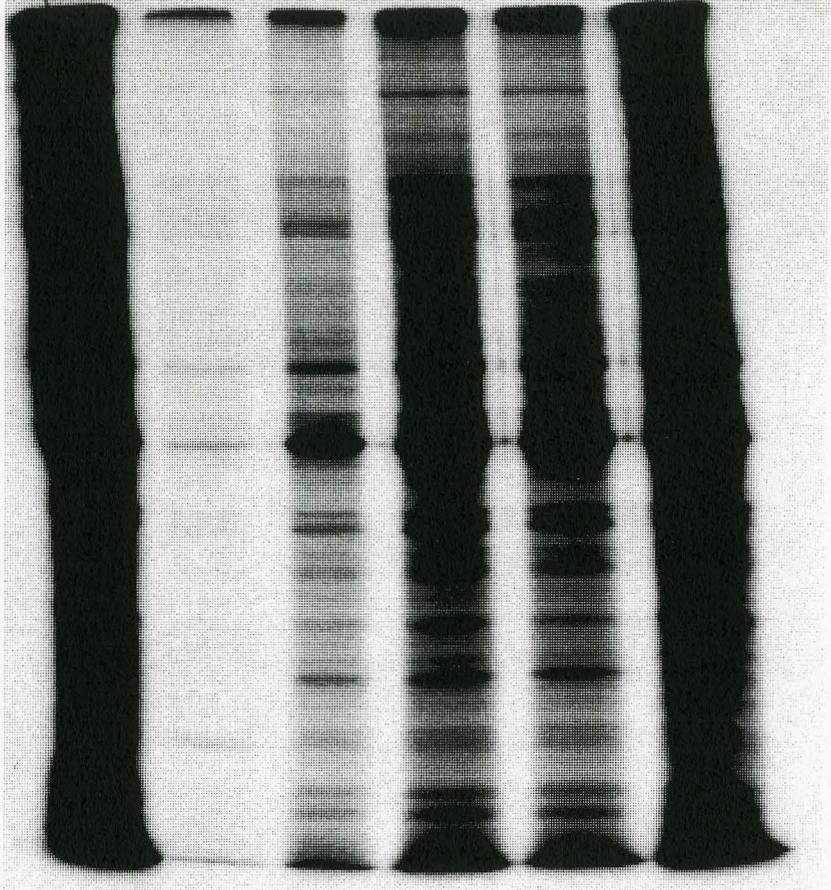
VP16 also seemed to have an effect on vhs activity in the VP16 null virus 8MA. 8MA causes enhanced shutoff relative to wild type virus, possibly because the VP16 provided to the virus is produced from the cellular genome (16-8) and may not undergo the same viral modifications as virally produced VP16. To test whether passage through the cell type 16-8 causes the altered shutoff phenotype seen in the 8MA virus, a virus in which the VP16 locus had been restored (8MAR) was passaged through 16-8 cells and compared with the same virus passaged through Vero cells (Fig. 3.1). 8MAR passaged through 16-8 showed a slight increase in shutoff, although not to the same degree as 8MA. This could be the result of an increased particle:PFU ratio caused by passage through 16-8 cells, or an effect caused by cellular VP16 provided in trans. Further experiments are necessary to distinguish between these possibilities.

The previously observed exaggerated shutoff of 8MA (Lam *et al.*, 1996) was confirmed in these experiments, and was shown to be a virion effect as no viral gene

Figure 3.1. Effects on shutoff of passage through the VP16 expressing cell line 16-8.

The virus 8MAR, which is a revertant to wild-type of the VP16 null virus 8MA, was passaged through the VP16-producing cell type 16-8 to produce the virus stock labeled 8MAR[16-8]. Above each lane the virus used to infect at 10 PFU/cell, and in brackets, the cell type that the virus was grown in. Vhs(-), a vhs null HSV-1 KOS mutant, has been described previously (Jones *et al.*, 1995). Lanes are labeled above by virus used to infect, and in brackets the cell type the virus was produced in. Vero cells were infected with virus in the presence of actinomycin D. Cells were labeled as described in materials and methods from 5-6 hrs postinfection, harvested, run on a 12% SDS-PAGE gel, and autoradiographed.

Mock
HSV-2 G [Vero]
8MA [16-8]
8MAR [Vero]
8MAR [16-8]
vhs(-) [Vero]



expression is required for exaggerated shutoff. The shutoff phenotype of 8MA does not seem to be a consequence of passage through 16-8 cells per se.

As another test of the hypothesis that VP16 contributes to the host shutoff phenotype, it was of interest to test if VP16 from HSV-2 could influence shutoff phenotype of a HSV-1 derived virus. The virus 8MA2R, containing the VP16 gene from HSV-2 (designated VP16-2) was constructed by cloning a 3.2 kb Sac I fragment containing the UL48 (VP16) gene (Burke et al. (1992), Genbank Accession #M57289) out of the HSV-2 G genome and recombined into the tk locus of the HSV-1 VP16 null virus 8MA. The structure of the tk and vhs loci were determined by Southern blot (Fig. 3.2). In Fig. 3.2b, viral DNA was probed with a plasmid containing tk sequence to confirm recombination of the 3.2 kb Sac I VP16-2 DNA fragment into the tk locus of 8MA2R. In Fig. 3.2d, Pst I / Hinc II-cut viral DNA was probed with a randomly labeled plasmid containing the vhs ORF to confirm that 8MA2R possessed a HSV-1 KOS vhs allele.

The production of VP16 during infection with 8MA2R was verified via Western blot of 12 h post-infection cell lysate with an antibody for a GST:VP16 fusion protein (Fig. 3.3a). The amount made is roughly similar to that made by other HSV virus strains, as might be expected due to the similarity between HSV-1 and HSV-2 VP16 promoters (Greaves and O'Hare, 1991). 8MA2R yielded wt-levels of virus upon harvest, grew in a noncomplementing cell type (Vero), and no distinct plaque morphology was observed,

Figure 3.2. Verification of 8MA2R Structure.

A. Schematic diagram of digests performed to verify the structure of the tk locus of the virus 8MA2R, which contains a 3.2 kb HSV-2 G fragment containing the UL48 gene.

B. Southern blot of Pvu II-cut viral DNA probed with random-labeled ptk173 plasmid, which contains the tk ORF. The name of the virus or plasmid DNA probed is listed above each lane. 8MA2R contains the HSV-2 G VP16 gene within the tk locus, and 8MAR is a revertant to wt of the VP16 null mutant 8MA. p14a is a plasmid containing the VP16-1 gene in the tk locus. The intact tk gene is represented by the 2041 bp band, and the tk locus containing the HSV-2 strain G VP16 gene is represented by the bands at 3610 and 1640. The HSV-1 VP16 gene within the tk locus is represented by the bands at 2560 and 2200. The band at 4363 corresponds to plasmid backbone. The first three and last three lanes are taken from two different exposures of the same blot for clarity.

C. Schematic diagram of digests performed to verify the structure of the vhs locus in the virus 8MA2R.

D. Southern blot of Pst I / Hinc II-cut viral DNA probed with pSP6vhs, which contains the vhs ORF. The names of the viral strain are listed above each lane. The HSV-1 vhs gene is represented by the single band at 2.2 kb (seen in HSV-1 strain KOS and 8MA2R), while the two bands at 1.3 and 1.0 kb represent the HSV-2 strain G vhs gene.

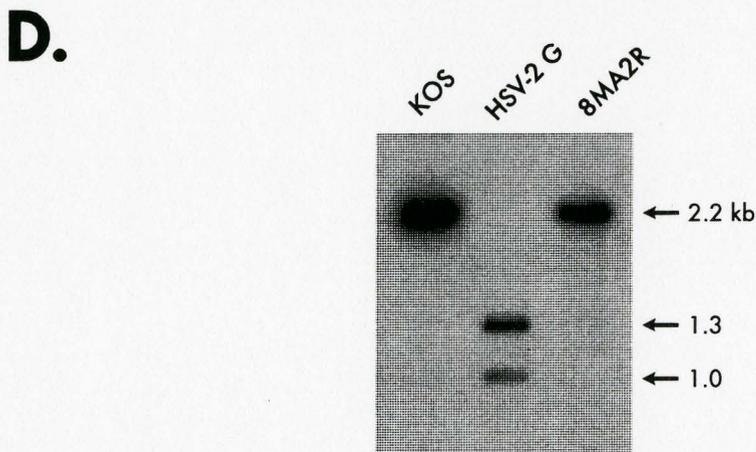
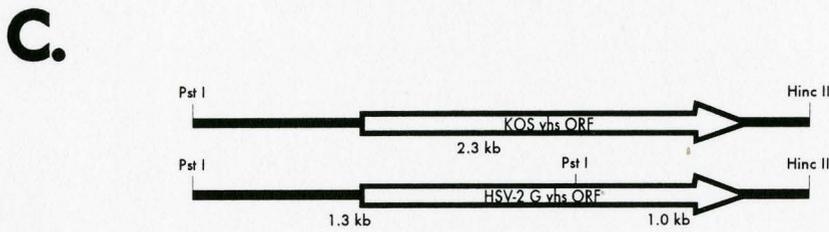
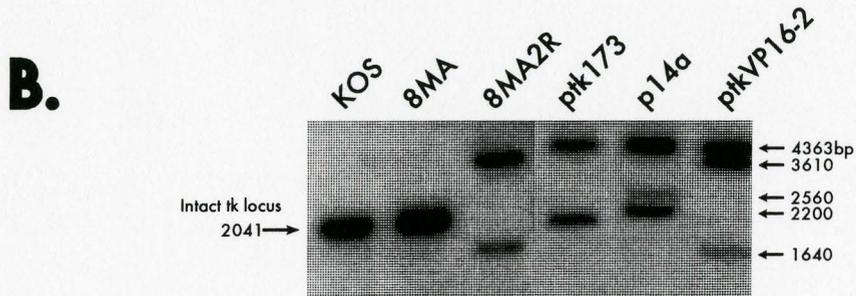
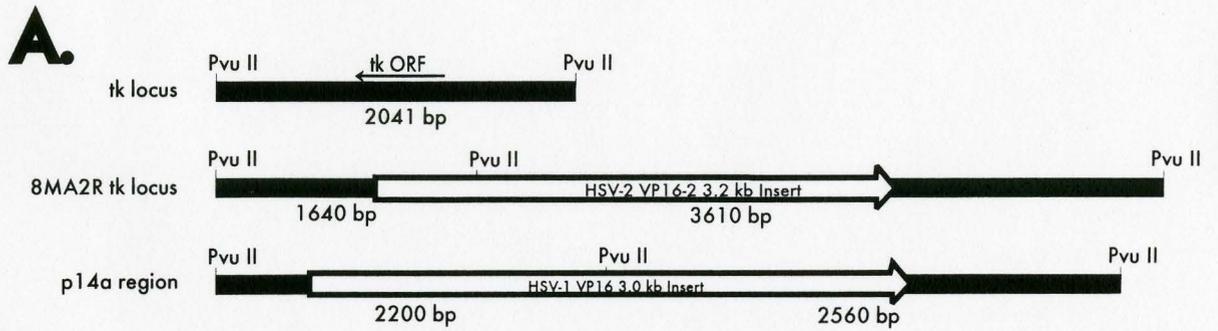
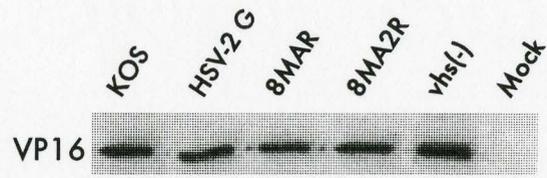
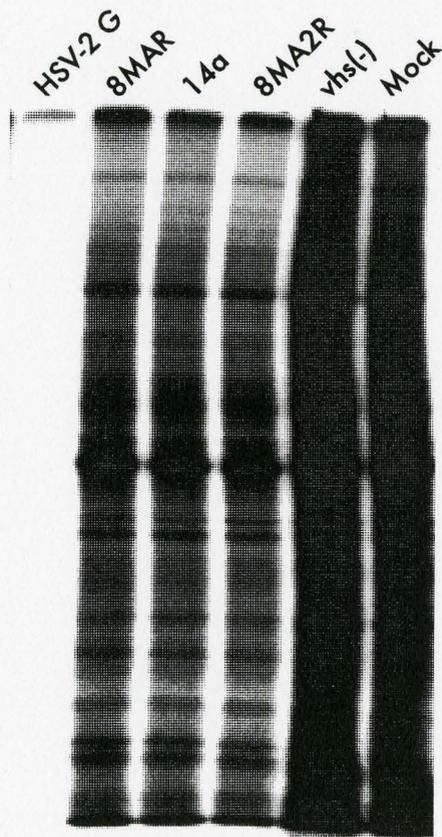


Figure 3.3. Substitution of VP16 from HSV-2 G does not affect shutoff phenotype.

A. Western blot of infected cell lysates 12 hrs PI to verify production of VP16-2 in 8MA2R. The names of the virus used to infect is listed above each lane. Vhs(-) contains an interruption cassette in the vhs locus. The primary antibody is a polyclonal rabbit antibody raised against a GST:VP16(aa 4-511) fusion used at 1:500 dilution. Secondary goat anti-rabbit antibody conjugated to alkaline phosphatase was used at 1:5000, chemiluminesced, and exposed to X-ray film for 10 seconds.

B. Vero cells were infected with virus in the presence of actinomycin D. Cells were labeled as described in materials and methods from 5-6 hrs postinfection, harvested, run on a 12% SDS-PAGE gel, and autoradiographed. The virus used is labeled above each lane. The virus 14a contains the HSV-1 VP16 gene within the tk locus, while 8MA2R contains the HSV-2 G VP16 gene within the tk locus.

A.**B.**

implying that essential functions of VP16-1 can be substituted for by VP16-2. The influence of VP16-2 upon shutoff seems negligible (Fig. 3.3b), as observed shutoff in the presence of a transcriptional block was comparable to that of 14a, a virus which contains the VP16-1 gene in the tk locus.

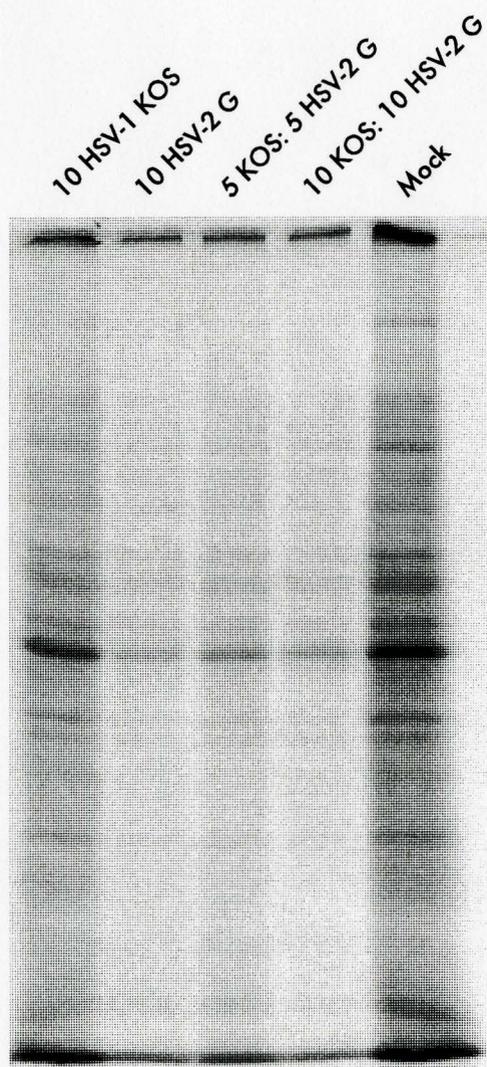
3.2. Vhs Intertypics

3.2.1. Lack of Dominance of HSV-1 Shutoff Phenotype in Mixed Infections

As several reports have detailed previously (Hill *et al.*, 1985; Fenwick and Everett, 1990b), HSV-1 “weak” shutoff seems to be dominant over “strong” shutoff of HSV-2 in mixed infections, and this is hypothesized to be a result of competition for host cellular factors required to effect shutoff. It was of interest to determine whether these results were replicable in our laboratory with the parental HSV strains used to generate various intertypic vhs constructs. Mixed infections were performed at both 10:10 MOI as well as at 5:5 MOI of HSV-1 strain KOS and HSV-2 strain G and the infected Vero cells labeled with ³⁵S-met to determine translation efficiency in the presence of a transcriptional block (Act D). This concentration of Act D has been shown to inhibit HSV IE gene product accumulation in previous experiments (not shown). No clear dominance of HSV-1 was observed under these conditions (Fig. 3.4), as mixtures of HSV-1 and HSV-2 were as effective as HSV-2 alone in shutting off host translation. The discrepancy between this result and previous results may be explained by HSV strain differences, the presence of transcriptional inhibition, the use of different cell types, or differences in virus production.

Figure 3.4. HSV-1 strain KOS and HSV-2 strain G do not cause shutoff interference in mixed infections.

Vero cells were infected with virus in the presence of actinomycin D. Cells were labeled as described in materials and methods from 4.5-5.5 hrs postinfection, harvested, run on a 12% SDS-PAGE gel, and autoradiographed. The MOI used for each sample is listed before the virus name above the lane.

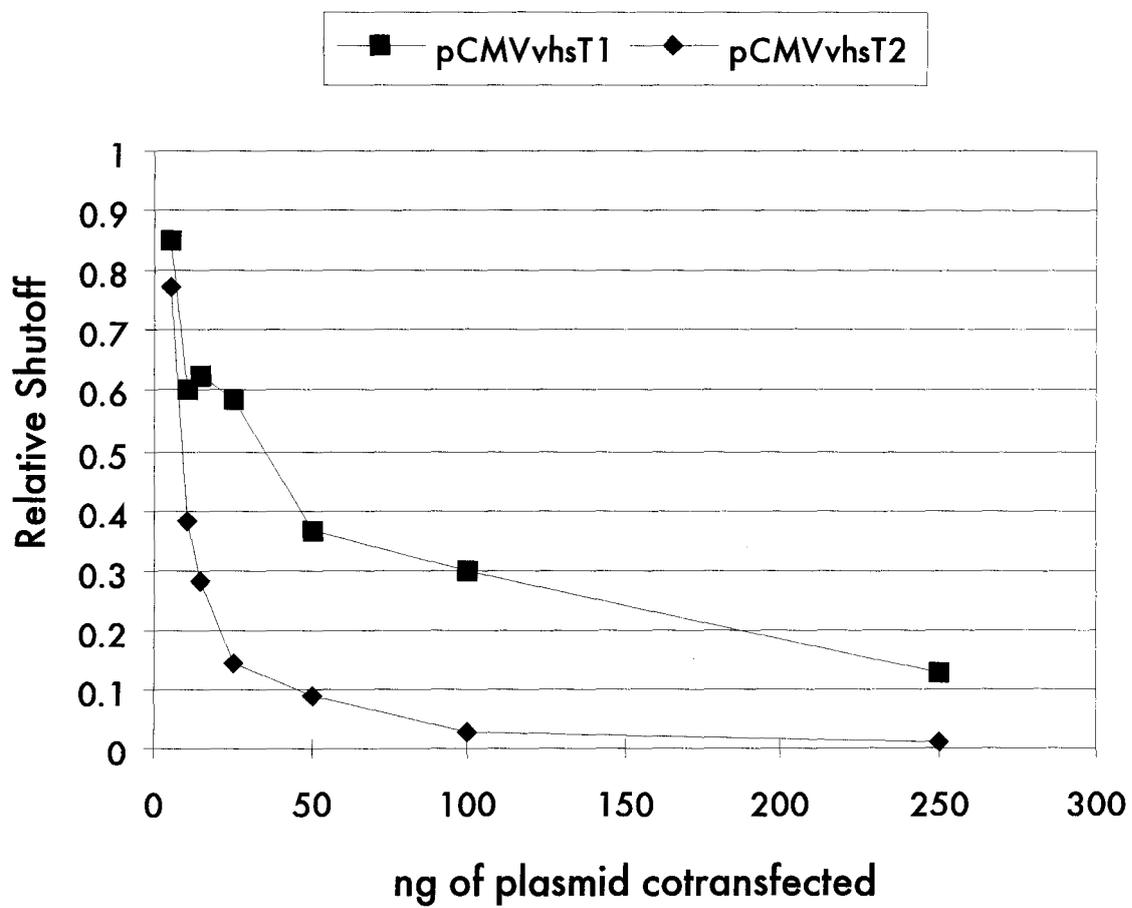


3.2.2. VhsT1 and VhsT2 Show Differing Phenotypes Upon Transfection

Early studies using transfected vhs seemed to indicate that the UL41 (vhs) ORF from various HSV strains varied in ability to shutoff a cotransfected reporter gene (Pak *et al.*, 1995), (Jones *et al.*, 1995). Also, preliminary data produced by Mabrouk Elgadi suggested that the vhsT1 and vhsT2 proteins possessed similar ability to cleave mRNA into secondary degradation products. To test whether the HSV-1 and HSV-2 vhs proteins differed in activity in a cotransfection system, plasmids containing the UL41 ORFs from HSV-1 KOS and HSV-2 G under the control of the CMV IE gene promoter were cotransfected with the reporter plasmid pRSV β -gal and the plasmid pCMV to standardize CMV promoter levels at 250 ng. As shown in Fig. 3.5, vhsT1 and vhsT2 possess different levels of activity in this assay. Quantitation of activity is difficult as transfected pCMVvhs constructs likely shut off their own transcripts as well; vhs which causes greater mRNA degradation will produce less vhs protein before coming to equilibrium between the amount of vhs produced and the amount of mRNA degradation that occurs. Thus, the difference in activity seen in Fig. 3.5 is likely an underestimate. It is also possible that differential rates of degradation of the two vhs proteins could influence this estimate, and this factor was not accounted for in this study. Nonetheless, it is evident from this result that in the absence of other viral proteins, vhsT1 and vhsT2 differ in shutoff ability *in vivo*.

Figure 3.5. Cotransfection assay for vhs activity.

Duplicate cultures of Vero cells were cotransfected with 15 μ g of the β -galactosidase expression vector pRSV β -gal, 15 μ g of pUC18 carrier DNA, and various amounts of pCMVvhsT1 or pCMVvhsT2. pCMV vector DNA was added to each sample to maintain a constant amount of HCMV promoter in each mixture. Cell lysates were prepared at 2 d posttransfection and levels of β -galactosidase activity determined as described under Materials and Methods. The level of β -galactosidase activity for each transfection was normalized to that measured in Vero cells transfected with 15 μ g pRSV β -gal, 15 μ g pUC18, and 250 ng pCMV vector (defined as 100% Relative Shutoff).



3.2.3. In vitro Assay of Vhs Activity

The procedure developed by Elgadi *et al.* (1996) was used to see if the degradation rate of intact mRNA varied between vhsT1 and vhsT2 in a more controlled in vitro system, and also to see if transfection results were corroborated in another system.

VhsT1 and vhsT2 transcripts were transcribed in vitro, and translated in a RRL system in the presence of ^{35}S -met. The amount of vhs present was estimated by running aliquots of the translation mix on SDS-PAGE, and quantitated via Phosphoimager. ^{35}S -met labeled protein was diluted in a twofold series, run on SDS-PAGE gel, and quantitated to ensure that this method returned accurate values along a representative range of dilution series. To test vhs activity, vhs amounts were doubly diluted with blanked reticulocyte lysate and an aliquot of the dilution quantitated. The remaining vhs dilution was incubated with gel purified, intact internally ^{32}P -labeled pCITE mRNA for 10 min at 30°C, and the mRNA extracted as described in Materials and Methods. The purified RNA was then run on a formaldehyde-agarose gel, transferred to Nytran membrane, and cross-linked. The membrane was then exposed to Phosphoimager cassette, and the exposure quantitated with ImageQuant software. Vhs protein amounts were compared to the amount of intact mRNA remaining after 10 min to provide an estimate of vhs activity.

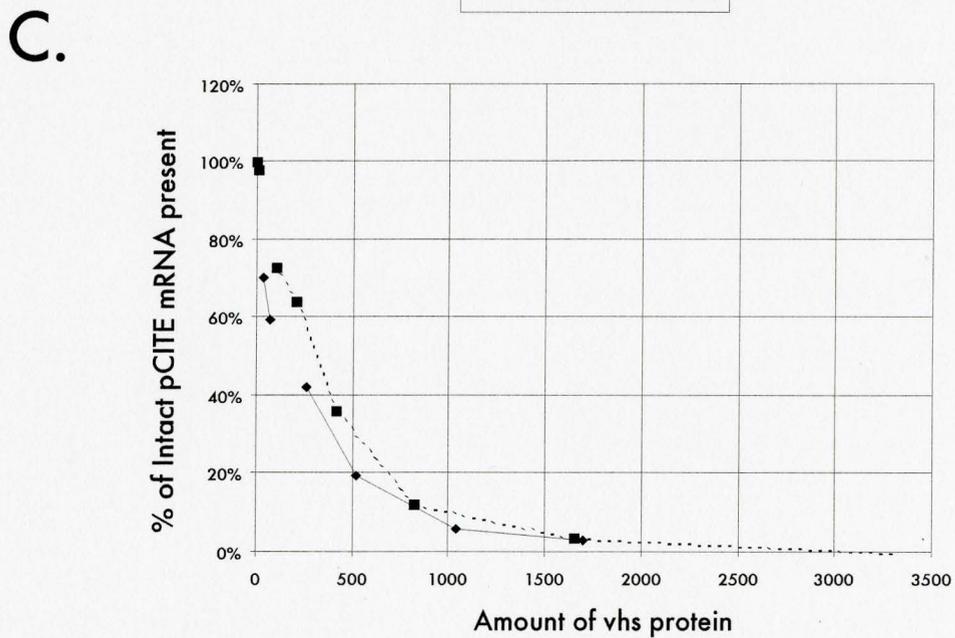
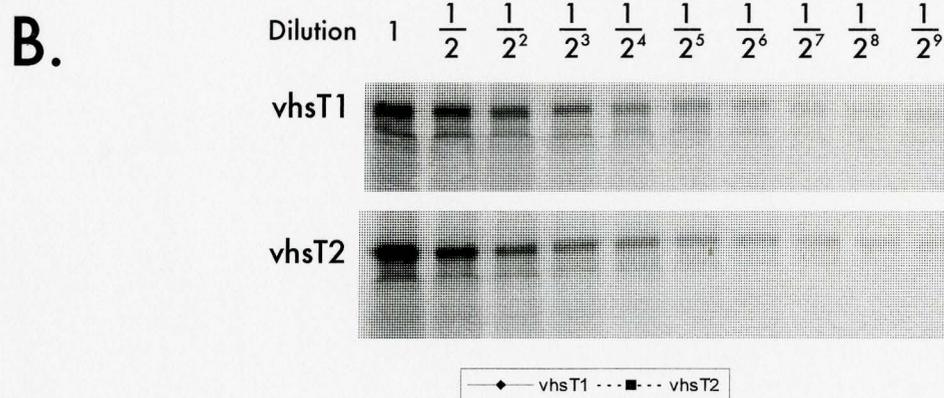
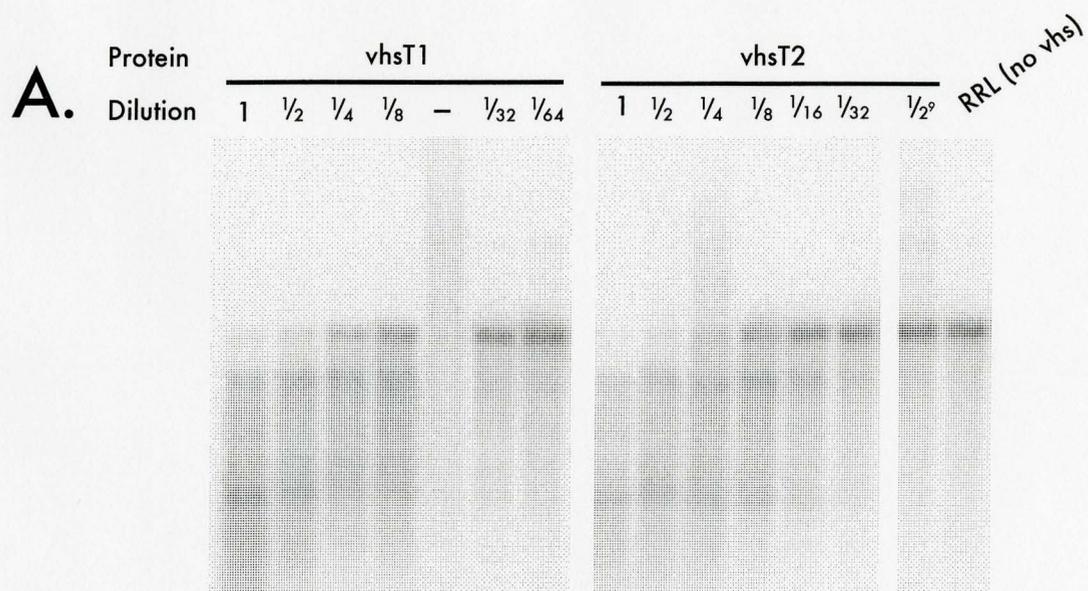
The results are diagrammed in Fig. 3.6. Both vhsT1 and vhsT2 contain an equal number of methionine residues and thus ^{35}S -met labeling can be expected to measure

Figure 3.6. Degradation of pCITE transcripts in the presence of vhsT1 and vhsT2.

A. Various dilutions of in vitro translated, ³⁵S-met labeled vhsT1 and vhsT2 were incubated at 30°C in rabbit reticulocyte lysate (RRL) (Novagen) with internally ³²P labeled pCITE mRNA transcripts.

B. Gels used to quantitate the amount of vhs present in degradation reactions. A portion of the 2-fold diluted vhs / blank RRL mixture was removed and run on 12% SDS-PAGE, fixed, treated with Enlightning (DuPont), dried, and quantitated via PhosphoImager exposure and ImageQuant software..

C. The amount of vhs present in the sample is graphed vs. the percentage of input pCITE transcript present intact after 10 m (the top band in Fig. A.), relative to blank RRL alone.



relative abundance of the two proteins quantitatively. Both proteins appear to cause a similar level of degradation when vhs is abundant, but this may be a result of limiting substrate (intact mRNA). As vhs is diluted further, the levels of degradation are seen to diverge slightly. Unfortunately, the accuracy of vhs quantitation becomes more subject to variation as the amount of vhs diminishes. Another difficulty is that when very little vhs activity is observed and there is an excess of substrate, small procedural mRNA losses may overwhelm minor vhs-induced losses.

Notwithstanding these limitations, these data demonstrate that the differences in activity between vhsT1 and vhsT2 seen in vivo were not replicated in this in vitro system.

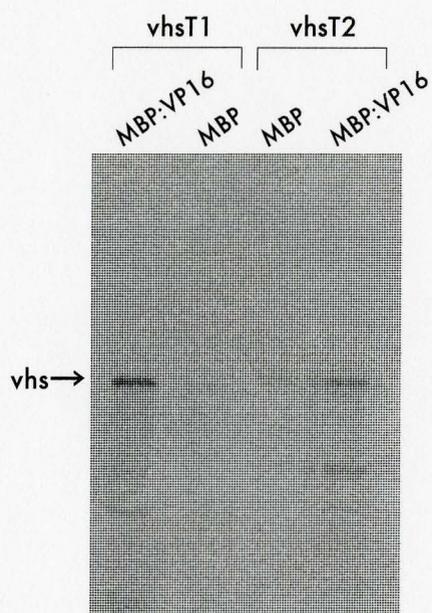
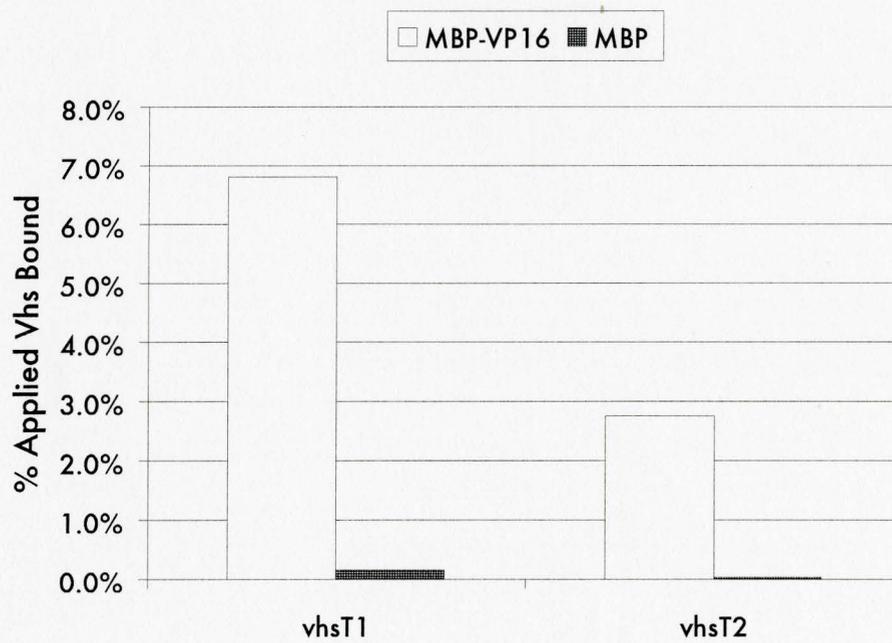
3.2.4 Binding of VhsT1 and VhsT2 to VP16 In vitro

Binding assays have previously been used to determine the ability of vhs derivatives and mutants to bind VP16 (Schmelter *et al.*, 1996; Smibert *et al.*, 1994). Bacterially-produced maltose binding protein (MBP):VP16 fusion proteins were bound to amylose-agarose beads, and used to test binding of vhs constructs to VP16 in vitro. VhsT1 and vhsT2 were translated in RRL and labeled with ³⁵S-met, and roughly equal counts of vhs protein was incubated with MBP:VP16 fusions as described in Materials and Methods. As seen in Fig. 3.7, vhsT1 displays a greater ability to bind to MBP:VP16 than vhsT2 in this assay. As shown above, viral mutants containing HSV-2 VP16 are not altered in shutoff phenotype and vhsT2 shows strong shutoff compared with vhsT1 in transfections. Together, these data suggest that VP16-vhs interactions may play a partial

Figure 3.7. In vitro binding to MBP-VP16 by vhsT1 and vhsT2.

A. Rabbit reticulocyte translated, ^{35}S -met labeled vhsT1 and vhsT2 were incubated with maltose binding protein (MBP)-VP16 fusion protein or MBP bound to amylose-agarose for 1 hour, washed as described in materials and methods, the beads boiled in 2XSB, spun down, and the supernatant loaded onto a 12% SDS-PAGE gel. The amount of vhs present was quantitated by exposure of the gel to PhosphoImager cassettes followed by quantitation using ImageQuant software. The top label indicates the ^{35}S -labeled protein applied (vhsT1 or vhsT2) and the bottom label indicates the binding resin used (MBP or MBP:VP16 amylose-agarose).

B. Graph of % applied vhs protein retained on MBP or MBP:VP16 amylose agarose, measured as described above.

A.**B.**

role in determining shutoff phenotype, but do not completely determine shutoff phenotype as vhs alleles differ in activity in the absence of VP16. If vhs-VP16 binding plays a role in regulating shutoff, vhs would be the active partner which determines binding strength, as VP16 substitution does not influence shutoff phenotype. The GST:VP16 protein used in this assay was bacterially produced, and thus may lack modifications important for determining vhs binding.

3.2.4. Characterization of the VhsT1 and VhsT2 Constructs within the Context of a Viral Infection

Activity differences have been noted in viruses possessing copies of the UL41 (vhs) allele from HSV-1 strain 17 and HSV-2 strain G (Fenwick and Everett, 1990b). These studies did not address the possibility that the native HSV-2 UL41 promoter may affect shutoff phenotype, or that locus-specific effects could also play a role. To more accurately control for these effects, vhs constructs were created and the recombinant alleles, which differed only in the vhs ORF, were inserted into the tk locus of the UL41-null vhs(-) virus.

The first viruses tested were the intertypic recombinants vhsT1 and vhsT2. The vhs ORF present in these viruses is diagrammed in Fig. 3.8. As seen in Fig. 3.9, the presence of the HSV-2 strain G vhs ORF within the recombinant virus vhsT2 confers strong shutoff ability upon the resultant virus which is comparable to that of the parental vhs strain, HSV-2 strain G.

Figure 3.8. Schematic diagram of the various intertypic vhs constructs tested in virus.

All vhs constructs listed here were placed antisense into the viral tk ORF to create the intertypic viruses. Black denotes UL41 coding sequence originally derived from HSV-2 strain G while white denotes UL41 coding sequence from HSV-1 strain KOS. Increasing height of the ORF bar indicates the increasing degree of homology between HSV-1 strain KOS vhs and other sequenced herpesviruses. The VP16 binding site indicated has been described previously (Smibert *et al.*, 1994).

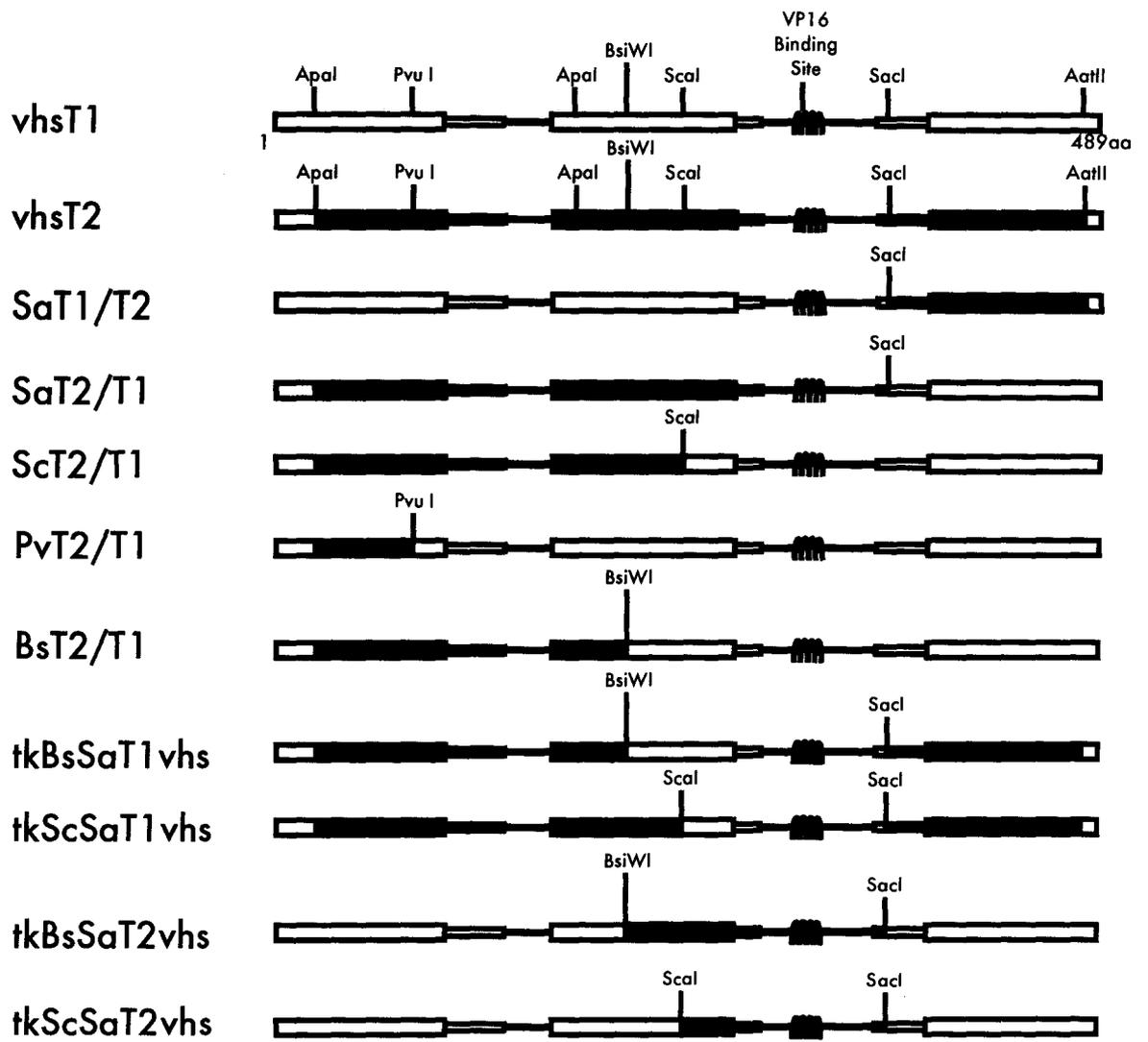
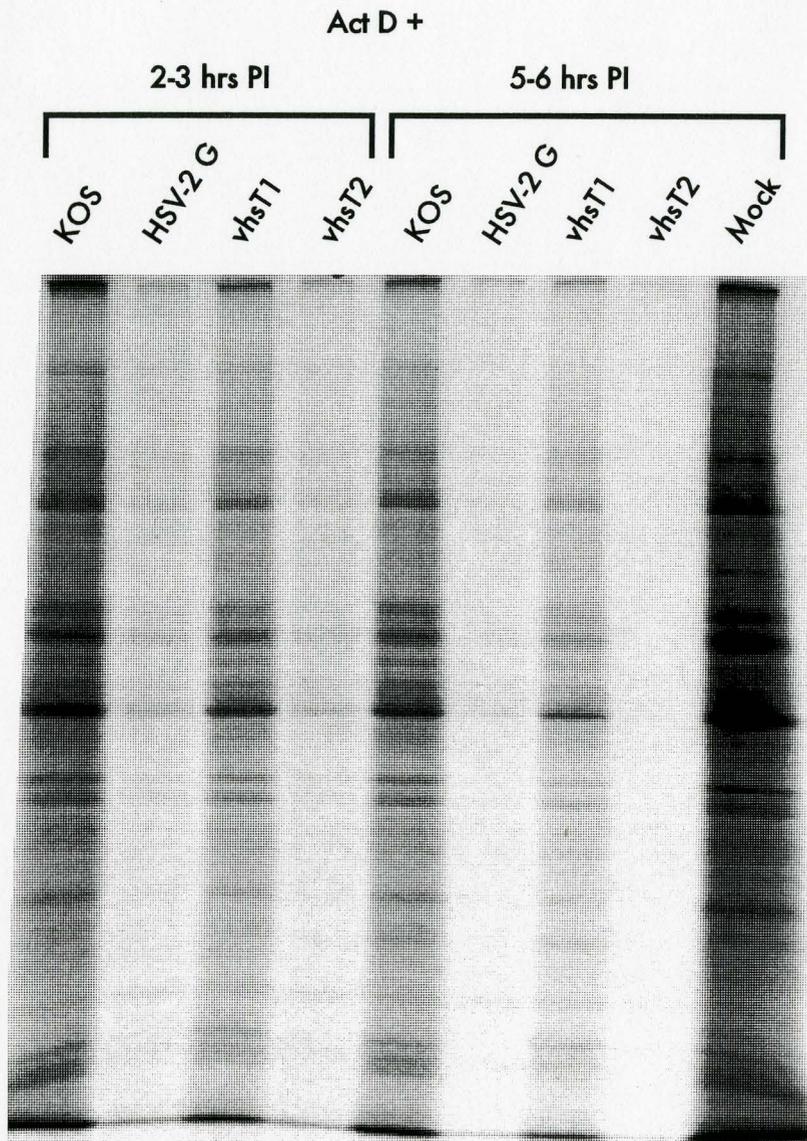


Figure 3.9. The vhs ORF determines shutoff phenotype in the context of a viral infection.

Vero cells were infected at 10 MOI in the presence of actinomycin D. Cells were labeled during the times indicated (PI = postinfection), harvested, run on a 12% SDS-PAGE gel, and autoradiographed.



To more accurately quantitate the shutoff abilities of vhsT1 and vhsT2, shutoff experiments were performed at a range of MOI to see if saturation levels of shutoff differed between the two viruses. As shown in Fig. 3.10, vhsT2 requires dramatically less virus to effectively wipe out translation by the infected cell and also saturates at a lower level of translation, making it unlikely that the effects seen are a result of differential incorporation of the vhs protein into the virion.

3.2.5. Localization of the regions responsible for shutoff phenotype

Further intertypic mutants were created and placed into the tk locus of the vhs null mutant Pvhs(-) (Jones *et al.*, 1995) to create the viruses diagrammed in Fig. 3.8. A series of intertypics spanned the vhs ORF (previously created by Karen Koop in our lab), and several others were created to switch regions of the vhs ORF known to bind VP16. The structure of the newly-created viruses was verified by Southern blot of digested viral DNA (Fig. 3.11). It was hoped that the strong shutoff phenotype could be localized to a region of the vhs ORF. These viruses were assayed for their ability to shutoff host protein synthesis in the presence of the transcriptional blocker Act D, and the levels of translation quantitated relative to protein synthesis levels in vhs(-) infected cells (Fig. 3.12). It does not seem that any easily definable region of the vhs locus is responsible for the HSV-2 strong shutoff phenotype. A level of activity comparable to that of vhsT2 could be conferred by the region present in SaT2/T1 or ptkBsSaT1 (Fig. 3.8). Switching of an identified VP16 binding domain (in the tkBsSaT1vhs/tkBsaT2vhs and tkScSaT1vhs/tkScSaT2vhs viruses) did not switch vhs phenotypes but resulted in an

Figure 3.10. Comparison and quantitation of shutoff activity in vhsT1 and vhsT2 at various MOI.

A. Vero cells were infected with virus at the MOI indicated in the presence of actinomycin D. Cells were labeled as described in materials and methods from 4-5 hrs postinfection, harvested, run on a 12% SDS-PAGE gel, autoradiographed, and exposed to a PhosphoImager cassette for signal quantitation. B. Graph of translation relative to mock infected (100% translation) vs. MOI of virus used to infect cells.

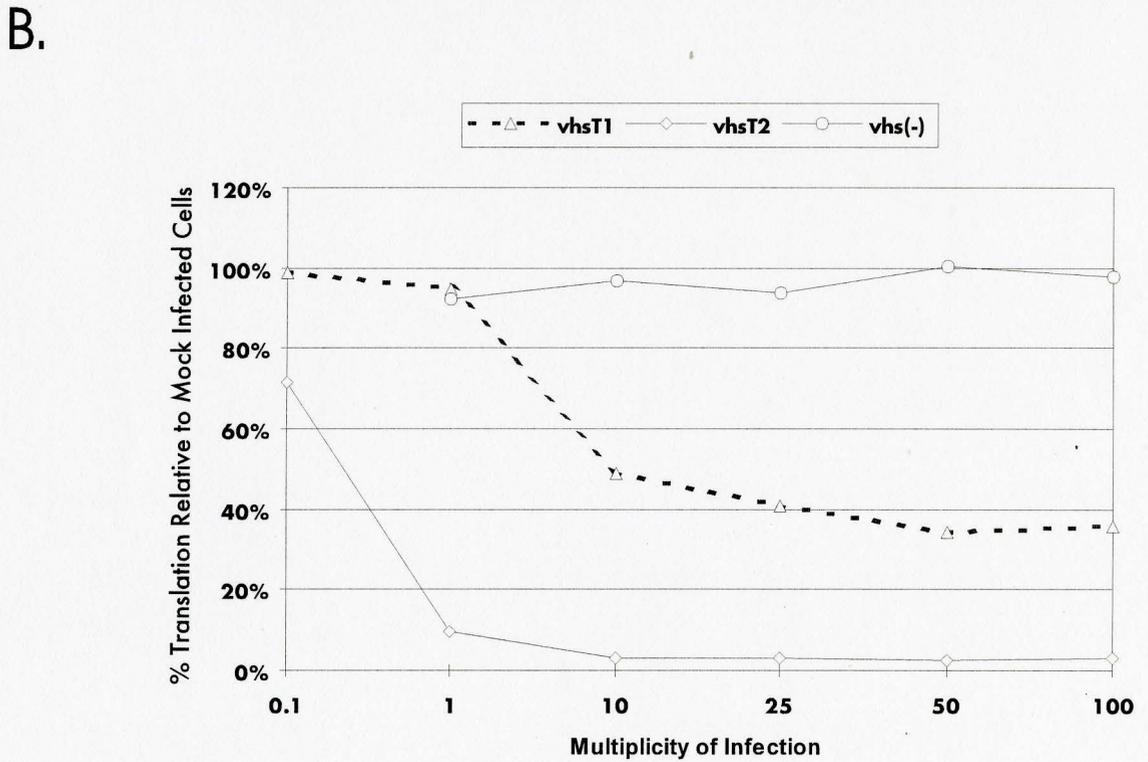
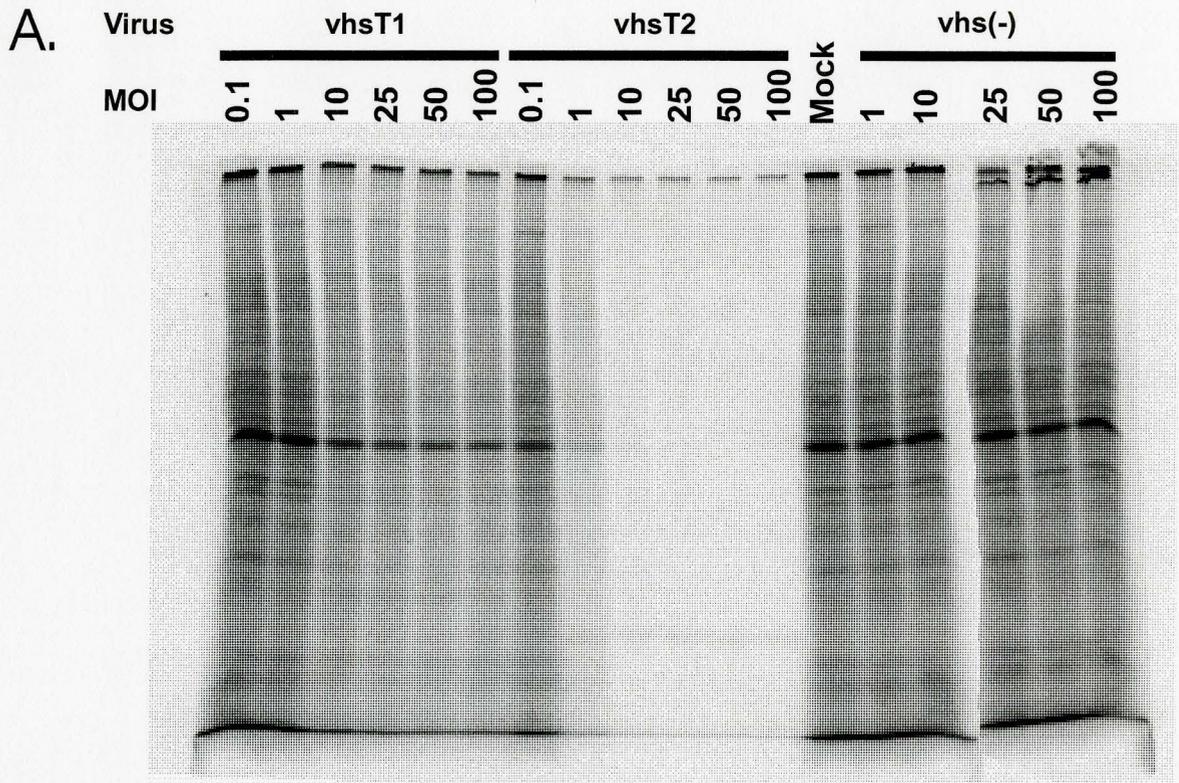
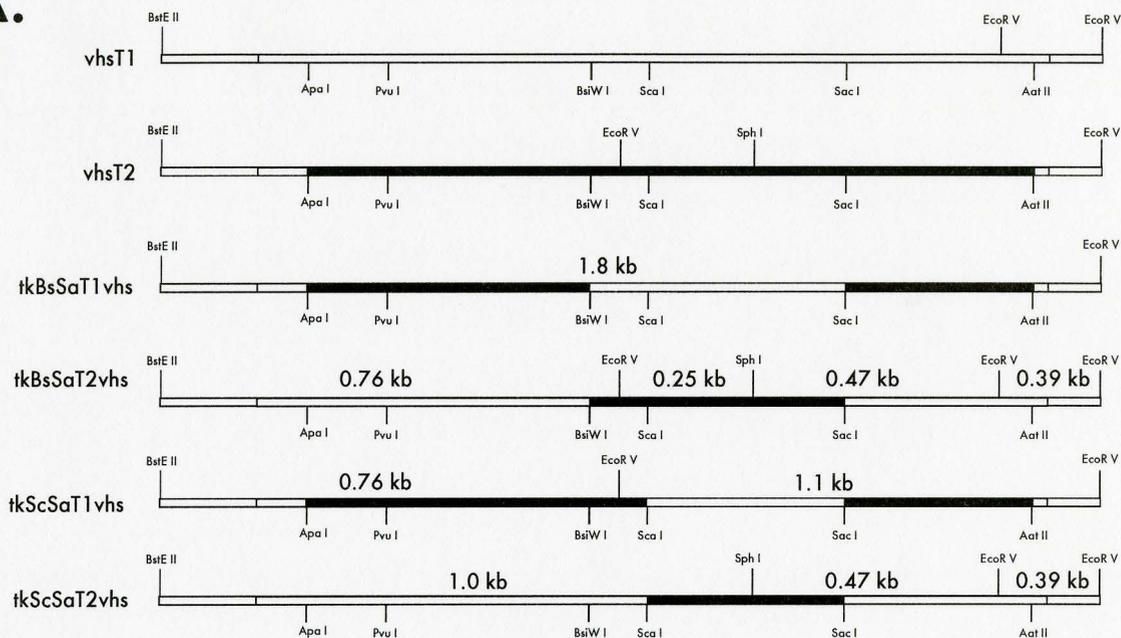


Figure 3.11. Verification of Intertypic Structure.

A. Schematic diagram of cut sites relative to the vhs ORF and fragment sizes produced. HSV-1 KOS sequence is denoted by white boxes and black boxes denote HSV-2 G vhs sequence. The size in kb of fragments generated by EcoR V / Sph I / BstE II digestion are noted between restriction sites.

B. Southern blot of viral DNA triply cut with EcoR V / Sph I / BstE II and probed with randomly labeled pSP6vhs (which contains the vhs ORF). All viruses produced bands of the expected sizes, with two unexpected bands at ~2 kb and 0.7 kb appearing in all viral DNA samples probed, likely caused by the disrupted UL41 locus in the vhs(-) strain background (Jones *et al.*, 1995).

A.



B.

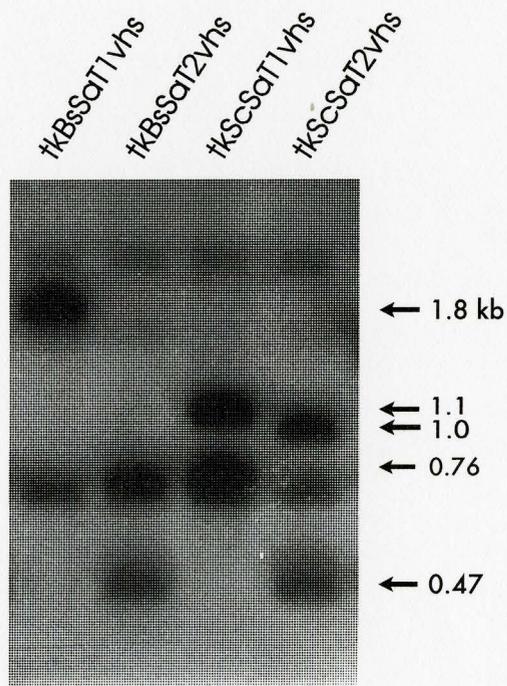
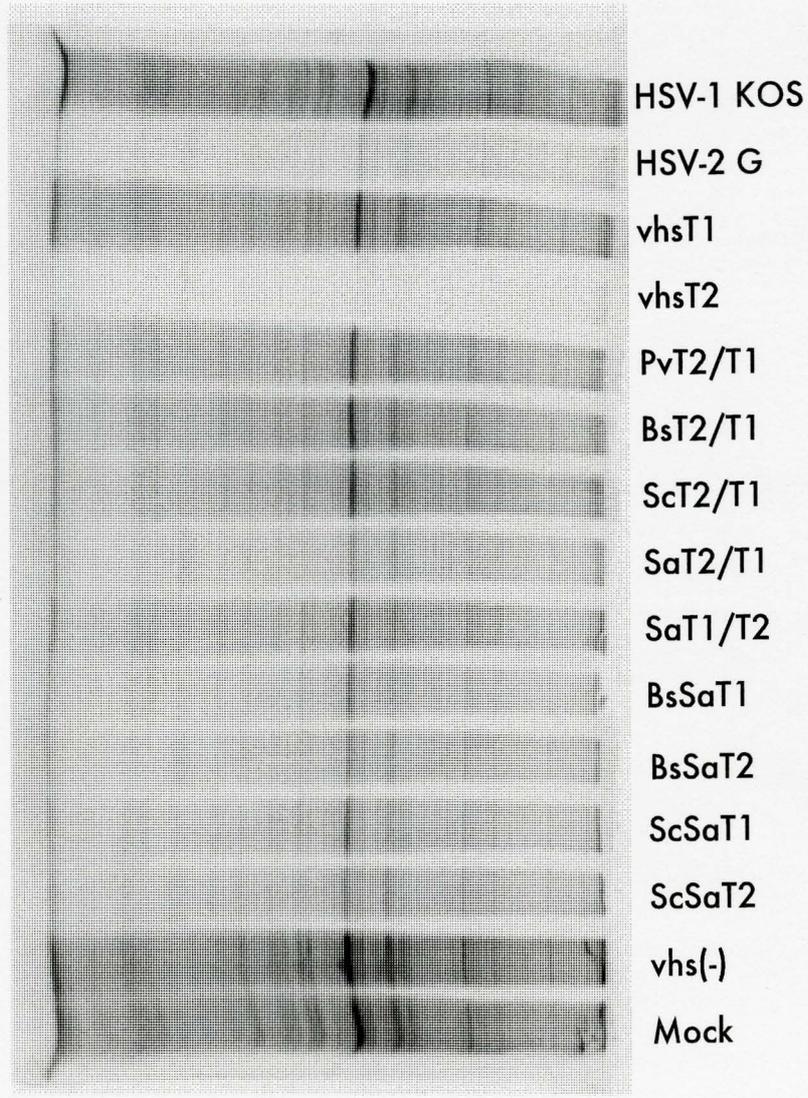


Figure 3.12. Comparison and quantitation of the shutoff activity of various vhs intertypics in Vero cells.

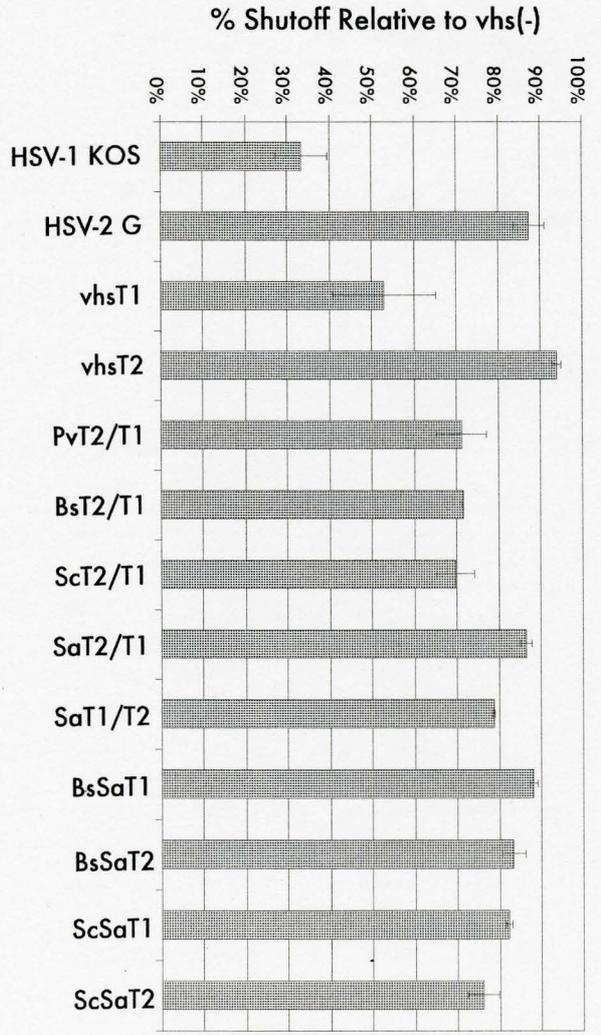
A. A representative gel depicting intertypic shutoff phenotype. Vero cells were infected with various viruses and labeled between 5-6 hrs PI. Samples were then run on a 12% SDS-PAGE gel, autoradiographed and exposed to a PhosphoImager cassette for signal quantitation.

B. Shutoff activity \pm standard error is graphed relative to vhs(-) infection (no shutoff).

A.



B.



intermediate shutoff phenotype, arguing that this region, and possibly VP16, does not play a critical role in modulating initial host shutoff phenotype.

Perhaps the clearest result of this analysis is that all of the intertypic recombinants analyzed showed enhanced shutoff relative to the vhsT1 construct, a result that is further discussed in the Discussion.

3.3. The role of UL13 in host shutoff

3.3.1. Construction of UL13(-) virus

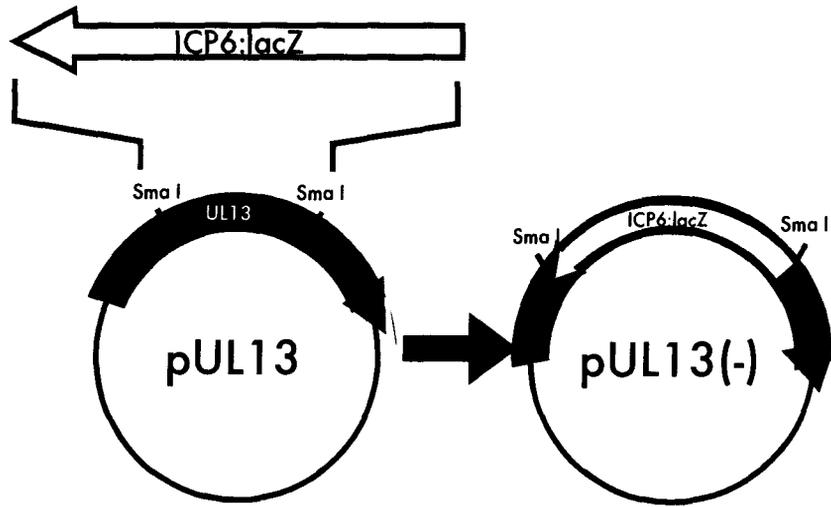
To check the effects of UL13 on vhs activity in a viral context, a UL13 null mutation in the HSV-1 KOS strain background was created for future generation of KOS strain double mutants. To create the virus the plasmid pUL13(-) was constructed as described above and cotransfected with KOS viral DNA in Vero cells (diagrammed in Fig. 3.13), and progeny virus selected via blue-white plaque screening. After several rounds of plaque picks, viral DNA was analyzed via Southern blot to determine genomic structure of the resultant virus. While construction of UL13(-) was occurring, several other UL13 null viruses and their progenitor strains were obtained from other sources. The viruses Δ UL13 (referred to as R7356) and parental HSV-1 strain F were obtained from Bernard Roizman (Purves and Roizman, 1992). The viruses D13lacZ and parental strain HSV-1 KOS1.1 were obtained from Stephen Rice (unpublished data). These genomic structure of these viruses was checked via Southern blotting to ensure their identity (Fig. 3.14).

Figure 3.13. Diagram of UL13(-) null virus construction.

A. An ICP6::lacZ fusion cassette (Goldstein and Weller, 1988) was ligated blunt into the Sma I – Sma I excision of plasmid pUL13.

B. Insertion of the disrupted UL13 gene into the HSV genome, depicting the orientation of the inserts within the genome..

A.



B.

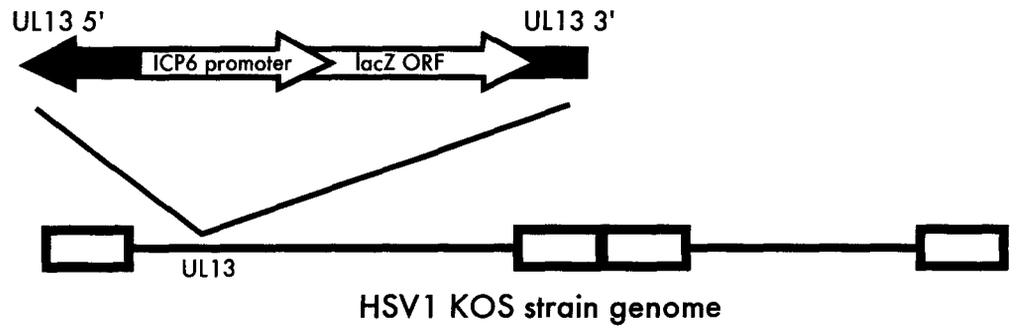
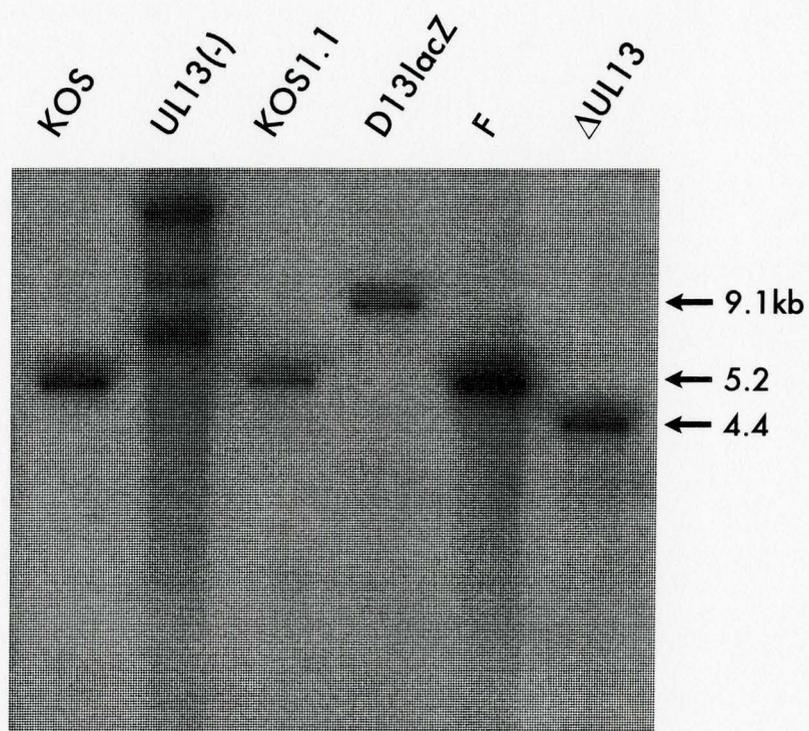


Figure 3.14. Verification of UL13 locus structure of viral mutants.

Viral DNA from the labeled virus DNA was prepared, digested with Bgl II, run on a 1% agarose gel, Southern blotted, and probed with randomly-labeled pUL13 plasmid. The band marked at 5.2 kb represents the wild type intact UL13 locus. The 4.4 kb fragment of Δ UL13 contains a 773 bp deletion in the UL13 gene. The 9.1 kb fragment of d13lacZ contains a 773 bp BstE II – Hind III deletion in the UL13 ORF as well as a 3.1 kb lacZ insertion in frame with the UL13 ORF. An expected 9.0 kb fragment of UL13(-) was not present; three unexpected bands were present, indicating a mutation in or rearrangement of the genome.



As seen in Fig. 3.14, the virus UL13(-) does not possess the expected restriction pattern for the UL13 insertion mutant expected from recombination following transfection. A different set of diagnostic digests (Bcl I – Kpn I) used for screening purposes were as expected (not shown) and thus this abnormality was not detected earlier during the screening process.

The unexpected restriction pattern observed for UL13(-) may be a result of viral genome recombination due to the presence of two ICP6 promoter sequences present within the ICP6:lacZ interruption cassette, but simple recombination between the ICP6:lacZ insert and the native ICP6 promoter would result in a lethal deletion of the region between both ICP6 promoters, which are present in the same orientation within the genome. Thus, the observed UL13(-) pattern may possibly be a result of more complex viral genome rearrangement, a mutation affecting restriction sites used in this analysis, or a non-homologous recombination of pUL13(-) sequence within the viral genome.

3.3.2. Assay of UL13 Null Virus Shutoff

Various UL13 mutants were tested at an MOI of 10 with and without Act D treatment in Vero cells (Fig. 3.15). The results diagrammed show some difference between wt and UL13 null viruses in shutoff, but the differences varied widely and were not replicable between trials. We reasoned that small (2-fold) variations in viral titer might be contributing to the variability in this assay, and decided to try other methods to determine shutoff levels. UL13 null virus was tested for shutoff ability in MEL cells,

Figure 3.15. Comparison of various UL13 mutants in Vero cells with and without transcriptional block.

Vero cells were infected with the labeled viruses at an MOI of 10. Act D (-) denotes cells infected without the presence of actinomycin D, while Act D(+) indicates presence of the drug throughout infection. Cells were labeled as described in materials and methods from 4-5 hrs postinfection, harvested, run on a 12% SDS-PAGE gel, and autoradiographed.

which show a strong shutoff upon infection of either HSV-1 or HSV-2 virions. Thus, if a shutoff difference between UL13 null virus and wt exists, it may be more easily seen in a cell type (MEL) which shows exaggerated shutoff. As seen in Fig. 3.16, both KOS and UL13(-) shut off host protein synthesis to some extent, but UL13(-) does not shutoff as strongly as KOS in MEL cells. We also decided to test wt and UL13 null viruses under a variety of MOIs in order to more clearly see shutoff or lack thereof in UL13 null mutants relative to wt. As shown in Figs. 3.17 and 3.18, the UL13 null virus appears to be mildly hampered but not deficient in shutoff ability at high MOI.

Figure 3.16. Shutoff efficiency of UL13 null virus in differentiated MEL cells.

Terminally differentiated MEL cells were infected with 10 PFU of each virus in the presence of actinomycin D. Cells were labeled from 5-6 hrs PI, harvested, and run on a 12% SDS-PAGE gel and autoradiographed.

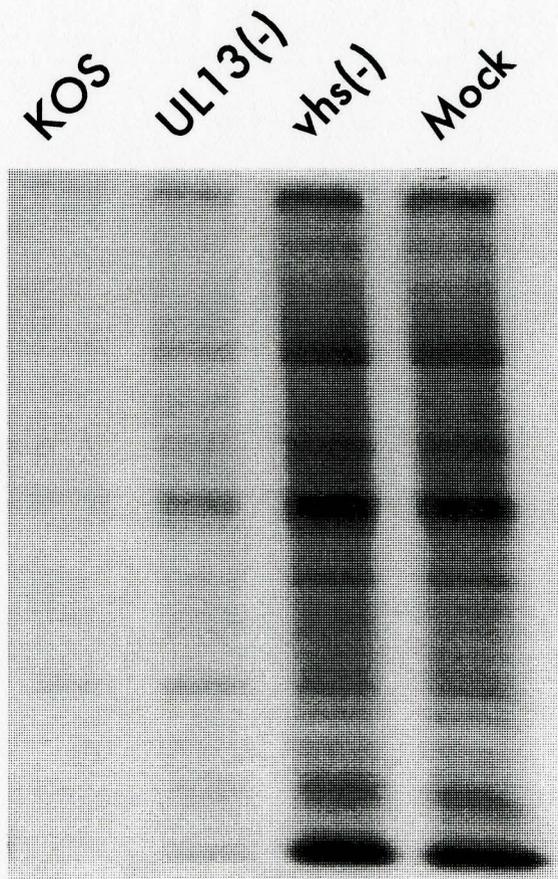


Figure 3.17. Effects of increasing MOI on shutoff in UL13 null mutants compared to wild type in Vero cells.

A. Vero cells were infected with virus at the MOI indicated in the presence of actinomycin D. Cells were labeled as described in materials and methods from 4-5 hrs postinfection, harvested, run on a 12% SDS-PAGE gel, autoradiographed, and exposed to a PhosphoImager cassette for signal quantitation. B. Graph of translation relative to mock infected vs. MOI.

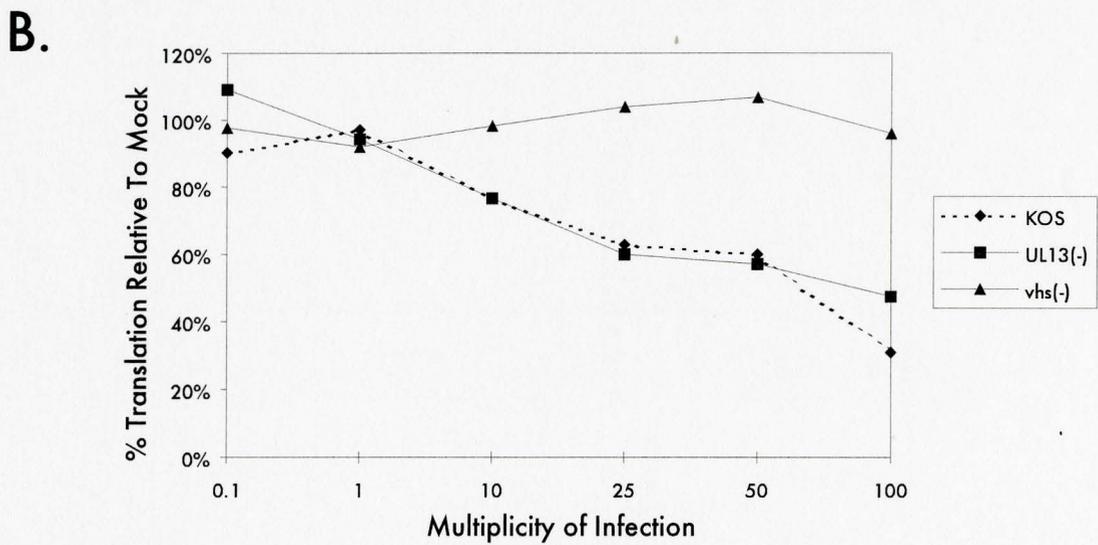
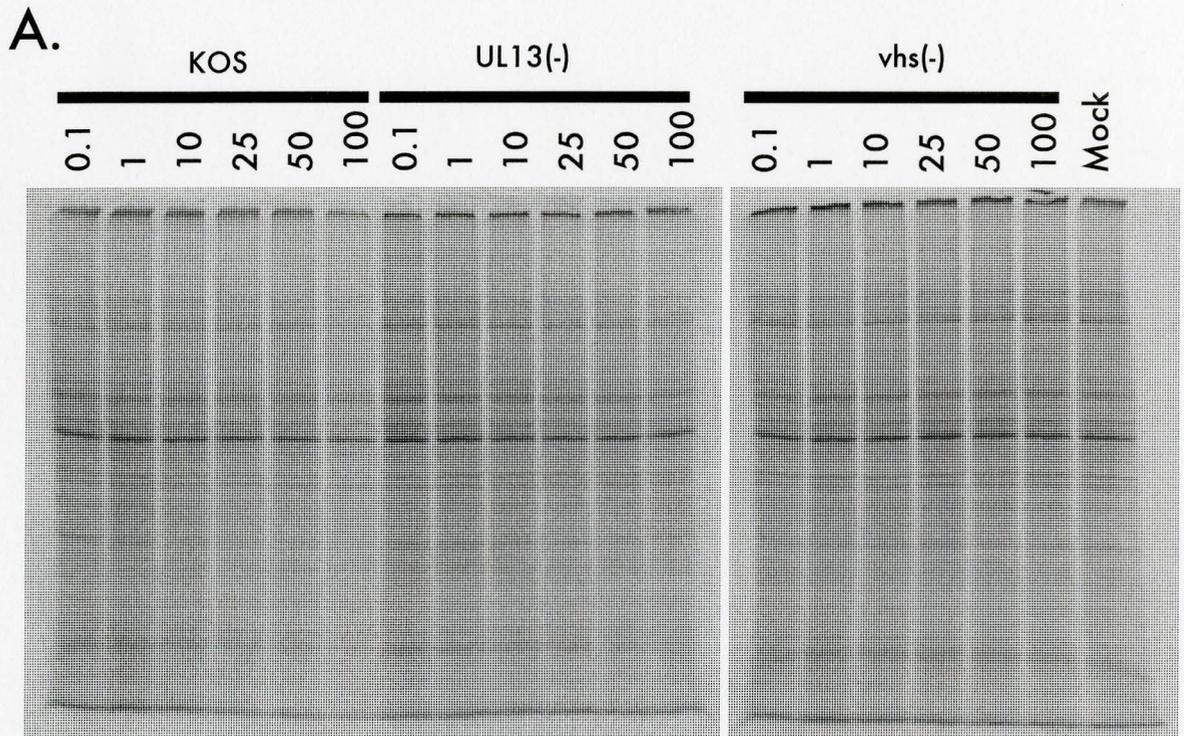
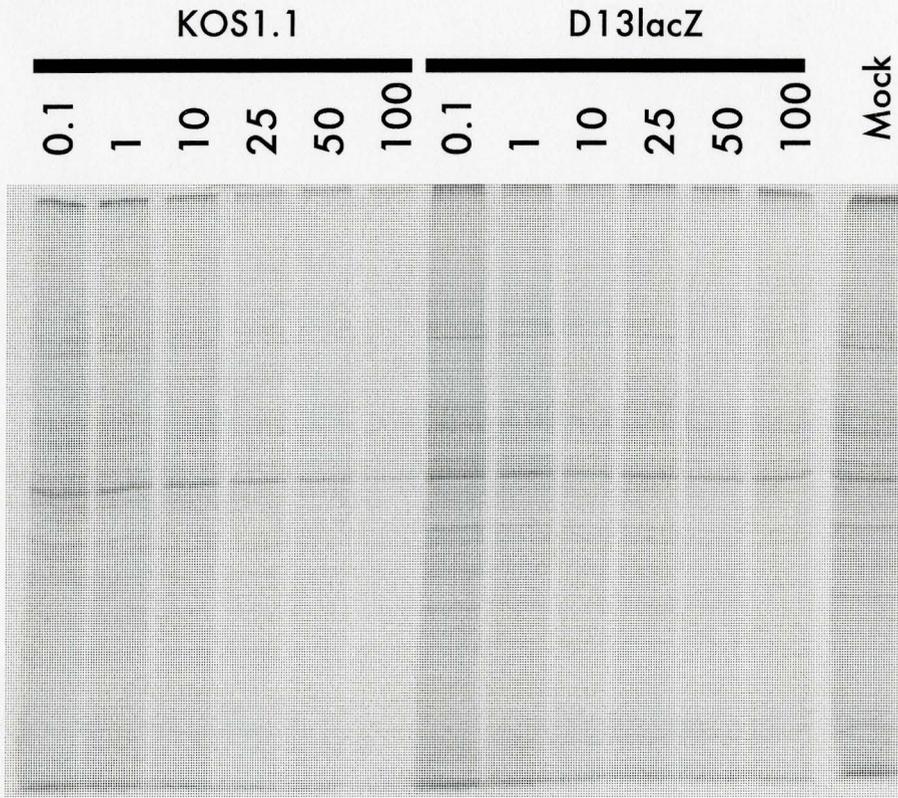


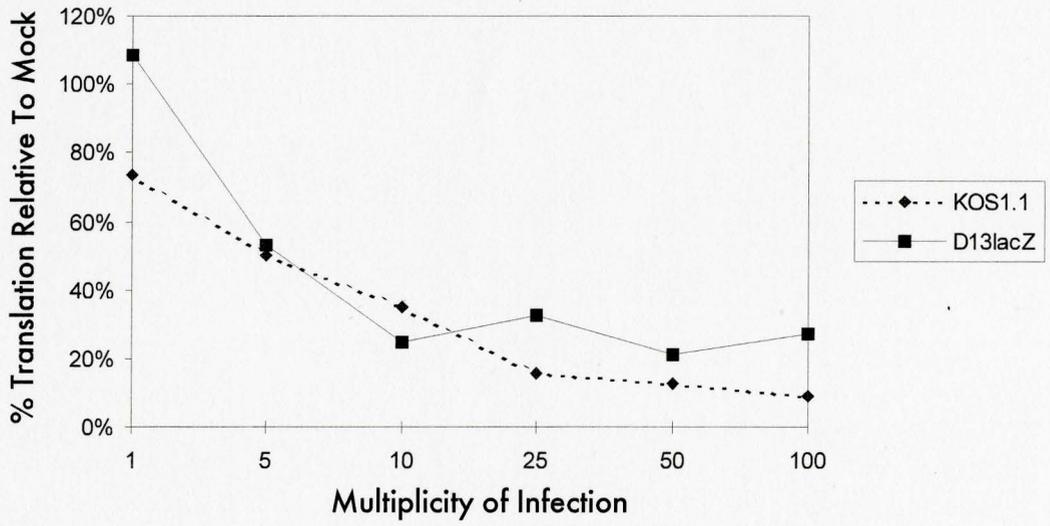
Figure 3.18. Effects of increasing MOI on shutoff in UL13 null mutants compared to wild type in Vero cells.

A. Vero cells were infected with virus at the MOI indicated in the presence of actinomycin D. Cells were labeled as described in materials and methods from 4-5 hrs postinfection, harvested, run on a 12% SDS-PAGE gel, autoradiographed, and exposed to a PhosphoImager cassette for signal quantitation. B. Graph of translation relative to mock infected vs. MOI.

A.



B.



IV. DISCUSSION

4.1 The Effect of VP16 on Early Shutoff

It seems clear that VP16 plays a role in regulation of the virion host shutoff protein during L gene expression (Lam *et al.*, 1996). It thus seemed reasonable to ask if VP16 also played a role early during infection. Several facts hinted that VP16 may play a role in regulation of early host shutoff. Fenwick and Everett found that the virus 17G41, a HSV-1 strain 17 virus containing both HSV-1 and HSV-2 UL41 alleles, shows strong shutoff in the presence of Act D. However, when tested in mixed infections, weak shutoff was still dominant over the strong 17G41 phenotype. This suggests that the manner in which vhs protein is presented in the cell affects shutoff phenotype, as dominance varies depending on whether the different vhs alleles are packaged within the same virion (17G41) or not (mixed infection). Interestingly, other researchers have analyzed the composition of 17G41 virions and found that both vhs alleles are packaged into the virion in similar amounts (McLauchlan *et al.*, 1992a). Therefore, the 17G41 virion appears to contain both vhs proteins at wild-type levels (resulting in a virion which contains both wt levels of HSV-1 vhs and wt levels of HSV-2 vhs, totaling double that of a wt virion) (McLauchlan *et al.*, 1992a).

The virus 8MA, a null mutant in the VP16 gene (Ace *et al.*, 1989) showed a stronger shutoff upon infection than wild-type virus (Lam *et al.*, 1996), in infections conducted without an actinomycin D transcriptional blockade. I have replicated this finding in the

presence of a transcriptional blockade, and shown that this effect is due at least in part to increased shutoff caused by the infecting virion, as the effect is still apparent when viral gene expression is blocked with Act D. 8MA must be grown in cells that express the wt VP16 protein (cell line 16-8), and thus the virion composition would be expected to be similar to that of wt virus. Thus, this result is surprising. Two possibilities suggest themselves as to why 8MA possesses a stronger shutoff phenotype: 8MA may suffer from an increased PFU/particle ratio (thus delivering more tegument protein per PFU) or the VP16 provided in trans to the virus does not substitute fully for cis produced VP16.

The observation that 8MAR harvested from 16-8 cells has a slightly increased shutoff has at least two possible interpretations. One possibility is that cellular VP16 does increase shutoff ability but viral VP16 produced from the 8MAR genome counteracts this phenotype, resulting in an intermediate phenotype. Another likely possibility is that the particle / PFU ratio in 8MA is increased, and passage through the 16-8 cell line increases the particle / PFU ratio in wt virus, leading to an increased dosage of tegument proteins, and consequently a stronger shutoff phenotype. The studies performed are not sufficient to distinguish between these two possibilities. Irrespective of these uncertainties, the data illustrate that the genotype of the VP16 locus can strongly influence virion host shutoff activity as shown by comparing shutoff of 8MA to 8MAR(16-8) (Fig.3.1).

Insertion of the HSV-2 strain G VP16 gene into the VP16-null virus 8MA produced the virus 8MA2R. If the strain of VP16 influenced shutoff phenotype, it would be expected that 8MA2R would display a strong shutoff phenotype. However, 8MA2R does not display a strong shutoff phenotype. This implies that VP16 strain alone cannot determine shutoff phenotype. These experiments do not rule out the possibility that vhs regulates early host shutoff activity via differential binding to VP16, as shown in Fig. 3.7, but they do rule out the possibility that VP16 is the determinant of host shutoff phenotype. Transfection result presented in this thesis and elsewhere (Pak *et al.*, 1995; Hamouda *et al.*, 1997; Everly and Read, 1997) indicate that vhs from HSV-2 strains is more effective at destabilizing reporter genes in the absence of VP16, implying that the vhs proteins do not require other viral partners to possess significant differences in activity. This evidence contradicts preliminary findings from in vitro degradation studies (Elgadi *et al.*, 1996) which showed that both vhsT1 and vhsT2 create secondary products from mRNA degradation at similar rates. In vitro measurements of intact mRNA loss presented in this thesis support these earlier findings, and argue that vhsT1 and vhsT2 alleles do not show a large difference in activity in a RRL system.

4.2 Vhs Allele Effects

It is well established that RRL translation is somewhat m7GpppG cap-independent (Pelham and Jackson, 1976; Bergmann *et al.*, 1979), and recent results show that this is due to a lack of cytoplasmic mRNA binding proteins such as the La autoantigen which render translation cap-dependent and prevent spurious initiation at internal sites when

added to RRL (Svitkin *et al.*, 1996). These or other differences between RRL and intact cellular systems may account for the conflicting results obtained from RRL and transfection systems. While vhs degrades mRNA in reticulocyte lysate, it is unclear whether vhs activity in this system is comparable to that found in transfected or infected cells. Thus, *in vitro* translation of vhs is subject to a number of complications that may affect activity estimates derived from experiments in RRL. The estimates of vhs activity obtained in the RRL system are of dubious reliability as most measures were obtained when concentration of substrate (intact mRNA) was possibly limiting, and thus activity would slow to appear similar as substrate concentration approaches zero. Estimates at higher concentrations of substrate suffer from inaccuracy in measurements of vhs protein concentrations, and the difficulty of determining relatively small changes in substrate to be significant.

Binding studies performed *in vitro* with vhsT1 and vhsT2 produced *in vitro* showed a small difference in binding to bacterially produced VP16 of HSV-1 (Fig. 3.7). However, it is doubtful that this binding difference is the sole cause of shutoff phenotype, as transfected vhsT1 and vhsT2 show different activity in the absence of VP16. The effect of VP16 may account for some discrepancies between our intertypic results and those of Everly and Read (1997) (discussed below).

Transfection of vhs from HSV-1 and HSV-2 has been performed previously (Pak *et al.*, 1995; Hamouda *et al.*, 1997) and showed a difference in the ability of transfected

vhs constructs to inhibit, respectively, CAT and HIV production. Pak *et al.* (1995) did not show significant differences in shutoff ability between HSV-2 strain 333 gene fragments containing the UL41 gene and HSV-1 strain KOS fragments upon transfection. Hamouda *et al.* (1997) assayed for the production of infectious HIV units in CMV-driven UL41 cotransfection, but did not control for the amount of CMV promoter present.

The transfection results presented in Fig. 3.5 support the hypothesis that vhs ORF composition determines shutoff phenotype in the absence of other viral factors, and that HSV-2 strain G vhs has a stronger shutoff phenotype. These findings have since been independently duplicated with several strains of HSV-1 and HSV-2 (Everly and Read, 1997)

To ask whether vhs ORF composition affected shutoff phenotype within the context of a viral infection, a portion of the HSV-2 strain G vhs ORF was transferred into the type 1 vhs ORF in the tk locus, producing the virus vhsT2. Experiments which switched UL41 genes between HSV strains have been performed previously (Fenwick and Everett, 1990b). These studies did not control for differences in promoter activity which may affect the amount of vhs present to be packaged into virions. To more clearly account for differences in promoter activity, as well as locus effects, the viruses vhsT1 and vhsT2 were constructed so that only the structure of the vhs ORF differed between constructs. VhsT2 clearly shows a strong shutoff while vhsT1 shows a weak shutoff phenotype over a range of MOI. Coupled with transfection data, this implies that vhs

ORF composition can determine shutoff phenotype, although packaging and particle:PFU variations and other virion factors could also affect shutoff. Interestingly, vhsT2 shows a slightly stronger shutoff phenotype than HSV-2 strain G, implying that other factors are probably influencing shutoff in this case. Viral studies provide a way to minimize transcriptional effects present in transfection assays, as vhs is packaged into the virion and no transcription is required for host shutoff. Previous studies have shown that vhs from HSV-1 strain 17 and HSV-2 strain G are packaged in similar amounts in viruses containing either gene, thus packaging is probably not influenced by host shutoff phenotype (McLauchlan *et al.*, 1992a). Shutoff phenotype is unlikely to directly influence late protein production and packaging as VP16 has been shown to abrogate the activity of late-produced vhs (Lam *et al.*, 1996).

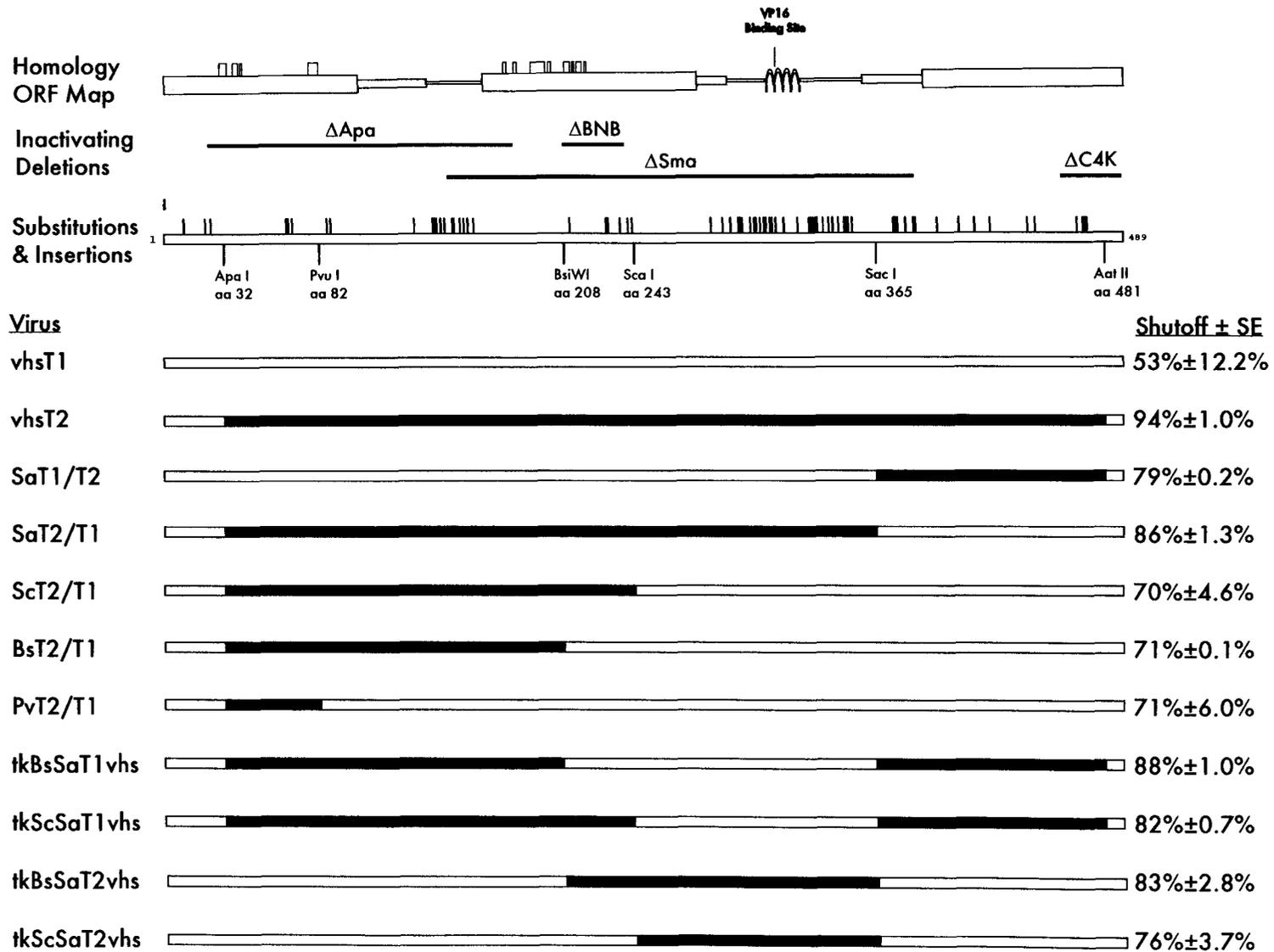
To account for possible inaccuracies in vhs protein dosage, infection at a range of MOIs was performed using the vhsT1 and vhsT2 viruses. If different shutoff phenotypes were an effect of varying particle:PFU ratios, one would expect the results from a translation vs. MOI curve to be superimposable, but this was not the case. Thus, the phenotypes observed are not a result of variable particle:PFU production among the viruses used. VhsT2 showed a large drop in translation even when infected with 0.1 PFU/cell, but as HSV-1 virions have at least a 25:1 particle:PFU ratio, several viral particles were delivered to each cell even at an MOI of 0.1. Recent studies have shown that the virus 17G41(41-), which contains the HSV-2 G UL41 gene in the tk locus, differs ~ threefold in particle:PFU ratio from its parental strain 17 (McLauchlan *et al.*, 1992a),

implying that vhs from HSV-2 does not drastically alter particle:PFU ratio in virus grown in BHK cells. Differential saturation of cells with HSV virions was not a likely explanation of these results, as up to 100 HSV PFU have been shown to enter a cell in a linear fashion (Sodeik *et al.*, 1997). It is still unclear whether vhs requires a cellular partner(s) to effect mRNA degradation, but it seems clear that the HSV-2 vhs ORF can confer a strong shutoff phenotype via the unknown mechanism in a viral background.

In an attempt to map the regions of the vhs ORF that determine strong shutoff phenotype, several other vhs constructs were constructed and tested to determine their shutoff phenotype. No clear localization of strong shutoff was determined, and it seems that strong shutoff activity is distributed in several regions along the vhs ORF. A map of aa substitutions and insertions between the KOS UL41 ORF and the HSV-2 G UL41 ORF is diagrammed in Fig. 4.1. While the vhsT2 construct lacks several amino acid changes present in the N-terminus region, it confers strong shutoff comparable to or exceeding that of HSV-2 G (Fig. 3.12). A relatively few changes in aa 32-82 (present in the virus PvT2/T1) confer an intermediate phenotype, similar to that of aa 82-243 (viruses BsT2/T1, ScT2/T1). Assuming no sequence changes in this region also downregulate shutoff efficiency, it would seem that the region from aa 82-243 does not contribute to increased shutoff in vhsT2, although a relatively large cluster of mutations are present in a small region between aa 82-208 and a large number of residues homologous to the FEN-1 exonuclease family are present (Doherty *et al.*, 1996). When the region is extended from aa 32-365, however, this confers an increase in shutoff level (virus

Figure 4.1. Mapping intertypic phenotype to aa differences and salient features of the vhs ORF.

The top figure, labeled homology ORF map, shows homology of KOS virus vhs protein sequence to a consensus vhs sequence (derived from KOS vhs, HSV-2 vhs, pseudorabies vhs, and equine herpesvirus vhs). The taller regions denote greater homology to consensus while shorter regions denote less homology. A region known to bind VP16 (Smibert *et al.*, 1994; Schmelter *et al.*, 1996) is marked. The small open blocks on the top of the vhs ORF denote vhs regions which share homology to the FEN-1 like endonuclease family (Doherty *et al.*, 1996) helix-loop-helix DNA binding domains. Inactivating deletions are arranged representatively with the bar denoting the region deleted in the deletion mutant (Jones *et al.*, 1995; Strelow and Leib, 1996b). Substitutions and insertions are noted by black lines above the amino acids in HSV-1 KOS vhs which differ from those in HSV-2 G vhs (alignment performed using the LALIGN program). Restriction sites used to create intertypics are shown on the underside and their amino acid location within the ORF is noted below. The viruses used in shutoff experiments are listed below and their vhs ORF are diagrammed below; open boxes represent HSV-1 KOS vhs sequence while black represents HSV-2 G vhs sequence. The relative shutoff efficiency \pm standard error is listed on the right beside each virus.



SaT2/T1). The C-terminal portion aa 365-481 alone confers relatively robust shutoff (virus SaT1/T2) with relatively few amino acid substitutions. This region has been shown to be necessary for vhs function previously (Strelow and Leib, 1996a), as shown in Fig. 4.1 (Inactivating Deletions, Δ C4K).

The viruses tkBsSaT1vhs and tkScSaT1vhs differ only in the inclusion of the region between aa 208-243, and counterintuitively the KOS aa sequence from 208-243 confers stronger shutoff than the HSV-2 G region. TkBsSaT1vhs showed the strongest shutoff of all intertypics screened excepting vhsT2. Conversely, the viruses tkBsSaT2vhs and tkScSaT2vhs show an opposite effect; inclusion of the KOS aa sequence from 208-243 downregulates shutoff slightly. This relatively small region is necessary for vhs function (Fig. 4.1, deletion Δ BNB), contains a relatively small number of amino acid changes, and contains some of the exonuclease motif mentioned previously (Doherty *et al.*, 1996).

These results agree with recent work using intertypic vhs chimeras in a cotransfection assay system (Everly and Read, 1997). Activity of several HSV-1 KOS / HSV-2 333 intertypic constructs driven by the CMV promoter in shutting off a cotransfected β -gal reporter plasmid was determined. The values obtained for shutoff in this study and in that of Everly & Read (1997) are presented in Table 4.1 for comparison. The values obtained in these two studies were obtained in quite different manners, and are probably not directly comparable due to assay differences. The % shutoff figure used

Table 4.1. Comparison of vhs intertypic results.

The left table summarizes results of infection of Vero cells with intertypic HSV recombinants. The columns, from left to right, indicate name of virus used, the HSV-2 G aa sequences exchanged into the KOS vhs ORF, and the % shutoff in a translation assay. The right table summarizes data taken from Everly and Read (1997) using a cotransfection shutoff assay. Columns, from right to left, indicate plasmid name, the HSV-2 333 aa sequences exchanged into the KOS vhs ORF, and the equivalents of DNA required to reduce β -gal activity to the same extent as UL41 from HSV-2 333 ([DNA]_{0.3}). Note that in the left table, increasing % shutoff indicates stronger vhs activity, while in the left table, decreasing [DNA]_{0.3} indicates stronger vhs activity. A precise definition of [DNA]_{0.3} is present in the text. Similar constructs are arranged on the same horizontal line for comparison. The construct xK/3/K/3 (135,208,365,489) contains a 32 aa N-terminal extension detailed in Everly and Read (1997).

Virus	HSV-2 G aa Sequence	% Shutoff	Plasmid	HSV-2 333 aa Sequence	[DNA] _{0.3}
vhsT1	-	53%±12.20%	KOS	-	40.3±5.9
vhsT2	(32-481)	94%±1.00%	333	(1-489)	1.0
PvT2/T1	(32-82)	71%±6.00%			
			3/K(135)	(1-135)	1.1±0.1
BsT2/T1	(32-208)	71%±0.10%	3/K(208)	(1-208)	1.8±0.5
ScT2/T1	(32-243)	70%±4.60%			
SaT2/T1	(32-365)	86%±1.30%	3/K(365)	(1-365)	0.7
tkScSaT2vhs	(243-365)	76%±3.70%	K/3/K(243,365)	(243-365)	46.1±12.9
SaT1/T2	(365-481)	79%±0.20%	K/3(365)	(365-489)	6.5±2.5
tkBsSaT1vhs	(32-208,365-481)	88%±1.00%	3/K/3(208,365)	(1-208,365-489)	0.8
tkBsSaT2vhs	(208-365)	83%±2.80%	K/3/K(208,365)	(208-365)	13.5±1.8
tkScSaT1vhs	(32-243,365-481)	82%±0.70%			
			xK/3/K/3(135,208,365)	(135-208,365-489)	9.3
			3/K/3(135,365)	(1-135,365-489)	0.8
			K/3/K(135,208)	(135-208)	41.6
			K/3/K(208,243)	(208-243)	14.5±1.8

in our intertypic shutoff experiments represents % of labeled protein amount present relative to vhs null mutant infection translated protein. The $[DNA]_{0.3}$ value used by Everly & Read (1997) is defined as the concentration of effector DNA (vhs construct) estimated to reduce reporter gene expression to 30% of control transfected cells, relative to the amount of HSV-2, 333 construct DNA needed to reduce reporter activity by the same amount (to 30%). As an example, a $[DNA]_{0.3}$ value of 1.8 for construct 3/K(208) indicates that 1.8 times as many moles of construct 3/K(208) DNA are needed to lower reporter expression to 30%, relative to construct HSV-2, 333 DNA (Everly and Read, 1997).

Intermediate shutoff in our assay appears to be ~70-80%, while it appears to be from ~6-15 $[DNA]_{0.3}$ in the transfection system. Many of the results, within the limits of comparison, agree between these two different assay systems. The N-terminal portion of vhs appears to effect intermediate or strong shutoff in both systems (virus PvT2/T1, plasmid 3/K(135)). Extension of this N-terminal fragment to aa 208 does not significantly increase shutoff in either system, implying that the aa from 135-208 are not important in conferring strong shutoff (virus BsT2/T1, plasmid 3/K(208), plasmid K/3/K(135,208)). The weak shutoff of plasmid construct K/3/K(135,208) supports this contention. The C-terminal region of vhs (aa 365-481) also contains regions capable of conferring intermediate shutoff upon the vhs ORF, as seen in virus SaT1/T2 and plasmid K/3(365). Constructs containing HSV-2 sequence interrupted by HSV-1 sequence at aa 208-365 both show shutoff close to or exceeding HSV-2 sequence alone (virus

tkBsSaT1vhs, plasmid 3/K/3(208,365). However, the complementary intertypic virus tkBsSaT2vhs and plasmid K/3/K(208,365) show strong or intermediate shutoff, respectively, implying that a region within aa 208-365 is also capable of increasing shutoff ability. The plasmid K/3/K(243,365) further localizes this region to aa 208-243. However, the intertypic plasmid K/3/K(243,365), which shows weak shutoff in cotransfection, shows intermediate shutoff in a similar viral construct (tkScSaT2vhs), possibly indicating that a condition present in virion infection of cells which is not present in transfection affects this region. While many of the viral constructs display intermediate phenotypes, many of the plasmids with similar composition display shutoff comparable to or exceeding HSV-2 (plasmid 333), such as 3/K(365), and 3/K/3(135,365) (Table 4.1).

Several factors may differ between the two assay systems which would account for these discrepancies: a) the viral system may be susceptible to large differences in particle:PFU ratio between viruses which may skew results; b) vhs autoregulation of its own production in a transfection system is not present in the viral system, where a fixed dose of vhs is delivered to the cell and in the presence of an Act D block no more is made; c) other viral factors, such as the numerous virion protein kinases present in the tegument or VP16, which are not present in a cotransfection system, are affecting shutoff; d) stability of the proteins may differ in the viral vs. the transfection system due to the different mode of delivery and the presence of other viral proteins; e) differences in packaging signals among vhs proteins may lead to dosage differences between intertypic

viruses . Large dosage differences extrapolated from Everly & Read's (1997) assay (40.3 X as much KOS vhs DNA is required to shut off equivalent to 333) are not present to the same extent in the viral system, which may indicate that 10 PFU may contain a partially saturating amount of vhs, or that other viral factors act to limit maximum vhs activity. The different saturation shutoff levels present in MOI titrations (Fig. 3.10) argues that viral vhs dosage does not correspond linearly with shutoff as is assumed in the transfection system of Everly and Read (1997).

VP16 has been shown to recruit vhs to the nucleus, where, presumably, it is not active (Frank Jones, unpublished data). This may represent another method of limiting vhs activity shortly after infection, although this has not been conclusively demonstrated as of yet.

It is possible that two or more regions of vhs act independently to effect shutoff and the final shutoff phenotype observed is a result of several activities of the vhs protein. Many aa regions which do not affect shutoff phenotype are likely essential to vhs, as several deletion mutants in these regions inactivate shutoff.

The data of Everly and Read (1997) led them to conclude that the regions conserved among α -herpesvirus UL41 homologues are responsible for the type-specific differences in vhs activity. The intertypic virus data supports the conclusion that these conserved regions (Fig. 4.1, top bar) are capable of conferring strong shutoff (clearly shown in PvT2/T1 and SaT1/T2, Fig. 4.1). However, the construct tkScSaT2vhs (which

contains a length of type 2 ORF between aa 243 – 365 spanning the highly variable VP16 binding region), still possesses a weak intermediate shutoff phenotype, indicating that this poorly conserved region can also influence shutoff ability. Differences in activity seen between SaT2/T1 and ScT2/T1 (Fig. 4.1) support the conclusion that the aa 243 – 365 region can also influence shutoff phenotype.

4.3 The role of UL13 in Virion Host Shutoff

In these studies, UL13 null viruses have been shown to have significant shutoff activity in MOI titration experiments. Barring the results obtained with the virus UL13(-), which may contain a genetic lesion of unknown type, viruses obtained from other labs showed fairly robust shutoff, contradicting the results obtained by Overton et al. (1994). However, substantial but reduced levels of shutoff (78% and 59% of mock translation) have been detected in UL13 null viruses (Ng *et al.*, 1997; Ng *et al.*, 1996), although amounts vary by 20% between these reports from the same lab using the same viruses and shutoff conditions. It should be noted that different parental strains were used in these experiments: HSV-1 SC16 (Overton *et al.*, 1994), HSV-1 FΔ305 (Ng *et al.*, 1997), HSV-1 KOS (this work), and HSV-1 KOS1.1 (a descendent of KOS, this work) may possess strain specific differences which alter shutoff ability in the absence of UL13.

UL13 and/or ICP22 function have been postulated to determine the accumulation of UL41 mRNA and consequently the packaging of vhs into the virion (Ng *et al.*, 1997). If this observation is accurate, the contradicting reports from Overton et al. (1994), Ng et

al. (1997), and the present series of experiments may be due to cell-type specific complementation of UL13 and/or ICP22 activity upon virion preparation. It has previously been shown that UL13 or ICP22 null viruses replicate poorly in BHK-21, RAT-1, and rabbit skin cells but are less restricted in Hep-2 human epidermoid carcinoma or Vero cells (Purves *et al.*, 1993). The viruses tested by Overton *et al.* (1994) were prepared in Vero cells, banded in glycerol gradients, and used to infect Vero cells as well as analyzed for virion composition. UL13 null and wt virions showed similar levels of vhs incorporation in this production system (Overton *et al.*, 1994). Ng *et al.* (1997) prepared virions in rabbit skin cells, banded virions in a dextran gradient for purification, infected Vero cells to test shutoff, and grew virions in rabbit skin cells to analyze virion composition. In both cases, UL13 viruses showed reduced but present levels of shutoff in Vero cells. Thus, the differences between the virion composition results reported by Overton *et al.* (1994) and Ng *et al.* (1997) could be due to restriction of γ gene expression in rabbit skin cells, leading to production of virions with reduced amounts of γ gene products. Virions grown in Vero cells, which complement the γ gene restriction to some extent (Purves *et al.*, 1993), probably do not suffer from this defect and thus produce relatively normal levels of vhs, which were detected in virions by Overton *et al.* (1994).

In the present study, virions were prepared in Vero cells, sonicated, cell debris centrifuged and removed, and the supernatant used to infect Vero cells. Particle:PFU ratios may have differed due to this preparation method, and if this resulted in an increase in the particle:PFU ratio, shutoff would also be expected to increase with dosage of

tegument proteins in L particles and/or defective virions. L particles have been shown to effect shutoff as well as intact virions (McLauchlan *et al.*, 1992a). This possible difference in virion preparation may explain the apparent robust shutoff observed in viral infection. If UL13 null viruses produce more defective particles, this would act to increase shutoff to that of wt HSV-1, as was observed.

Ng *et al.* (1997) also present evidence that vhs protein accumulates to a lesser extent in Vero cells 24 h after infection, and show that vhs mRNA accumulates to a different extent in rabbit cells 24 h after infection. These results, by themselves, do not contradict Overton *et al.* (1994) as differences in accumulation in late mRNA in UL13 or ICP22 null virus infections can be explained by the somewhat slower growth cycle of these mutants in rabbit skin cells (no viral gene controls were used to demonstrate other viral mRNA levels in rabbit cells). The differential accumulation of vhs protein in UL13 null virus infections has been noted previously by Overton *et al.* (1994), in which the authors noted that the amount of vhs synthesized was reduced, but found that UL13 null virions had equivalent amounts of vhs. Thus, differences in amount of vhs produced during infection do not necessarily translate into virion composition differences.

Supporting the data of Overton *et al.* are those of D. Leib (unpublished results) which show a lack of mRNA degradation during infection in the absence of transcriptional inhibitors using a similar viral construct. However, as viral transcription is occurring during this assay, it is difficult to ascribe lack of shutoff to the lack of UL13

when lack of UL13 may be influencing other viral gene product accumulation and viral protein effects upon the cell.

Many questions remain about vhs-induced shutoff of the host cell. Whether VP16 plays a role in diminishing shutoff in a viral infection is still unclear, although it is clear that VP16 from HSV-2 G does not change shutoff phenotype. VP16 may still play a role early during shutoff, but this is difficult to test directly as VP16 null mutants are lethal. The fact that VP16 from HSV-2 G can functionally substitute for type 1 VP16 argues that the two proteins share all essential viral functions and can substitute structurally for each other.

Efforts to determine the vhs ORF regions which control shutoff phenotype were partially successful although the map of regions derived from these experiments was not simple, with multiple and redundant vhs regions conferring an increase in shutoff activity. The precise mechanism by which vhs induces shutoff is not clear, but these intertypic mutants may assist in later attempts to determine whether cellular factors are involved directly in shutoff by correlating shutoff activities with putative mediators of mRNA degradation, for example.

The influence of UL13 on vhs activity is still in question, although it seems certain that UL13 mutants possess some shutoff activity, the reasons for the conflicting results discussed above are still unclear. Further tests of virion vhs content in compatible cell lines are required to determine whether varying vhs dosage explains the results, or

some further effect is present. The putative effect of ICP22 and/or UL13 which is being complemented in Vero cells but not in BHK cells is still undetermined. Further work to investigate this factor, and the function of ICP22 and/or UL13 which is complemented could show precisely what regulatory pathways influence γ gene expression and possibly vhs activity or packaging.

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