

REGULATION OF VIRAL AND CELLULAR
GENE EXPRESSION IN CELLS INFECTED
WITH HERPES SIMPLEX VIRUS

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Abstract

When herpes simplex virus (HSV) infects a cell, viral genes are expressed to high levels, while cellular gene expression is dramatically inhibited. The major goal of the work described in this thesis was to understand the molecular basis for differential expression of viral and cellular genes in HSV infected cells. A two part-approach was taken to examine this problem. First, I compared the expression of virally-transduced and endogenous β -globin genes in HSV infected cells. These experiments were prompted by previous work which demonstrated that an HSV transduced rabbit β -globin gene is efficiently expressed in infected Vero cells, a result that was in apparent conflict with previous experiments demonstrating that HSV infection inhibits the expression of the mouse β -globin gene resident in mouse erythro leukemia cells. This approach demonstrated that HSV products inhibit the expression of an endogenous β -globin gene while transactivating the expression of a virally transduced β -globin gene. These results illustrate that the selective inhibition of cellular gene expression plays an important role in the preferential expression of genes associated with the viral genome. Therefore, the second approach involved a detailed investigation of the herpes simplex virus vhs gene product, one of the viral functions that inhibits cellular gene expression. I have confirmed that the vhs effect requires the HSV UL41 open reading frame. In addition, I identified the vhs protein as a 58kDa phosphoprotein that is packaged into virions. Using several approaches I demonstrated that the vhs protein interacts with the HSV transcription factor VP16. In addition, I have data which suggests this interaction downregulates the

vhs function and that this downregulation might play a key role in the preferential expression of viral genes in the HSV infected cell.

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HCMV	human cytomegalovirus
Hepes	(N-[hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HMBA	hexamethylene bisacetamide
HSV	herpes simplex virus
ICP	infected cell polypeptide
IE	immediate early
kbp	kilobase pair
kDa	kiloDaltons
L	late
M	molar
μg	microgram
mg	milligram
min	minute
μL	microlitre
MEL	mouse erythroleukemia
mL	millilitre
MOI	multiplicity of infection
mm	millimetre
mM	millimolar
μM	micromolar
MOPS	3-[N-morpho]linopropane-sulfonic acid
mRNA	messenger ribonucleic acid
ng	nanogram
NP40	nonidet P-40
nm	nanometre

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List of Abbreviations

Act D	actinomycin D
dATP	deoxyadenosine 5'-triphosphate
ATP	adenosine 5'-triphosphate
BMV	Brome mosaic virus
BSA	bovine serum albumin
bp	base pair
dCTP	deoxycytidine 5'-triphosphate
CPE	cytopathic effect
cpm	counts per minute
CTP	cytidine 5'-triphosphate
Cx	cycloheximide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E	early
EDTA	ethylenediaminetetraacetate
dGTP	deoxyguanosine 5'-triphosphate
gB	glycoprotein B
gC	glycoprotein C
gD	glycoprotein D
gI	glycoprotein I
GTP	guanosine 5'-triphosphate
h	hour
PAA	phosphonacetic acid
Pipes	piperazine-N,N'-bis[2-ethane-sulfonic acid]
PRV	pseudorabies virus
PMSF	phenylmethanesulfonyl fluoride
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
TBP	TATA-binding protein
TCA	trichloroacetic acid
tk	thymidine kinase
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
ts	temperature sensitive
dTTP	deoxythymidine 5'-triphosphate
U _L	unique long
U _S	unique short
UTP	uridine 5'-triphosphate
UTR	untranslated region
VIC	VP16-induced complex
vhs	virion-host shutoff
wt	wild-type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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Introduction

1.1 Herpes Simplex Virus: General Introduction

The human pathogen, herpes simplex virus (HSV), is a member of the alpha herpesvirus subfamily. In general, HSV initially infects an individual at mucosal surfaces where it undergoes lytic replication. The replicated virus can then travel to ganglia via the sensory nerves that innervate the site of infection. At these ganglia, the virus becomes latent in the neuronal cell body where it will remain for the lifetime of the host. Upon appropriate stimuli, the latent virus can reactivate, traveling back along the sensory nerves to the initial site of the infection (reviewed by Whitley, 1990).

The HSV particle consists of an icosahedral nucleocapsid that contains the HSV genome. The particle is surrounded by a cell derived lipid bilayer, the envelope, that contains several virally encoded glycoproteins. The space between the envelope and the nucleocapsid contains several viral proteins and is referred to as the tegument (virion structure is reviewed by Roizman and Sears, 1990).

The HSV genome is a 150 kbp linear double stranded DNA that is divided into two unique components: U_L and U_S (for unique long and unique short). Both U_L and U_S are flanked by distinct inverted repeats, referred to as the *b* sequence and *c* sequence, respectively. The genome terminates in direct repeats, referred to as the *a* sequence and an inverted copy of the *a* sequence is found between the *b* and *c* repeats at the U_L/U_S junction. The number of *a* sequences at the U_L/U_S junction and the U_L termini is variable while one copy

of the *a* sequence is found at the U_S termini. The U_L and U_S sequences freely invert relative to one another and as such, four isoforms of the HSV genome exist (genome structure is reviewed by Roizman and Sears, 1990).

The HSV infectious cycle is initiated by the attachment to, and subsequent penetration of, the virion into the target cell and these processes are both mediated by viral glycoproteins. Penetration is thought to involve the fusion of the viral envelope with the cell plasma membrane, presumably releasing the contents of the tegument and the nucleocapsid into the cell (attachment and penetration are reviewed by Roizman and Sears, 1990). The nucleocapsid then travels to nuclear pores where it injects the viral genome into the nucleus (Tognon *et al.*, 1981; Batterson *et al.*, 1983).

After injection of the HSV genome into the nucleus, viral genes are transcribed by the host RNA polymerase II in a regulated fashion (Alwine *et al.*, 1974; Honess and Roizman, 1974; Ben-Zeev *et al.*, 1976; Costanzo *et al.*, 1977; reviewed by Wagner, 1985; Roizman and Sears, 1990). Thus the sequential expression of viral genes is described as a cascade. The first genes to be expressed are the immediate early (IE or α) genes which encode the infected cell proteins (ICPs) 0, 4, 22, 27, and 47. Initially, IE genes were defined by their expression in the absence of protein synthesis and evidence suggests that IE proteins are involved in driving the expression of the remaining HSV genes (Honess and Roizman, 1974, 1975). Following expression of IE proteins, early (E, or β) genes are expressed. Their expression requires IE proteins but does not require viral DNA synthesis. Many E gene products are involved in replicating viral DNA, for example, the viral DNA polymerase and thymidine kinase (tk) are encoded by E genes. Finally, late (L or γ) genes are expressed.

L genes require IE gene products and viral DNA synthesis in cis to obtain maximal levels of expression (Johnson and Everett, 1986; Mavromara-Nazos and Roizman, 1987). L genes can be divided into two subclasses, leaky late and true late (or γ_1 and γ_2). Leaky late genes are expressed in the absence of viral DNA replication and their expression is stimulated by viral DNA synthesis. In contrast, the expression of true late genes is much more dependent on viral DNA replication. Several late gene products, including glycoprotein C (gC), are viral structural proteins.

The cascade of viral gene expression initiated in HSV infected cells has offered researchers a model system, of moderate complexity, in which to study eukaryotic gene expression. These investigations have concentrated on two components of this cascade: the trans-acting HSV encoded proteins that regulate viral gene expression and the cis-acting DNA elements that drive the expression of individual viral genes.

1.2 HSV Promoters

Many investigations have identified the cis-acting DNA sequences required to express IE, E, and L genes. These studies have demonstrated that IE promoters contain binding sites for cellular transcription factors, making them strong constitutive promoters (reviewed by Thompson and McKnight, 1992). IE promoters also contain multiple copies of the nucleotide sequence TAATGARATTC (where R is a purine) which renders them sensitive to transcriptional activation by the virion protein, VP16 (see below).

Investigations into E gene expression have employed the tk and glycoprotein D (gD) promoters as models. Initially, the activity of the tk promoter was examined in the absence of viral regulatory proteins. These studies compared the activity of the wild type tk promoter to various mutated tk promoters in *Xenopus* oocytes and *in vitro* transcription reactions (McKnight *et al.*, 1981; McKnight and Kingsbury, 1982; Jones *et al.*, 1985). The essential regions of the tk promoter were also defined using a cellular transformation assay (McKnight *et al.*, 1981). These analyses suggested that the tk promoter consists of a TATA box linked to two upstream Sp1 binding sites separated by a CAATT box. Subsequent work analyzed the tk promoter sequences required for HSV products to activate tk expression. Initially, Smiley *et al.* (1983) mapped the tk promoter sequences required for super-infecting virus to activate a tk gene integrated into the cellular genome. Eisenberg *et al.* (1985) then determined the tk promoter sequences required for super-infecting virus to activate a transiently transfected tk gene. Finally, Coen *et al.* (1986) mapped the promoter sequences required to express the tk gene resident in the HSV-1 genome at the tk locus. These studies concluded that similar sequences are required for tk transcription in the presence or absence of HSV activators. In other words, no sequences are specifically required for HSV regulators to activate tk expression. Similarly, activation of the HSV-1 gD gene (another HSV E gene) by either viral products or the SV40 virus enhancer requires identical gD promoter sequences (Everett, 1984a). This study demonstrated that the gD promoter consists of a TATA box linked to two Sp1 binding sites and implies that the gD promoter lacks virus specific transactivation sequences.

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1.3.1 VP16

The ability of VP16 to activate IE gene expression was first recognized in studies designed to map IE gene promoters. These studies involved the construction of cell lines containing the HSV tk gene linked to the upstream region of various HSV IE genes (Post *et al.*, 1981; Mackem and Roizman, 1982 a, b; Batterson and Roizman, 1983; Cordingly *et al.*, 1983; Lang *et al.*, 1984; Preston *et al.*, 1984). The expression of the tk gene in these cell lines, like the expression of the tk gene under the control of its own promoter, is activated by HSV infection. However, unlike the wild type tk gene, these IE-tk fusions are activated by HSV infection in the absence of viral protein synthesis. This result suggests that the activation of these IE-tk fusions is mediated by a component of the infecting virus particle. This virion protein was identified by Campbell *et al.* (1984) who found a fragment of HSV DNA that specifically transactivates co-transfected IE promoters. This fragment encodes a 65kDa protein, known as VP16 (also known as ICP25, α -TIF, Vmw65, UL48), which is a virion phosphoprotein located in the tegument (Spear and Roizman, 1972; Heine *et al.*, 1974; Roizman and Furlong, 1974).

Identification of the VP16 response elements was initiated by Mackem and Roizman (1982b), who discovered multiple copies of the sequence TAATGARATTC (where R is a purine) in IE promoters. Subsequent mutational analysis demonstrated that this sequence is required for activation of IE promoters by either superinfecting virus or co-transfected VP16 (Kristie and Roizman, 1984; Preston *et al.*, 1984; Gaffney *et al.*, 1985; Bzik and Preston,

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Investigations which mapped the promoters of two HSV late genes, gC and US11, revealed relatively small promoters, consisting of sequences around the cap site, including the TATA box (Homa *et al.*, 1986; Johnson and Everett, 1986; Homa *et al.*, 1988). As with E promoters, no sequences are specifically required for HSV products to activate either promoter.

The results described above suggest that E and L promoters lack HSV specific activation elements. This implies that viral regulatory functions activate E and L gene expression through the cellular transcription factors that bind to E and L promoters. This model is supported by results demonstrating that some heterologous promoters are activated by viral regulatory proteins (see below).

1.3 HSV Regulatory Proteins

The HSV genetic program is driven by HSV proteins that regulate viral gene expression. HSV differs from many other DNA viruses in that some of its regulatory proteins are delivered into the cell as part of the virus particle. The best characterized of these virion regulatory proteins is VP16, which transactivates IE gene expression. Notably VP16 activates the expression of the IE infected cell proteins (ICPs) 4, 0, 27, and 22. These IE proteins in turn regulate viral gene expression in the infected cell. In the following section, I will review the current knowledge pertaining to the regulation of HSV gene expression by VP16 and ICPs 4, 0, 27, and 22.

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1986). Surprisingly, VP16 is unable to bind to calf thymus DNA or the TAATGARATTC sequence (Marsden *et al.*, 1987; McKnight *et al.*, 1987; O'Hare *et al.*, 1988; Preston *et al.*, 1988). However, an activity that bound to the TAATGARATTC motif was identified in uninfected cell extracts (Kristie and Roizman, 1987). Subsequently, VP16 was shown to interact with the TAATGARATTC sequence in the presence of an uninfected cell extract (I will refer to this complex as VIC for VP16 induced complex) (McKnight *et al.*, 1987). McKnight *et al.* (1987) were able to eliminate binding of both the uninfected cell factor and VP16 to the TAATGARATTC element using specific competitor DNA. These results imply that VP16 binds to the TAATGARATTC motif indirectly, by interacting with a cellular factor that binds directly to the TAATGARATTC element. Also, several correlative studies have suggested that this indirect interaction is essential for the transactivation of IE gene expression by VP16 (Ace *et al.*, 1988; Gerster and Roeder, 1988; O'Hare *et al.*, 1988; O'Hare and Gooding, 1988; Preston *et al.*, 1988). O'Hare *et al.* (1988) identified the cellular TAATGARATTC binding factor as the ubiquitous octamer binding protein Oct-1. They also demonstrated that an Oct-1 binding site from the immunoglobulin light chain gene confers VP16 inducibility on an otherwise unresponsive gene. The Oct-1 protein binds to the ATGCTAAT sequence that overlaps the 5' portion of the TAATGARATTC motif. On its own, the Oct-1 binding site is not responsive to VP16, presumably because the GARATTC portion of the TAATGARATTC motif is required to incorporate VP16 into VIC (Gerster and Roeder, 1988; Kristie *et al.*, 1989; O'Hare *et al.*, 1988).

Recent work has shown that VP16 has a low affinity for the TAATGARATTC sequence (Kristie and Sharp, 1990). Although this DNA binding activity is

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eliminated by mutation at either the 5' or 3' end of the TAATGARATTC motif, the relevance of this DNA binding activity to VP16 function is unknown (Kristie and Sharp, 1990).

The results described above suggest that VP16, Oct-1, and DNA are required for VIC formation and additional work has suggests that at least one other host cell factor is required (Gerster and Roeder, 1988; apRhys *et al.*, 1989; Katan *et al.*, 1990). This factor, referred to as HCF, binds to VP16 in the absence of DNA (Kristie and Sharp, 1990; Xiao and Capone, 1990) and the gene encoding this protein has recently been cloned (Wilson *et al.*, 1993).

VP16, like other transcriptional activators, contains two functional domains, one involved in promoter recognition and another required to activate transcription. Mutational analysis has shown that the promoter recognition domain of VP16 is located within amino acids 1-388 as these residues drive VIC formation (Triezenberg *et al.*, 1988; Greaves and O'Hare, 1989; Werstuck and Capone, 1989 a, b). These experiments also demonstrated that the carboxy terminal region of VP16 is required for transactivation but not for VIC formation. These results suggest that the transactivation domain of VP16 is located in the carboxy terminus of the protein. Sadowski *et al.* (1988) and Cousens *et al.* (1989) tested this hypothesis by linking the carboxy terminus of VP16 to a heterologous DNA binding domain that lacks a transactivation region. The resulting fusion proteins are potent transactivators and therefore these results confirm that the transactivation domain of VP16 is located in the carboxy terminus .

A large number of studies have investigated how the carboxy terminus of VP16 activates transcription. This intense interest stems from observations that

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function to a lytic infection could only be assessed using a VP16 protein that is structurally competent but transactivation deficient. This was accomplished by Ace *et al.* (1989) who constructed an HSV-1 mutant, in1418, which encodes a transactivation defective VP16. This defect results from an in-frame linker insertion that disrupts VIC formation. The linker however, does not compromise the structural function of VP16 as it does not impair rescue of the HSV-2 ts VP16 mutant. As expected, in1418 expresses decreased quantities of HSV IE mRNAs. For example, ICP0 and ICP27 mRNA levels are reduced 4-5x, ICP22 mRNA levels are reduced 2x, while ICP4 mRNA levels are not affected. in1418 also displays a high particle to PFU ratio despite a wild type number of defective particles. This suggests that in1418 has difficulty initiating a productive infection at low MOI. This hypothesis is consistent with data demonstrating that in1418 displays a reduction in viral protein synthesis at low MOI. Thus, VP16 is a bifunctional protein involved in virus assembly and the transactivation of IE gene expression.

1.3.2 ICP4

ICP4, a 175kDa nuclear phosphoprotein, is arguably the most important HSV encoded transcription factor (Pereria *et al.*, 1977; Hay and Hay, 1980; Wilcox *et al.*, 1980; Faber and Wilcox, 1986a). The ICP4 coding sequences are located entirely in the *c* repeat and as such, the gene encoding ICP4 is diploid (Marsden *et al.*, 1978; Preston *et al.*, 1978; Clements *et al.*, 1979; Anderson *et al.*, 1980). The isolation of HSV ts mutants which map to the ICP4 gene demonstrated that ICP4 is essential for virus replication. The phenotypes of

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the VP16 transactivation domain, like several other transactivation domains, is highly acidic (for examples see Giniger and Ptashne, 1987; Ma and Ptashne, 1987; Hope *et al.*, 1988). This common characteristic implies that acidic activators might function through a single mechanism. Since the carboxy terminus of VP16 is a very potent transactivation domain, many researchers have employed it as a model for acidic activator function. These studies have suggested that the transactivation domain of VP16 activates transcription by interacting with TFIID, TFIIB, TFIIA, TAFII40 (a recently cloned TFIID associated factor), and/or some as yet unidentified transcriptional adapter (Berger *et al.*, 1990; Stringer *et al.*, 1990; Flanagan *et al.*, 1991; Ingles *et al.*, 1991; Lin *et al.*, 1991; Lin and Green, 1991; Timmers and Sharp 1991; White *et al.*, 1991; Wang *et al.*, 1992; Zhu and Pryures, 1992; Goodrich *et al.*, 1993; Roberts *et al.*, 1993). This large number of potential targets may relate to data suggesting that the carboxy terminus is composed of two functionally distinct transactivation domains (Regier *et al.*, 1993; Walker *et al.*, 1993). Further work has demonstrated that one domain of the carboxy terminus interacts with TFIIB while the other domain interacts with the TFIID associated factor TAFII40 (Goodrich *et al.*, 1993; Roberts *et al.*, 1993).

What role does VP16 play in the infectious cycle? VP16 is one of the most abundant HSV virion proteins, and thus it is not surprising that an early study, which employed an HSV-2 temperature sensitive (ts) lethal VP16 mutant, demonstrated that it plays a critical role in virion assembly (Ace *et al.*, 1988). Consistent with this, an HSV-1 mutant virus, deleted for the VP16 coding sequences, is not viable and only grows on cells that supply VP16 in trans (Weinheimer *et al.*, 1992). Thus, the importance of the VP16 transactivation

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these mutants suggest that ICP4 is required to repress the synthesis of IE proteins and mRNAs, and to activate the synthesis of E and L proteins and mRNAs (Watson and Clements, 1978; Preston, 1979; Dixon and Schaffer 1980; Preston 1981). Furthermore, ICP4 appears to be continuously required for transcription of E and L mRNAs (Dixon and Schaffer, 1980; Watson and Clements, 1980). The essential nature of ICP4 and its involvement in regulating viral gene expression was confirmed by the construction of HSV mutants that bear large deletions in both copies of the ICP4 coding sequences (DeLuca *et al.*, 1985; Paterson and Everett, 1990). Subsequent use of ICP4 deletion mutants in nuclear run-on assays has demonstrated that ICP4 modulates viral gene expression at the transcriptional level (Godowski and Knipe, 1986; DeLuca and Schaffer 1988; Sheppard and DeLuca, 1989 and 1991).

Since ICP4 is required to activate E and L transcription, researchers have assayed ICP4 function in the absence of other viral proteins. Using a cell line which constitutively expresses ICP4, Persson *et al.* (1985) demonstrated that ICP4, on its own, activates the expression of several E genes located in the virus chromosome. In addition, ICP4 activates the expression of both E and L promoters in transient transfection experiments (Everett, 1984b; DeLuca and Schaffer, 1985; Everett, 1985; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985 a, b; Quilan and Knipe, 1985; Everett, 1986; Gelman and Silverstein, 1986; Mavromara-Nazos *et al.* 1986; Shapria *et al.*, 1987; Everett, 1988b). The relevance of these transient experiments to ICP4 function in virus was confirmed by DeLuca and Schaffer (1985) who demonstrated that a ts ICP4 allele is ts for activation in transient transfections. ICP4 also transactivates the expression of several heterologous promoters in transient transfections assays.

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Sensitive promoters include the rabbit β -globin promoter, the HPV16 early region, the SV40 early region, the adenovirus E2a promoter, and the HIV LTR (Everett, 1984b and 1985; Tremblay *et al.*, 1985; Everett, 1988b; McCusker and Bacchetti, 1988; Albrecht *et al.*, 1989; Spessot *et al.*, 1989; Bachenheimer and Elishiekh, 1990). In addition, ICP4 activates the expression of some cellular genes located at their normal chromosomal loci (Latchman *et al.*, 1987; Kemp and Latchman, 1988). In contrast, some promoters are not activated by ICP4. For example, the HSV ICP6 promoter is not activated by ICP4 in either the viral genome or in transient co-transfections (Inchauspe and Ostrave, 1989; Sze and Herman, 1992; Desai *et al.*, 1993). Similarly, the Chinese hamster apt gene is not expressed when inserted into the viral genome, suggesting that it is not activated by ICP4 (Tackney *et al.*, 1984).

ICP4 also affects the expression of IE promoters in transient transfections. Alone, ICP4 inhibits the activity of all IE promoters (O'Hare and Hayward, 1985b; Gelman and Silverstein, 1987; Resnick *et al.*, 1989). Similarly, at high concentrations, ICP4 inhibits the activation of IE promoters by ICP0. However, when low concentrations of ICP4 are used, ICP0 and ICP4 cooperate to activate IE promoters (Gelman and Silverstein, 1987; Resnick *et al.*, 1989). Thus ICP4 is able to activate and repress IE promoters.

Given that ICP4 regulates transcription, it is not surprising that ICP4 has an intrinsic DNA binding activity. Initial experiments demonstrated that ICP4 from crude cell extracts bound to DNA-cellulose (Powell and Purifoy, 1976; Hay and Hay, 1980). Subsequently, ICP4 purified by Freeman and Powell (1982) lost the ability to bind to DNA. This led to suggestions that ICP4 bound to DNA indirectly through host cell factors. However, several recent studies employing

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The ICP4 DNA binding function appears to play a role in repressing ICP4 gene transcription and this repression is mediated by an ICP4 binding sequence located over the mRNA cap site. This site is required for ICP4-mediated downregulation of the ICP4 promoter in transient transfections (O'Hare and Hayward, 1987; Roberts *et al.*, 1988). These data are consistent with experiments where deletion of the ICP4 gene cap site region, in virus, results in overexpression of ICP4 (DeLuca and Schaffer, 1988).

A large body of evidence suggests that the ICP4 DNA binding function is required for ICP4-mediated transactivation. For example, the ts ICP4 protein encoded by the HSV-1 mutant, tsK, is also ts for DNA binding (Paterson *et al.*, 1990). This establishes a correlation between the ICP4 DNA binding function and the ICP4 transactivation function. This correlation is reinforced by the observation that many mutations in ICP4 which impair DNA binding also impair transactivation in transient co-transfection assays and in lytic infections (DeLuca and Schaffer, 1988; Patterson and Everett, 1988 a, b; Sheppard *et al.*, 1989). Moreover, the addition of ICP4 binding sites to a reporter gene potentiates the activation of the reporter by ICP4 in both transient transfection assays and *in vitro* transcription reactions (Tedder *et al.*, 1989; Tedder and Pizer, 1988). In addition, efficient expression of the UL49.5 gene during lytic infection requires an ICP4 binding site located in the 5' untranslated leader (Romanelli *et al.*, 1992)

Although the results described above suggest that ICP4-mediated transactivation requires sequence-specific DNA binding, evidence to the contrary also exists. For example, Paterson *et al.* (1990) demonstrated that four ts ICP4 proteins, which drive expression of some early proteins at the non-

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highly purified ICP4 have demonstrated that ICP4 binds directly to DNA as a dimer (Michael *et al.*, 1988; Kattar-Cooley and Wilcox, 1989; Michael and Roizman, 1989; Wu and Wilcox, 1990). In addition, ICP4 binds to specific DNA sequences and these binding sites have been identified in the promoter regions and mRNA coding sequences of several HSV genes (Beard *et al.*, 1986; Faber and Wilcox, 1986b; Kristie and Roizman, 1986 a, b; Muller, 1987; Faber and Wilcox, 1988; Michael *et al.*, 1988; Tedder *et al.*, 1989). The first sites identified contained the consensus sequence ATCGTC which was later shown to be insufficient for sequence-specific DNA binding by ICP4 (Beard *et al.*, 1986; Faber and Wilcox, 1986b; Kristie and Roizman, 1986 a, b; Muller, 1987; Roberts *et al.*, 1988; Pizer *et al.*, 1991). Subsequent investigations also identified sites that lacked this consensus sequence which led to the classification of sites as either consensus or non-consensus (Kristie and Roizman, 1986 b; Michael *et al.*, 1988; Tedder *et al.*, 1989). Thus ICP4 might bind DNA through two distinct mechanisms. However, methylation interference studies suggest that similar G residues are required for ICP4 to bind to consensus and non-consensus sites (Michael and Roizman, 1989). This implies that ICP4 might bind to DNA through a single mechanism. By comparing several consensus and non-consensus sites, DiDonato *et al.* (1991) derived a more degenerate consensus sequence for the ICP4 binding site (RTCGTCNNYNSG, where Y is a pyrimidine, S is a C or G, and N is any base) which can accurately predict sites and non-sites. Using this consensus sequence the authors suggest that the HSV genome contains approximately 550 ICP4 binding sites. A similar ICP4 binding site consensus sequence was identified by Everett *et al.* (1991a) using an independent approach.

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permissive temperature, fail to bind DNA *in vitro* at the non-permissive temperature. Also, Sheppard and DeLuca (1991) isolated an HSV-1 mutant which expresses an ICP4 protein that does not bind DNA, but can activate viral gene expression. This ICP4 mutant contains an inactivating insertion at amino acid 320 and an alanine to valine substitution at amino acid 342 which in turn, rescues ICP4 function. These two studies suggest that specific DNA binding does not play a role in transactivation. However, these altered proteins may bind DNA *in vivo* although they do not bind DNA *in vitro*. Recent work investigated the function of proximal ICP4 binding sites in HSV promoters that are located at their normal chromosomal loci. Smiley *et al.* (1992) demonstrated that three ICP4 binding sites in the vicinity of the gD promoter are not required for normal expression of gD mRNA. Also, work by Imbalzano *et al.* (1990) suggests that ICP4 binding sites in the tk promoter are not required for normal tk expression. These results therefore suggest that the ICP4 binding sites in these promoters do not regulate gD or tk transcription, implying that specific DNA binding does not play a role in transactivation by ICP4. However, other ICP4 binding sites in the HSV genome might allow for normal expression of these genes in the absence of proximal ICP4 binding sites.

How does ICP4 activate transcription? A number of investigations suggest that ICP4 functions through the TATA box sequence and the TATA binding factor TFIID. For example, the pseudorabies virus (PRV) IE protein, (an ICP4 homologue), stimulates transcription *in vitro* and this ability is abrogated by the addition of excess TFIID (Abmayr *et al.*, 1988). In addition, the PRV IE protein requires TFIID to alleviate nucleosome-mediated inhibition of transcription *in vitro* (Workman *et al.*, 1988). These data suggest that the PRV IE protein

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stimulates transcription by facilitating the formation of a complex containing TFIID. Consistent with these *in vitro* data are results demonstrating that the adenovirus E1b TATA box is necessary and sufficient for PRV IE protein-mediated transactivation (Wu and Berk, 1988). Similarly, ICP4 transactivates minimal promoters containing only a TATA box and a cap site (Johnson and Everett, 1986; Mavromara-Nazos *et al.*, 1986; Shapira *et al.*, 1987). In addition, the SV40 early promoter is activated by ICP4 and the degree of this activation is dependent upon the precise sequence of the TATA region (Everett, 1988b). Moreover, the *aprt* gene, which lacks TATA homology, is not expressed when inserted into the HSV genome (Tackney *et al.*, 1984). More recent experiments demonstrate that ICP4 functionally substitutes for the transcription factor Sp1 (Imbalzano *et al.*, 1991). Because Sp1 activates transcription through TFIID (Pungh and Tjian 1990), it is possible that ICP4 also functions through TFIID. In addition, the ability of ICP4 to transactivate a given promoter is inversely proportional to the ability of that promoter to interact with TBP (TATA binding protein, the DNA binding component of TFIID) (Imbalzano and DeLuca, 1992). All of the above indirect evidence suggests that ICP4 activates transcription by recruiting TFIID to the target promoter. It is therefore significant that ICP4 directly interacts with TBP (Smith *et al.*, 1993). In these experiments ICP4 and TBP, when bound to their respective DNA binding sites, cooperatively interact with TFIIB to form a tripartite complex. Mutational analysis suggests that ICP4 must participate in this tripartite complex in order to transactivate transcription (Smith *et al.*, 1993). This implies that the ICP4 DNA binding function might play an essential role in the ICP4 transactivation function. Given that ICP4 and TBP are bound to DNA in this complex, the DNA between the ICP4 binding site and

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Construction of viral mutants bearing deletions in both copies of the ICP0 gene has allowed researchers to assess the role of ICP0 in the infectious cycle (Stow and Stow, 1986; Sacks and Schaffer, 1987). Surprisingly, ICP0 is not required for viral replication in tissue culture and the expression of viral proteins in ICP0 mutants is comparable to wild type virus during high multiplicity infections (Stow and Stow, 1985; Sacks and Schaffer, 1987). Although these results suggest that ICP0 plays no role in a viral infection, ICP0 mutants do have an elevated particle to PFU ratio (Stow and Stow, 1985). This elevation cannot be attributed to a large number of defective particles, since plaquing efficiency is rescued by infection of cells that supply ICP0 in trans (Sacks and Schaffer, 1987). These results suggest that ICP0 is involved in initiating a productive infection. Inasmuch as a VP16 transactivation deficient HSV mutant is rescued by ICP0 overexpression, ICP0 might initiate an infection by activating IE gene expression (Ace *et al.*, 1989). ICP0 is also required for normal synthesis of viral proteins and mRNAs during lytic infections at low MOI (Everett, 1989; Cai and Schaffer, 1992; Chen and Silverstein, 1992). This requirement applies to genes in all three kinetic classes with the exception of ICP4 which is expressed normally. ICP0 is also necessary for efficient production of virus from infectious viral DNA (Cai and Schaffer, 1989). This result might suggest that ICP0 is required to transactivate IE genes in the absence of the virion protein, VP16. This hypothesis is consistent with data demonstrating that infectious DNA from an ICP0 deletion mutant is defective for ICP4 expression (Cai and Schaffer, 1992). In summary, ICP0 is required for normal viral gene expression at both low MOI and in the absence of VP16. ICP0 is also required for a wild type particle to PFU ratio. Mutagenesis has correlated the ability of ICP0 to

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the TATA box must loop out. This looping may explain why the ICP4 DNA binding activity is required for transactivation while proximal ICP4 binding sites are dispensable for promoter activation in virus: the function of proximal ICP4 binding sites could be replaced by more distal sites. This looping may also relate to the ability of ICP4 to bend DNA when bound to an ICP4 binding site (Everett *et al.*, 1992).

1.3.3 ICP0

ICP0, like ICP4, plays a pivotal role in activating HSV gene expression during a lytic infection. This protein is a 110kDa nuclear phosphoprotein (Periera *et al.*, 1977; Hay and Hay, 1980; Ackemann *et al.*, 1984) that is encoded in the *b* repeats and as such, its gene is diploid (Preston *et al.*, 1978; Clements *et al.*, 1979; Watson *et al.*, 1979; Anderson *et al.*, 1980; Mackern and Roizman, 1980, 1982b; Rixon *et al.*, 1984). The ability of ICP0 to activate gene expression was first recognized in transient assays. In these experiments, ICP0 activated the expression of representative HSV promoters from each kinetic class, on its own or synergistically with ICP4 (Everett, 1984b; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985 a, b; Quilan and Knipe, 1985; Everett, 1986; Mavromara-Nazos *et al.*, 1986; Gelman and Silverstein, 1987; Shapira *et al.*, 1987). In addition, ICP0 transactivates heterologous promoters (Everett, 1985; O'Hare *et al.*, 1986; Mosca *et al.*, 1987; Everett, 1988a; McCusker and Bacchetti, 1988). This led to speculation that ICP0 is a promiscuous transactivator however, there are heterologous promoters that do not respond to ICP0 (Mosca *et al.*, 1987; Inchauspe and Ostrave, 1989).

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transactivate gene expression in transient transfections with the rescue of ICP0 mutants as assayed by several criteria (Cai and Schaffer, 1989; Chen and Silverstein, 1992; Everett, 1989). This suggests that the ability of ICP0 to transactivate gene expression in transient assays is relevant to ICP0 function in virus. However, given the ability of ICP0 to activate E and L gene expression in transient transfections assays, it is paradoxical that ICP0 fails to activate E and L gene expression in an ICP4 deletion virus (DeLuca *et al.*, 1985; Paterson and Everett, 1990).

ICP0 also allows for efficient infection of cells at different phases of the cell cycle. Cai and Schaffer (1991) have demonstrated that the plaquing efficiency of an ICP0 mutant varies with the stage of the cell cycle, while the plaquing efficiency of wild type virus does not. They also demonstrated that the peak plaquing efficiency for an ICP0 mutant occurs after release from G₀, late in G₁. These results suggest that ICP0 allows for viral replication independent of the cell cycle phase and that a cellular factor, expressed after the G₀ to G₁ transition, substitutes for ICP0. This hypothesis is interesting given that ICP0 induces expression of the cellular transcription factor, AP1 (Jang *et al.*, 1991). Since AP1 activity is expressed early in the G₀ to G₁ transition, AP1 might induce the expression of a cellular factor that optimizes HSV plaquing efficiency (Lamph *et al.*, 1988; Rauscher *et al.*, 1988).

How does ICP0 activate transcription? To investigate this question, Shapira *et al.* (1987) mapped the ICP0 response element in the gC gene. They located this element to -34 to +29 suggesting that either the TATA box or the 5' UTR (untranslated region) is required for ICP0 activation. Consistent with the possibility that ICP0 activates transcription through the 5' UTR, Ralph and

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Schaffer (1993) mapped ICP0 response elements to the 5' UTRs of the ICP0, ICP8, and UL10 genes. However, the ability of ICP0 to bind to TBP may suggest ICP0 activation also involves the TATA box (Yao and Schaffer, 1993). To understand how ICP0 activates transcription, several groups have identified critical regions required for its function. These investigations demonstrated that a cysteine/histidine rich region within amino acids 105-222 is required for transactivation in the presence or absence of ICP4 (Everett, 1988a; Everett, 1989; Chen *et al.*, 1991). This region bears some similarity to a Zn finger (reviewed by Berg, 1990), and has homology to several proteins that are thought to interact with DNA (Freemont *et al.*, 1991). Therefore it is not surprising that ICP0 binds Zn as well as single stranded and double stranded DNA-cellulose (Everett *et al.*, 1991b; Vaughan *et al.*, 1992). However, ICP0 does not bind to DNA in solution (Everett *et al.*, 1991b).

Mutational analysis has also been employed to understand synergistic activation by ICP0 and ICP4. These studies demonstrated that similar regions of ICP0 are required for transactivation in the presence or absence of ICP4; however, the sequences required for synergism with ICP4 are more extensive (Everett, 1987, 1988a). These results suggest that transactivation by ICP0 +/- ICP4 might be mechanistically similar. Several recent studies suggest that ICP0 may directly influence ICP4 function. For example, ICP0 influences the nuclear distribution of ICP4 and the mobility of ICP4 on an SDS polyacrylamide gel (Su and Knipe, 1989; Mullen and Hayward, 1993). Also, Yao and Schaffer (1993) demonstrated that ICP0 binds directly to ICP4. These results may or may not be relevant to the synergistic activation of gene expression by ICP4 and ICP0.

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and E gene expression is distinct from the requirement for true late gene expression.

ICP27 also regulates the expression of genes transiently transfected into cells. This approach however, has generated some contradictory results. Various investigations have shown that ICP27 downregulates the expression of reporter genes, driven by several HSV promoters, in the absence or presence of ICP4 and/or ICP0 (Block and Jordan, 1988; Sekilovich *et al.*, 1988; Hardwick *et al.*, 1989; Su and Knipe, 1989; McMahan and Schaffer, 1990). Other constructs containing reporter genes linked to HSV promoters were unaffected by ICP27 (Gelman and Silverstein, 1985; O'Hare and Hayward, 1985a; Everett, 1986; Mavromara-Nozos *et al.*, 1986; Rice and Knipe, 1988; Su and Knipe, 1989). In contrast, the expression of a reporter gene linked to the HSV alkaline exonuclease promoter is enhanced by ICP27 in the presence of ICP4, alone or in combination with ICP0 (Sekilovich *et al.*, 1988; Su and Knipe, 1989). The use of gB promoter/reporter genes in transient assays has led to some directly contradictory results. Sekilovich *et al.* (1988) demonstrated that the expression of a gB promoter/reporter gene is unaffected by ICP27 alone. Other studies suggest that a gB promoter/reporter gene is stimulated by ICP27 (Rice and Knipe, 1988; Su and Knipe, 1989). Yet another study demonstrated that ICP27 enhanced the activation of a gB promoter/reporter gene mediated by ICP4 or ICP0 but did not effect the activity of this construct on its own (McMahan and Schaffer, 1990). In contrast, Sekilovich *et al.* (1988) suggested that ICP27 inhibits the activation of a gB promoter/reporter gene mediated by a combination of ICP4 and ICP0. Use of VP5 promoter/reporter genes in transient assays has also generated contradictory results. Several studies have shown

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1.3.4 ICP27

ICP27 is a 63kDa nuclear phosphoprotein (Periera *et al.*, 1977; Hay and Hay, 1980; Ackermann *et al.*, 1984) required for viral replication (Sacks *et al.*, 1985; McCarthy *et al.*, 1989; Rice and Knipe, 1990). ICP27 is required for normal expression of viral genes in a lytic infection. For example, at the non-permissive temperature, ts mutants bearing lesions in ICP27 coding sequences overexpress both ICP4 and ICP27 mRNA while the levels of glycoprotein 3 and glycoprotein C mRNA are significantly reduced (Sacks *et al.*, 1985; Rice and Knipe, 1988; Smith *et al.*, 1992). The isolation of ICP27 null viruses demonstrated that ICP27 is required for: the expression of several true late proteins, the stimulation of leaky late protein expression, the downregulation of IE and E protein expression, and the stimulation of viral DNA synthesis (McCarthy *et al.*, 1989; Rice and Knipe, 1990). Some of these protein synthesis defects are reflected in altered mRNA levels. For instance, ICP27 mRNA (an IE mRNA) and ICP8 mRNA (an E mRNA) are overexpressed in an ICP27 null mutant whereas, ICP5 mRNA (a leaky late mRNA) is underexpressed and gC mRNA (a true late message) is undetectable (Rice and Knipe, 1990; Rice *et al.*, 1993). The phenotypes of several ICP27 mutant viruses (Sacks *et al.*, 1985; Rice and Knipe, 1990) suggest that ICP27 is a multifunctional protein consisting of several distinct regulatory domains. For example, mutagenesis has separated the ability of ICP27 to regulate leaky late gene expression from the activation of true late gene expression and both are separate from the ability of ICP27 to stimulate viral DNA synthesis. In addition, the downregulation of IE

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that a VP5 promoter/reporter gene is activated by ICP27 in the presence of ICP4 and ICP0 (Everett, 1986; Sekilovich *et al.*, 1988; Hardwicke *et al.*, 1989). In contrast, others have shown that a VP5 promoter/reporter gene is repressed by ICP27 in the presence of ICP4 or ICP0 (McMahan and Schaffer, 1990).

The potential basis for these conflicting results has recently emerged in reports by Sandri-Goldin and co-workers. In these experiments the cis-acting sequences required for ICP27-mediated activation and repression were determined. Initially, it was found that a CAT gene under the control of Moloney murine leukemia virus 5' and 3' LTR is activated by ICP27 (Chapman *et al.*, 1992). This activation is not dependent on the 5' LTR since CAT expression is also activated if the reporter gene is driven by the SV40 promoter. In contrast, if the 3' LTR is replaced by the SV40 polyadenylation signal, ICP27 does not activate CAT expression. These results demonstrate that ICP27 activates gene expression through sequences at the 3' end of the target gene. Further work has demonstrated that ICP27 activates gene expression through specific polyA signals while, the ability of ICP27 to repress gene expression requires introns within the target gene (Sandri-Goldin and Mendoza, 1992). These results suggest that ICP27 modulates gene expression at the level of splicing and polyadenylation. Therefore, previous results obtained from transient expression assays were likely influenced by the polyadenylation signals utilized by the reporter and the presence or absence of introns within the target gene. This might explain the contrary results obtained in transient assay for ICP27 function.

How are the activities that ICP27 displays in a transient transfection relevant to ICP27 function in virus? Experiments that utilized an ICP27 ts mutant suggest that ICP27 regulates viral gene expression at the post-transcriptional

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level (Smith *et al.*, 1992). This is consistent with the activities that ICP27 displays in transient assays. In addition, recent reports have identified an ICP27-dependent activity that stimulates utilization of L polyadenylation signals *in vitro* (McLauchlan *et al.*, 1989; McLauchlan *et al.*, 1992). This suggests that the ICP27 activation function identified in transient assays may mimic ICP27-dependent activation of late gene expression. In contrast, ICP27-mediated repression of IE and E genes during the lytic cycle might not relate to ICP27-mediated repression of spliced genes in transient assays. For example, in a lytic infection, most HSV genes repressed by ICP27 do not contain introns. Also, deletion of both introns from the ICP0 gene has no effect on ICP0 expression (Everett, 1991). Thus, ICP27 may not regulate the level of spliced viral mRNAs. However, ICP27 does inhibit the expression of several spliced cellular transcripts during lytic infection (Hibbard *et al.*, 1993). Therefore, this aspect of ICP27 function in virus might be relevant to ICP27-mediated repression of spliced mRNAs in transient assays.

Little is known about how ICP27 regulates the post-transcriptional processing of mRNA, however ICP27 does associate with both single stranded DNA and chromatin (Hay and Hay, 1980; Vaughan *et al.*, 1992). ICP27 also induces redistribution of small nuclear ribonucleoproteins that are required for splicing, and this re-organization may inhibit the expression of spliced mRNAs (Phelan *et al.*, 1993). Mutational analysis has demonstrated that the carboxy terminus of ICP27 is required for both the repression and activation functions of ICP27 in transient transfections and in virus (Hardwicke *et al.*, 1989). This region of the protein is a cysteine/histidine rich domain that binds Zn *in vitro* (Vaughan *et al.*, 1992), but the functional significance of this activity is unknown.

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cell types and this activity requires phosphorylation of ICP22 either directly or indirectly by the UL13 protein kinase.

1.4 Inhibition of Host Macromolecular Synthesis

HSV infection, like many other viral infections, induces a profound inhibition of host macromolecular synthesis (reviewed by Fenwick, 1984). Early studies demonstrated that HSV infection decreases the incorporation of tritiated uridine into rRNA, host RNA and total RNA (Roizman *et al.*, 1965; Hay *et al.*, 1966; Flanagan, 1967; Wagner and Roizman, 1969). HSV also initiates a decrease in protein synthesis early in infection, and this is paralleled by a breakdown in polysomes (Roizman *et al.*, 1965; Sydiskis and Roizman, 1966, 1967, 1968; Nishioka and Silverstein, 1977; Fenwick and Walker, 1978; Nishioka and Silverstein, 1978). In addition, HSV infection inhibits cellular translation (Spear and Roizman, 1968; Shimonon *et al.*, 1969; Honess and Roizman, 1973) and this effect is more potent with some strains of HSV-2 than with strains of HSV-1 (Powell and Courtney, 1975; Pereira *et al.*, 1977; Morse *et al.*, 1978; Fenwick *et al.*, 1979).

HSV infection also decreases the functional stability of host mRNA. In experiments where HSV-1 infected Vero cells were enucleated by treatment with cytochalasin B, the synthesis of host proteins was reduced relative to mock infected enucleated cells (Fenwick and Roizman, 1977). Subsequently, direct analysis confirmed that HSV destabilizes cellular mRNAs. Thus, HSV-1 induces degradation of pre-existing globin mRNA and inhibits globin protein synthesis in mouse erythroleukemia (MEL) cells (Nishioka and Silverstein,

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ICP27 also alters the electrophoretic mobility of ICP4 in both transient assays and during lytic infection (Su and Knipe, 1989; McMahan and Schaffer, 1990). Thus ICP27 might also modulate viral gene expression through ICP4.

1.3.5 ICP22

ICP22 is a 68kDa nuclear phosphoprotein that associates with cell chromatin and is required for normal expression of some viral genes in a cell type specific manner (Pierera *et al.*, 1977; Hay and Hay, 1980; Ackermann *et al.*, 1984). Intact ICP22 is not required for viral replication in tissue culture since a viral mutant expressing the amino terminal two-thirds of ICP22 is viable (Post and Roizman, 1981). This mutant exhibits a cell type specific growth impairment, especially at low MOI, which correlates with increased E protein expression and reduced L protein expression (Sears *et al.*, 1985). For example, the ICP22 mutant synthesizes normal amounts of gC, VP16, and ICP27 but the levels of US11, ICP0, and ICP35 proteins are reduced (Purves *et al.*, 1993). In addition, the reduction in US11 and ICP0 protein levels are reflected at the level of mRNA (Purves *et al.*, 1993). Evidence also suggests that ICP22 activity is modulated by the HSV-encoded protein kinase UL13, as a UL13 mutant and an ICP22 mutant exhibit similar phenotypes. For example, the UL13 mutant displays decreased quantities of US11 and ICP0 mRNA while gC, VP16, and ICP27 protein levels are normal (Purves *et al.*, 1993). In addition, Purves *et al.* (1992) demonstrated that ICP22 has an altered electrophoretic mobility in the UL13 mutant. This again suggests that UL13 might modulate ICP22 function. In summary, ICP22 optimizes the expression of a subset of HSV genes in some

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1977). Also, the relative concentration of globin mRNA to polyA⁺ mRNA is not affected by HSV infection (Nishioka and Silverstein, 1977). These data indicate that a general degradation of cellular mRNA occurs in HSV-1 infected MEL cells. In addition, Schek and Bachenheimer (1985) demonstrated that both HSV-1 and HSV-2 induce mRNA degradation in Vero cells. They also demonstrated that the HSV-2 mRNA degradation function is more potent than the HSV-1 function.

A large body of evidence suggests that a virion component(s) is, in part, responsible for HSV-mediated host shutoff. For example, HSV-2-induced polysome disaggregation, inhibition of host protein synthesis, and degradation of cellular mRNA do not require *de novo* viral gene expression in any cell type tested (Fenwick and Walker, 1978; Fenwick *et al.*, 1979; Hill *et al.*, 1983; Schek and Bachenheimer, 1985). Thus, all these phenomena are mediated by a virion component(s), and therefore they may be mechanistically linked. For example, an HSV-2 virion protein might trigger cellular mRNA degradation which in turn would inhibit cellular translation and induce polysome disaggregation. Although experiments have also demonstrated that HSV-1 carries a virion component(s) involved in host shutoff, results with HSV-1 have varied with the cell type employed. In Vero cells, HSV-1 inhibits host protein synthesis and induces mRNA degradation in the absence of viral gene expression (Fenwick *et al.*, 1979; Fenwick and Clark, 1982; Read and Frenkel, 1983; Schek and Bachenheimer, 1985). Therefore, in Vero cells an HSV-1 virion component(s) inhibits cellular translation and induces cellular mRNA degradation. Similarly, in MEL cells, HSV-1 infection inhibits cellular translation in the absence of viral gene expression (Nishioka and Silverstein, 1978; Hill *et*

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al., 1983). However, HSV-1-mediated degradation of cellular mRNA requires viral gene expression in MEL cells (Nishioka and Silverstein, 1978). Thus, in MEL cells a virion component inhibits cellular translation while a product expressed *de novo* is required to induce mRNA degradation. Together these results suggest that HSV-1-mediated host shutoff is mechanistically different in Vero cells and MEL cells, however the basis for this difference is currently not understood.

Several investigations suggest that HSV-1-induced host shutoff occurs in two distinct phases. For example, in MEL cells, UV irradiated HSV-1 induces polysome disaggregation and inhibits cellular translation but does not degrade cellular mRNA (Nishioka and Silverstein, 1977 and 1978). Thus, in MEL cells, the first phase of host shutoff (early shutoff) is mediated by a virion component(s) that induces polysome disaggregation and inhibits host translation. The second phase, referred to as late shutoff, requires viral gene expression and is responsible for mRNA degradation. A two phase shutoff system is also suggested by investigations that utilized a ts HSV-1 mutant, tsB7 (Fenwick and Clark, 1982). These experiments demonstrated that tsB7 contains two distinct ts lesions, one which impairs viral replication and another which impairs virion-induced inhibition of host translation. tsB7 also encodes a non-ts function, expressed *de novo* in the infected cell, that inhibits host protein synthesis. This investigation therefore suggests that HSV-1 contains two independent functions that inhibit cellular translation, one associated with the virus particle and one expressed *de novo* in the infected cell. Additional evidence for a two phase host shutoff effect was provided by Read and Frenkel

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thesis, an exhaustive discussion of these more recent findings is deferred to the Results and Discussion sections.

In addition to inhibiting host cell translation and degrading cellular mRNA, HSV infection also inhibits cellular transcription. Early reports demonstrated that HSV infection inhibits the incorporation of tritiated uridine in RNA (Roizman *et al.*, 1965; Hay *et al.*, 1966; Flanagan, 1967; Wagner and Roizman, 1969). Subsequent experiments demonstrated that HSV-1 infection of a polyoma virus transformed cell line decreases the incorporation of labeled uridine into polyoma specific RNA (Pizer and Beard, 1976). However, since this study employed 30 minute pulses the data does not conclusively demonstrate that HSV infection inhibits cellular transcription. More conclusive evidence was provided by Stenberg and Pizer (1982) who employed short pulses of tritiated uridine to examine the synthesis of adenovirus specific E1a and E1b mRNA in HSV-1 infected 293 cells. These experiments demonstrated that the transcription of adenovirus genes resident in 293 cells is suppressed by HSV infection. Stenberg and Pizer (1982) also demonstrated that the HSV-1 ts mutant, tsK, inhibits adenovirus specific transcription in 293 cells at the non-permissive temperature. Since tsK expresses only IE proteins at the non-permissive temperature, these results suggest that a component of the virion and/or an HSV IE protein are responsible for this inhibition. Other work suggests that ICP22 (an HSV IE protein) is required to inhibit the transcription of some cellular genes as assayed in nuclear run-on experiments (Kemp and Latchman, 1988). In summary, the above results suggest that an HSV IE protein may induce a general inhibition of cellular transcription.

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(1983) who isolated an HSV-1 KOS mutant, designated vhs1 (virion host shutoff). This mutant (discussed in more detail below) is defective for the inhibition of host translation in the absence of viral gene expression but can inhibit cellular translation if viral genes are expressed. Thus the results of Fenwick and Clark (1982) and Read and Frenkel (1983) argue that a component of the virus particle and a viral product expressed *de novo*, inhibit cellular translation.

Isolation of an HSV-1 mutant defective for virion-induced inhibition of host translation has proven an important step towards understanding HSV-mediated host shutoff. This mutant, designated vhs1, is viable, and thus virion-mediated inhibition of host translation is not required for viral replication in tissue culture (Read and Frenkel, 1983). Using marker rescue, the vhs1 mutation was mapped to the UL41 open reading frame of the viral genome. This open reading frame is predicted to encode a 55kDa protein (Frink *et al.*, 1981; Kwong *et al.*, 1988; McGeoch *et al.*, 1988). Recent studies have shown that the UL41 open reading frame is required for virion-induced mRNA degradation (Kwong and Frenkel, 1987; Strom and Frenkel). In addition, the UL41 product destabilizes both cellular and viral mRNAs (Kwong and Frenkel, 1987; Oroskar and Read, 1987; Strom and Frenkel, 1987; Oroskar and Read, 1989). Separate investigations by Fenwick and co-workers have provided evidence that the vhs activity of virions is down-regulated during infection (Fenwick and Owen, 1988; Fenwick and Everett, 1990 a,b). Taken in combination, these data imply that modulation of mRNA stability by the vhs function plays an important role in the HSV regulatory cascade. Because these reports were published while the present work was in progress, and bear directly on the issues addressed in this

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1.5 Project Rationale

When this project was initiated, it was clear that the HSV genetic program involved activation of viral gene expression and a dramatic inhibition of cellular gene expression. Although several viral regulatory proteins that drive this program had been identified, the basis for preferential expression of viral genes was not well understood. The activation of IE gene expression by VP16 did exhibit sequence-specific transactivation that would favor viral IE transcription. However, no virus specific sequence elements that mediate activation by HSV regulatory proteins have been identified in either HSV E or L promoters. These results therefore exclude a straight forward explanation for the selective activation of most HSV genes.

Previous experiments from this lab investigated the preferential expression of HSV genes by inserting a copy of the rabbit β -globin gene into the HSV genome (Smiley *et al.*, 1987). When this recombinant, designated L7/14, was used to infect Vero cells, the inserted β -globin gene was efficiently expressed, under the control of its own promoter, as a viral early gene. These results suggest that the preferential expression of viral genes does not rely on sequence-specific differentiation between viral and cellular promoters or mRNAs. However, the expression of the rabbit β -globin gene borne by L7/14 was surprising as HSV inhibits the expression of the mouse β -globin gene resident in MEL cells (Nishioka and Silverstein, 1977, 1978). To explore this apparent contradiction, I have characterized the expression of the rabbit and mouse β -globin genes in L7/14 infected MEL cells. In these experiments, L7/14 inhibited expression of the endogenous mouse β -globin gene while HSV

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products induced expression of the transduced rabbit β -globin gene. These results provide a dramatic demonstration that HSV products can have opposing effects on the expression of homologous genes located in the cellular and viral genomes. These results also confirm that the preferential expression of viral genes does not rely on sequence-specific differentiation between viral and cellular promoters or mRNAs.

The work presented in this thesis illustrates that preferential expression of HSV genes involves selective inhibition of cellular gene expression and the preferential activation of genes associated with the viral genome. The major goal of this work was to understand the molecular processes that underlie the differential behavior of viral and cellular genes in HSV infected cells. To this end, I investigated the HSV-mediated inhibition of mouse β -globin gene expression in MEL cells. I have determined that mouse β -globin gene expression is inhibited by two processes: the inhibition of mouse β -globin gene transcription and the degradation of pre-existing cytoplasmic mouse β -globin mRNA. Further investigations revealed that degradation of mouse β -globin mRNA required the HSV virion host shutoff function (vhs). I have confirmed that the vhs function is encoded by the UL41 open reading frame and have identified the vhs (UL41) protein as a 58kDa virion phosphoprotein.

As discussed above, the vhs function is thought to degrade both viral and cellular mRNAs. This lack of specificity suggests that viral mRNAs might only accumulate if they are spared the full force of the vhs effect. This point is especially relevant as the vhs function imposes a very short half-life on cellular mRNAs. The work of Fenwick *et al* suggests that a viral protein downregulates the vhs function (Fenwick and Owen, 1988; Fenwick and Everett, 1990 a,b).

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Materials and Methods

2.1 Bacterial Cultures

Liquid cultures of *E. coli* strains HB101 and MV1190 were grown in either Luria Broth (1% bactotryptone-0.5% yeast extract-85.5 mM NaCl-10 mM Tris-HCl (pH 7.6)-0.4% glucose) or terrific broth (1.2% bactotryptone-2.4% yeast extract-0.4% glycerol-88.7 mM potassium phosphate (7.4)) at 37°C under continuous agitation. For growth of HB101 on solidified medium, LB supplemented with 1.5% bactoagar was used and plates were incubated at 37°C. Identical culture conditions were used for *E. coli* strain N4830-1 with the exception that all incubations were carried out at 30°C. Where appropriate both solid and liquid media were supplemented with 40 μ g/mL of ampicillin.

2.2 Manipulation and cloning of plasmid DNA

All of procedures used in the manipulation and cloning of plasmid DNA, including restriction enzyme digestion, agarose gel electrophoresis, and ligation of DNA fragments, are described in Sambrook *et al.* (1989).

2.3 Ethanol precipitation of nucleic acids

RNA and DNA was precipitated from the appropriate salt solution (0.3 M sodium acetate (pH 5.2) or 0.15 M NaCl) by the addition of 2.5 or 3 volumes of ethanol, for DNA and RNA respectively. Large volumes of precipitate (greater than 1.5 mL) were collected by centrifugation at 12,000 rpm in a Beckman JA-

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Thus the action of this protein might dampen the vhs effect and allow for the accumulation of viral mRNAs. This downregulator would therefore play a pivotal role in selectively inhibiting the expression of cellular genes. To identify the vhs downregulator, I have searched for HSV proteins that bind to the vhs protein. These investigations have demonstrated that the HSV transcription factor, VP16, binds to the vhs protein and evidence presented here suggests that VP16 downregulates the vhs function. Based on the results presented here, I propose a model involving downregulation of the vhs function by VP16, to explain the preferential expression of genes associated with the HSV genome.

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20 rotor at 4°C for 20 min. For smaller volumes the precipitate was collected by centrifugation in a microcentrifuge (Eppendorf) at maximum speed for 15 min. After centrifugation, samples were generally washed with 70% ethanol followed by 95% ethanol and dried in a Speed-Vac lyophilizer (Savant).

2.4 Preparation of competent *E. coli*

Fifty mL log phase cultures of *E. coli* strain HB101 were pelleted by centrifugation at 2800 rpm for 10 min at 4°C in an IEC table top centrifuge. The resulting pellet was resuspended in 20 mL of 50 mM 3-[N-morpholino] propane-sulphonic acid (MOPS) (pH 7.0)-10 mM RbCl. Bacterial cells were then recentrifuged and the pellet was resuspended in 20 mL of 50 mM MOPS (pH 6.5)-70 mM CaCl₂-10 mM RbCl. After a final centrifugation, the bacterial pellet was resuspended in 2 mL of 50 mM MOPS (pH 6.5)-70 mM CaCl₂-10 mM RbCl₂ and stored at 4°C for future use. Transformation of competent *E. coli* was achieved by incubation of 100 μ L of competent cells with 100 ng of plasmid DNA or a 20 μ L ligation reaction (containing 100 ng of vector) at 4°C for 30 min. After heat shocking this mixture at 42°C for 2 min, transformants were selected by plating the transformation mixture on the appropriate selective plates.

2.5 Small scale preparation of plasmid DNA

Small scale isolation of plasmid DNA was performed using a modification of the technique described by Birnboim and Doly (1979). 1.5 mL saturated cultures of *E. coli* were pelleted in a microcentrifuge (Eppendorf) at maximum

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speed for 30 seconds. The bacterial pellet was resuspended in 150 μ L of 50 mM Tris-HCl (pH 8.0)-10 mM EDTA and the cells were lysed by the addition of 150 μ L of 1% SDS-0.2 mM NaOH. Protein and chromosomal DNA were removed by the addition of 150 μ L of 3 M potassium acetate (pH 5.5) and subsequent centrifugation in a microcentrifuge (Eppendorf) at maximum speed for 15 min. The supernatant was extracted with phenol-chloroform (prepared as described in Sambrook et al., 1989), and the plasmid DNA was precipitated with ethanol. The plasmid was then redissolved in 64 μ L of H₂O containing 20 μ g of RNase A and generally 16 μ L of this was suitable for restriction enzyme analysis.

2.6 Large scale purification of plasmid DNA

Large scale purification of plasmid DNA was achieved using either Quiagen columns as per the manufacturer's instruction or the LiCl/polyethyleneglycol method. For purification using the LiCl/polyethyleneglycol method, saturated 1 L cultures of *E. coli* were pelleted by centrifugation at 5000 rpm in a JA10 rotor (Beckman) for 15 min at 4°C. The bacterial pellet was then resuspended in 20 mL of 25 mM Tris-HCl (pH 8.0)-50 mM glucose-10 mM EDTA-2 mg/mL lysozyme and incubated for 5 min at room temperature. The cells were then lysed by the addition of 40 mL of 1% SDS-0.2 M NaOH and incubation on ice for 10 min. Protein and chromosomal DNA were removed by the addition of 30 mL of 3 M potassium acetate (pH 5.5) and subsequent incubation for 10 min on ice. Debris was then removed by centrifugation at 15,000 rpm in a Beckman JA-20 rotor at 4°C for 15 min. After warming to room temperature, the nucleic

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HSV genes were used in this work including 16-8 cells (Weinheimer, et al., 1992) which contain the HSV-1 VP16 gene, E5 cells (DeLuca et al., 1985) which contain the HSV-1 ICP4 gene, and VD60 cells (Ligas and Johnson, 1988) which contain the HSV-1 gD gene. These transformed lines were also maintained in complete α -MEM. Mouse L cell lines 1A and 4 (Friedman et al., 1988) were maintained in Dulbecco's modified Eagles medium supplemented with 2mM L-glutamine (Gibco)-100 U/mL penicillin G (Gibco)-100 μ g/mL streptomycin sulphate (Gibco)-1.36 mg/mL hypoxanthine-0.44 μ g/mL methotrexate-9.7 μ g/mL thymidine. All Vero cell lines and mouse cell lines were also supplemented with 5% and 10% fetal bovine serum (FBS) (Bocknek) respectively and maintained at 37°C in a humidified atmosphere supplemented with 5% CO₂. Vero cells and mouse L cells were maintained in 150 cm² tissue culture flasks (Corning) containing 25 mL of medium while MEL cells were maintained in 500 mL Erlenmeyer flasks containing 200 mL of medium.

2.8 Propagation and titration of viral strains

HSV-1 strains KOS, and F, and the HSV-2 strain 333 were propagated on monolayers of Vero cells. The HSV-1 mutants PAA'5 (Hall et al., 1984), L7/14 (Smiley et al., 1987), vhs1 (Read and Frenkel, 1983), hrR3 (Goldstein and Weller, 1988a) were also propagated on monolayers of Vero cells. The HSV-1 mutants d120 (DeLuca et al., 1985), FgDb (Ligas and Johnson, 1988), and 8MA (Weinheimer, et al., 1992) were propagated on E5 cells, VD60 cells, and 16-8 cells respectively. Ten to twenty confluent 150 cm² flasks were infected with 10⁶ PFU/flask in 10 mL of complete α -MEM and 2 hours post-inoculation

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acids present in the supernatant were precipitated by the addition of 0.6 volumes of isopropanol and the precipitate was collected by centrifugation at 3400 rpm for 30 min at room temperature in an IEC table top centrifuge. The precipitate was then redissolved in 5 mL of H₂O and RNA was precipitated by the addition of 5 mL of 5 M LiCl. The RNA precipitate was removed by centrifugation at 12,000 rpm in a Beckman JA-20 rotor at 4°C for 15 min. The supernatant was precipitated with ethanol and the resulting pellet was redissolved in 500 μ L of H₂O. Residual RNA was removed by the addition of RNase A to a final concentration of 50 μ g/mL and incubation at 37°C for 15 min. Plasmid DNA was then precipitated by the addition of an equal volume of 13% polyethyleneglycol-1.6 M NaCl and the precipitate was collected by centrifugation in a microcentrifuge (Eppendorf) at maximum speed for 15 min. The pellet was then resuspended in 500 μ L of H₂O, extracted with phenol-chloroform, and subsequently precipitated with ethanol. The DNA was then dissolved in H₂O and quantified by determining absorbance at 260 nm in an Ultraspec II spectrophotometer (Pharmacia LKB).

2.7 Mammalian Cell Culture

African green monkey kidney (Vero) cells and mouse erythroleukemia (MEL) cells (line 745aJG6, provided by A. Bernstein, Mount Sinai Research Institute, Toronto, Ontario, Canada) were maintained in complete α -MEM which consists of α -minimal essential medium (α -MEM) (Gibco) supplemented with 2mM L-glutamine (Gibco)-100 U/mL penicillin G (Gibco)-100 μ g/mL streptomycin sulphate (Gibco). Several Vero cell lines that were transformed with various

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infections were overlaid with 15 mL of complete α -MEM supplemented with 10% FBS. After the majority of the cells exhibited total cytopathic effect (CPE), they were pelleted by centrifugation at 2800 rpm for 10 min at 4°C in an IEC table top centrifuge. The cell pellet was resuspended in 1 mL of complete α -MEM per 150 cm² flasks and cells were then disrupted with a Braun-Sonic 2000 sonicator. Cell debris was removed by centrifugation at 3000 rpm for 15 min in an IEC table top centrifuge at 4°C. 0.25-0.5 mL aliquots of the supernatant were then stored in cryovials (Nunc) at -70°C.

Virus stocks were titrated using ten-fold dilutions ranging from 10⁻³ to 10⁻⁸ prepared in complete α -MEM. 0.8 mL of each dilution was used to infect 35 mm confluent dishes of the appropriate cell line and 2 hours post-inoculation infections were overlaid with 2 mL of complete α -MEM supplemented with 10% FBS and 0.05% human immune globulin (Connaught Laboratories). After plaques developed, monolayers were stained with 1% crystal violet in 61% ethanol-8.7% formalin-4.3% acetic acid and plaques were counted using a light microscope.

2.9 Infection of Vero cells and L cells

In experiments involving infection of Vero cells and L cells, infections were carried out in 10 mL, 3 mL, 1 mL, and 0.5 mL of complete α -MEM for 150 mm dishes (Nunc), 100 mm dishes (Corning), 60 mm dishes (Corning), 35 mm dishes (Corning), respectively. One and one half hours after inoculation cells were overlaid with complete α -MEM supplemented with 10% FBS (the approximate volume of overlay medium was 2 fold more than the initial

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inoculum). Where indicated, cells were maintained in 10 µg/mL of actinomycin D, 100 µg/mL of cycloheximide, or 200 µg/mL of phosphonoacetic acid (PAA) 20 min prior to and throughout the infection.

2.10 Induction and infection of MEL cells

MEL cells were diluted to a density of 2×10^4 cells/mL and when the cells reached a density of 1.5×10^5 cells/mL terminal erythroid differentiation was induced by the addition of hexamethylene bisacetamide (HMBA) to a final concentration of 5 mM. Three and one half days post-induction cells were pelleted by centrifugation at 2800 rpm for 10 min at 4°C in an IEC table top centrifuge, resuspended in complete α -MEM and recentrifuged. Cells were then resuspended to a final concentration of 2×10^7 cells/mL in complete α -MEM and infected with virus. One and one half hours post-inoculation cells were diluted to a concentration of 1×10^6 cells/mL with complete α -MEM supplemented with 10% FBS. The same procedure was followed for infection of undifferentiated MEL cells, omitting the HMBA inducer. Where indicated, cells were maintained in 10 µg/mL of actinomycin D or 100 µg/mL of cycloheximide, 20 min prior to and throughout the infection.

2.11 RNA extraction

Cytoplasmic RNA for primer extension, S-1 nuclease analysis, and Northern blots was prepared by the method of Berk and Sharp (1977). Wherever possible, solutions used in the extraction and analysis of RNA were treated with

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microcentrifuge (Eppendorf) for 15 min. After removal of the supernatant, 0.3 mL of elution buffer was mixed with the acrylamide pellet and the sample was recentrifuged. The supernatants were pooled and spun again to remove any residual acrylamide. The DNA was precipitated from the supernatant with ethanol, dissolved in 0.4 mL of 0.3 M sodium acetate (pH 5.2), then reprecipitated with ethanol.

2.13 S-1 nuclease analysis

A modification of the procedure of Berk and Sharp (1977) was used to quantify levels of mouse and rabbit β -globin mRNA. DNA probes were 5' end labeled with $\gamma^{32}\text{P}$ -ATP and strand separated as described below. RNA was initially precipitated from 0.4 mL of 0.3 M sodium acetate with ethanol and resuspended in 30 µL of 0.4 M NaCl-40 mM Pipes (pH 6.4)-10 mM EDTA-50% recrystallized, deionized formamide. After the addition of the appropriate single stranded ^{32}P 5' end labeled probe (10,000-30,000 Cerenkov counts per minute), RNA was hybridized at 42°C for 8 to 16 hours. Following hybridization, 0.3 mL of ice cold 150 mM NaCl-50 mM sodium acetate (pH 4.6)-5 mM ZnSO_4 -70 units of S-1 nuclease (BMC) was added and the mixture was incubated at 37°C for 1 h. The reaction was then stopped by the addition of 50 µL of 4 M ammonium acetate and 30 µL of 0.2 M EDTA followed by extraction with phenol-chloroform. The aqueous phase was then precipitated with ethanol, resuspended in 300 µL of 0.3 M sodium acetate and reprecipitated with ethanol. The resulting pellet was resuspended in 10 µL of sequencing gel loading dye, boiled for 2 min, and resolved on a 1xTBE-8% urea sequencing gel. The

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diethylpyrocarbonate and autoclaved prior to use. 150 mm dishes of Vero cells were washed with phosphate buffered saline (PBS), trypsinized with 1 mL of 100x trypsin/EDTA (Gibco), diluted in PBS, and pelleted by centrifugation at 2800 rpm in an IEC table top centrifuge for 10 min at 4°C. MEL cells were pelleted by centrifugation at 2800 rpm for 10 min at 4°C, resuspended in PBS, and repelleted. Cell pellets (containing 1×10^7 - 2×10^7 cells) were resuspended in 0.5 mL of ice cold isotonic buffer (0.15 M NaCl-10 mM Tris-HCl (pH 7.8)-1.5 mM MgCl_2) and lysed by the addition of 0.5 mL of ice cold isotonic buffer plus 2% NP40. After mixing, cells were incubated on ice for 5 min and nuclei were pelleted by centrifugation at 5000 rpm in a Beckman JA-20 rotor at 4°C for 5 min. The supernatant was removed and combined with an equal volume of urea/SDS (7M urea-0.35 M NaCl-0.01 M Tris-HCl (pH 7.8)-1% SDS). Protein was removed by extensive extraction with phenol-chloroform. The aqueous phase from the final phenol-chloroform extraction was precipitated with ethanol and the RNA pellet was washed four times with ethanol/0.4 M sodium acetate (2.5:1), dried under vacuum, and resuspended in 0.5 mL of H_2O . RNA was subsequently quantified by measuring absorbance at 260 nm in an Ultraspec II spectrophotometer (Pharmacia LKB).

2.12 Elution of DNA fragments from polyacrylamide gels

DNA bands were excised from polyacrylamide gels, mashed with a teflon plunger, and incubated with 0.6 mL of elution buffer (500 mM ammonium acetate-10 mM magnesium acetate-1 mM EDTA-0.1% SDS) at 37°C for 12 to 16 h. The acrylamide was removed by centrifugation at maximum speed in a

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digestion products were visualized by exposure of the gel to XAR5 x-ray film (Kodak) at -70°C with intensifier screens.

The mouse β -globin probe was prepared using the 152 bp Hae III to Dde I fragment spanning the mouse β -globin transcription start site. This fragment was dephosphorylated with calf intestinal alkaline phosphatase (Sambrook et al., 1989) and labeled with $\gamma^{32}\text{P}$ -ATP (10 mCi/mL, 3000 Ci/mmol, New England Nuclear) and T4 polynucleotide kinase (New England Biolabs). The two strands were denatured by boiling for 2 min in sequencing gel loading dye (80% deionized recrystallized formamide, 1x TBE (0.089 M Tris-borate-0.089 M boric acid-0.002 M EDTA), 0.1% bromophenol blue, and 0.1% xylene cyanol) and separated on a 1xTBE-8% urea sequencing gel. The slower migrating band, which represents the mRNA complementary strand, was eluted from the gel and used as the probe fragment. This probe was hybridized to 1 µg of cytoplasmic RNA from MEL cells or 10 µg of cytoplasmic RNA harvested from Vero cells infected with the βM recombinant.

The rabbit β -globin probe was a 320 bp Bst NI (previously described by Everett, 1983) which was dephosphorylated with calf intestinal alkaline phosphatase (Sambrook et al., 1989) and labeled with $\gamma^{32}\text{P}$ -ATP (10 mCi/mL, 3000 Ci/mmol, New England Nuclear) and T4 polynucleotide kinase (New England Biolabs). The two strands of this probe were separated by melting the double stranded fragment with NaOH at a final concentration of 0.17 M followed by electrophoresis on a 1xTBE-8% polyacrylamide gel (prepared from an 40% acrylamide-0.8% bisacrylamide stock solution). The faster migrating of the two fragments, which represented the mRNA complementary strand was purified

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from the gel and used as the probe fragment. This probe was hybridized to 10 µg of RNA harvested from Vero cells infected with the L7/14 recombinant.

2.14 Primer extension analysis

Primer extension analysis (Jones et al., 1985) employed the following synthetic oligonucleotides purchased from the Central Facility of the Institute for Molecular Biology, McMaster University: gD, 5'-CCCCATACCGGAACGCACCACACAA-3' (Watson et al., 1983), predicted extension product of 80 to 90 nucleotides; rabbit β-globin, 5'-CCGCAGACTTCTCCTCACTGGACAG-3', predicted extension product of 87 nucleotides (Efstratiades et al., 1977); mouse β-globin, 5'-AAGAGAACAGCAGCCTTCTCAGCATC-3', predicted extension product of 92 nucleotides (Konkel et al., 1978). Typically, 40 ng of oligonucleotide was labeled for 1 h at 37°C with 5 µL of γ³²P-ATP (10 mCi/mL, 3000 Ci/mmol, New England Nuclear) using 20 units of T4 polynucleotide kinase (New England Biolabs) and the buffer supplied with the enzyme in a final volume of 50 µL. Unincorporated isotope was removed by repeated ethanol precipitation and the labeled oligonucleotide was resuspended in 100 µL of H₂O. 10 µg RNA was precipitated from 0.4 mL of 0.3 M sodium acetate with ethanol, dried for 1 hour under vacuum, and resuspended in 8 µL of 10 mM Tris-HCl (pH 7.9)-1 mM EDTA containing approximately 10⁵ Cerenkov counts per minute of ³²P 5' end labeled oligonucleotide. After the addition of 2 µL of 10 mM Tris-HCl (pH 7.9)-1 mM EDTA-1.25 M KCl, the reaction was hybridized for 1 h at 60°C and then allowed to cool to room temperature. Reverse transcription was carried out at

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resuspended in 100 µL of nuclear storage buffer (25% glycerol-5.0 mM magnesium acetate-0.1 mM EDTA-5.0 mM DTT-50.0 mM Tris-HCl (pH 8.0)-400 units/mL RNasin) and stored in liquid nitrogen.

For run-on transcription assays, 10⁷ nuclei in 100 µL of nuclear storage buffer were incubated at 30°C for 35 min with 30 µL of 3.3 mM each of ATP, CTP, and GTP, 40 µL of 0.6 M KCl-12.5 mM magnesium acetate, 25 µL of α-³²P-UTP (10 mCi/mL, 3000 Ci/mmol, New England Nuclear), and 5 µL of H₂O. The reaction was terminated by the addition of DNase I to a final concentration of 20 µg/mL and 100 µg of *E. coli* tRNA, followed by incubation at 37°C for 10 min. Tris-HCl (pH 7.8), EDTA, SDS, and protease K were then added to final concentrations of 10 mM, 5 mM, 0.5%, and 300 µg/mL respectively, and the reaction was incubated at 37°C for 20 min. At this point a 10 µL aliquot of each reaction was precipitated in 1 mL of 5% trichloroacetic acid (TCA)-30 mM sodium pyrophosphate and the precipitate was collected onto nitrocellulose filters and washed extensively with 5% TCA-30 mM sodium pyrophosphate. These filters were then dried and Cerenkov counts were determined in a Beckman scintillation counter. This value was used to determine the relative rates of transcription in each sample; typically 1x10⁷ cpm were incorporated per 10⁷ nuclei, with infected and uninfected samples giving similar rates of incorporation. The remainder of the reaction was extracted with phenol-chloroform and TCA precipitated onto nitrocellulose filters as described above. RNA was eluted from the nitrocellulose filters by incubation in 1 mL of 1% SDS-10 mM Tris-HCl (pH 7.5)-5 mM EDTA for 10 min at 65°C. The elution was repeated and the elutes were pooled, extracted with phenol-chloroform, and ethanol precipitated.

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37°C for 1 h after the addition of 25 µL of 20 mM Tris-HCl (pH 8.7)-10 mM MgCl₂-5 mM DTT-0.33 mM of each dNTP-10 µg/mL actinomycin D-10 units of AMV reverse transcriptase (Life Sciences). The reaction was terminated by the addition of 300 µL of 95% ethanol and the resulting ethanol precipitate was collected by centrifugation. The pellet was resuspended in 10 µL of sequencing gel loading dye, boiled for 2 min, and 3 µL of the sample run on a 1xTBE-8% urea sequencing gel. The extension products were then visualized by exposure of the gel to XAR5 x-ray film (Kodak) at -70°C with intensifier screens.

2.15 Nuclear run-on assay

The isolation of nuclei, run-on transcription, and hybridization of transcripts to single-stranded DNA were performed as previously described by Marzluff and Huang, (1984) with some modifications, while the isolation of run-on RNA was performed as described in Greenberg and Bender (1989). All solutions and equipment used in the isolation of nuclei were pre-cooled to 4°C before use. 5 x 10⁷ MEL cells were first pelleted by centrifugation at 2800 rpm in an IEC table top centrifuge and resuspended in 1.0 mL of buffer I (0.32 M sucrose-3.0 mM CaCl₂-2.0 mM magnesium acetate-0.1 mM EDTA-0.1% triton X-100-1.0 mM DTT-10 mM Tris-HCl (pH 8.0)-40 units/mL RNasin (Promega Biotech)). The cells were lysed with 10 strokes of a Dounce homogenizer using a pestle B and subsequently the cells were diluted with 2 mL of buffer II (2.0 M sucrose-5.0 mM magnesium acetate-0.1 mM EDTA-1.0 mM DTT-10.0 mM Tris-HCl (pH 8.0)-40 units/mL RNasin). The cell lysate was then layered over 1.8 mL of buffer II and spun at 37,000 rpm for 45 min in an SW50.1 rotor. The nuclear pellet was

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The transcription of mouse β-globin and HSV tk genes were detected by hybridization of labeled RNA to single-stranded M13 probes immobilized to nitrocellulose filters. The mouse β-globin probe consisted of a 3.5 kbp Eco RI-Xba I fragment containing the entire mouse β-globin gene and 1.0 kbp of 5'-flanking sequences cloned between the Eco RI and Xba I sites of M13mp19. The HSV tk probe consisted of a 1.3 kbp Eco RI-Sma I fragment of HSV-1 KOS DNA containing 80 bp of 5'-flanking sequences inserted between the Eco RI and Sma I sites of M13mp19. The transcription of HSV ICP0 gene was detected by hybridization of labeled RNA to the denatured plasmid pIGA15 (Gelman and Silverstein, 1985). The plasmid was denatured by the addition of NaOH to a final concentration of 0.2 M and incubation at room temperature for 5 min. The NaOH was neutralized by the addition of sodium acetate (pH 5.2) to a final concentration of 0.3 M.

Single stranded M13 DNA was harvested from the supernatant of *E. coli* strain MV1190 infected with the appropriate M13 recombinant. One liter of infected culture was centrifuged at 5000 rpm for 15 min in a Beckman JA10 rotor and the phage in the supernatant were precipitated by the addition of 250 mL of 20% polyethylene glycol-2.5 M NaCl. After incubation for 15 min at room temperature, the phage precipitate was pelleted by centrifugation at 8000 rpm for 20 min in a Beckman JA10 rotor. The phage pellet was resuspended in 24 mL of 10 mM NaCl-10 mM Tris-HCl (pH 7.5)-1 mM EDTA and reprecipitated by the addition of 3 mL of 20% polyethylene glycol-2.5 M NaCl. After incubation at room temperature for 15 min, the precipitate was collect by centrifugation at 12,000 rpm in a Beckman JA20 rotor for 15 min. The phage pellet was resuspended in 5 mL of 0.1 M NaCl-8 mM MgSO₄-50 mM Tris-HCl (pH 7.5)-

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0.1% gelatin. After the addition of solid KCl to a final concentration of 1 M, the solution was incubated on ice for 30 min. The resulting precipitate was removed by centrifugation at 12,000 rpm in a JA20 rotor and the supernatant was mixed with an equal volume of urea/SDS. After exhaustive phenol-chloroform extraction, the aqueous phase was precipitated with ethanol and DNA yields were quantified by determining absorbance at 260 nm in a Ultraspec II spectrophotometer (LKB).

Slot-blots bearing immobilized DNA were prepared as follows: 20xSSC (1xSSC is 0.15 M NaCl and 0.015 M sodium citrate) was added to both denatured plasmid DNA and single stranded M13 DNA to a final concentration of 6x and 10 µg per slot was applied to nitrocellulose filters. The DNA was immobilized by baking the filters at 80°C for 2 h, or by UV crosslinking using a Stratalink 2400 (Stratagene) as per the manufacturer's instructions. The filters were prehybridized and hybridized in 5xSSC-0.1% SDS-1 mM EDTA-10 mM Tris-HCl (pH 7.5)-0.02% Ficoll-0.02% polyvinylpyrrolidone-0.02% bovine serum albumin (BSA)-250 µg/mL of *E. coli* tRNA-250 µg/mL of salmon sperm DNA-50% formamide. Following prehybridization for 5 h and hybridization for 36 h (both at 52°C), filters were washed extensively in 2xSSC-0.1% SDS at 68°C and treated with 10 µg/mL of RNase A for 20 min at 37°C, followed by further washes at room temperature with 2xSSC-0.1% SDS. Filters were then air dried and exposed to Kodak XAR5 film with intensifier screens at -70°C.

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2.17 Preparation of infectious viral DNA

HSV recombinants were constructed using methods described in Smiley (1980) and Smiley et al. (1981). Infectious HSV DNA was prepared from 10 150 cm² tissue culture flasks infected at a MOI of 10 PFU/cell. After complete CPE was observed (24-48 h post-infection), cells were pelleted by centrifugation in an IEC table top centrifuge at 2800 rpm for 10 min at 4°C and lysed by the addition of 10 mL of 0.2 M EDTA (pH 8.0)-0.5% SDS. Protein was removed from the extracts first by treatment with 100 µg/mL of proteinase K for 14 h at 37°C followed by extraction with phenol. Finally, the extract was exhaustively dialyzed at 4°C against 0.1xSSC and the infectious DNA was stored at 4°C.

2.18 Transfection of infectious DNA and isolation of recombinant viruses

For the rescue of constructs into the viral genome, 1 µg of the appropriate plasmid was combined with 20 to 40 µL of infectious HSV DNA, 250 µL of 1% HEPES-1.6% NaCl-0.07% KCl-0.02% Na₂HPO₄-0.2% dextrose-(pH 7.1), and 10 µg of salmon sperm DNA in a final volume of 475 µL. Calcium phosphate precipitates were formed by the addition of 25 µL of 2.5 M CaCl₂ followed by incubation at room temperature for 20 min. The precipitate was then added to 35 mm dishes of subconfluent monolayers of Vero cells and 4 to 6 h later the precipitate was removed and the cells were treated for 1 min with 1.0 mL of 20% glycerol in complete α-MEM supplement with 10% FBS. Cells were then washed extensively and returned to an incubator. After complete CPE was

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2.16 Northern blots

For analysis of RNA by Northern blots 10 µg/lane of cytoplasmic RNA in 4.5 µL of H₂O was mixed with 2 µL of 10x MOPS running buffer (0.2 M MOPS (pH 7.0)-50 mM sodium acetate-10 mM EDTA), 3.5 µL of 37% formaldehyde, and 10 µL of formamide, followed by incubation at 65°C for 15 min. After the addition of 4 µL of RNA loading buffer (1 mM EDTA (pH 8.0)-0.25% bromophenol blue-0.25% xylene cyanol-50% glycerol), the samples were electrophoresed through a 1.0% agarose gel containing 1.1% formaldehyde and 1x MOPS running buffer. After electrophoresis, the gel was soaked for 30 min in 20xSSC and transferred to nitrocellulose using a Stratagene Pressure Blotter as per the manufacturer's instructions. The RNA was cross-linked to the nitrocellulose by UV crosslinking using a Stratalink 2400. The oligonucleotide, 5'-CGGCTGCAGTCCGGGGCGCAACT-3', was purchased from the Central Facility of the Institute for Molecular Biology, McMaster University and is complementary the HSV-1 glycoprotein B mRNA sequences extending from +35 to +60. This oligonucleotide was end labeled with γ³²P-ATP as described above, and hybridized to the filter in 250 mM sodium phosphate (pH 7.2)-7% SDS-1% BSA-1 mM EDTA for 8 to 16 h at 60°C. The unhybridized probe was removed by washing the blot twice in 2x SSC-0.1% SDS at 60°C and twice in 0.1x SSC-0.1% SDS also at 60°C. The results of the blot were then visualized and quantified using a phosphorimager (Molecular Dynamics) and imagequant software.

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observed (5 to 7 days later), cells were harvested and disrupted with a Braun-Sonic 2000 sonicator. The recombinant carrying the mouse β-globin gene was plaque purified and structure was verified by Southern blotting of viral DNA. Recombinants carrying the lacZ-ICP6 cassette were identified by overlaying isolated plaques with F11 medium (Gibco) supplemented with 2mM L-glutamine (Gibco), 100 U/mL penicillin G (Gibco), 100 µg/mL streptomycin sulphate (Gibco), 5% FBS, 300 µg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and 0.5% agarose. Blue plaques, which developed 4 to 16 h after overlay, were plaque purified based on the blue plaque phenotype and their structure was confirmed by Southern blot analysis.

2.19 Nick translation

Typically 1 µg of plasmid DNA was combined on ice with 5 µL of 10x nick translation buffer (0.5 M Tris-HCl (pH 7.5)-0.1 M MgSO₄-1 mM DTT-0.5 mg/mL BSA), 1 µL of a solution consisting of 20 mM dATP, dTTP, and dGTP and 5 µL of α-³²PdCTP (3000 Ci/mole; 10 mCi/mL; New England Nuclear) in a final volume of 46.5 µL. 2.5 µL of DNase I (10 ng/mL) was then added followed by 10 units of *E. coli* DNA polymerase I. The reaction was then incubated on ice for 1 h at 16°C. Unincorporated nucleotides were then removed by passage of the reaction over a G-50 Sephadex spun column as described in Sambrook et al. (1989). The probe was then denatured by boiling prior to addition to the probe solution.

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2.20 Random priming reactions

Plasmid DNA (200 ng) was combined with 2 μ L of pd(N)6 DNA random primers (Pharmacia 27-2166-01) in a final volume of 38 μ L. The plasmid-primer mixture was boiled for 5 min and then placed on ice for 5 min. Random priming was initiated by the addition of 6 μ L of 10x Klenow buffer, 3 μ L each of 1mM dATP, dGTP, and dTTP, 5 μ L of α -³²PdCTP (3000 Ci/mmol; 10 mCi/mL; New England Nuclear), and 20 units of Klenow (New England Biolabs). After incubating the mixture at room temperature for 2 h, unincorporated nucleotides were removed by passage of the reaction over a G-50 Sephadex spun column as described in Sambrook et al. (1989). The probe was then denatured by boiling prior to addition to the hybridization solution (see below).

2.21 Southern blot analysis of viral DNA

Viral DNA for Southern blot analysis (Southern, 1975) was prepared by lysing infected cells with 10 mM Tris-HCl (pH 7.8)-5 mM EDTA-0.5% SDS. Extracts were deproteinized by treatment with 300 μ g/mL of protease K at 37°C for 3 h and subsequent extraction with phenol-chloroform. DNA was then precipitated with ethanol and redissolved in H₂O.

Restriction enzyme digested DNA was resolved through 1% agarose horizontal slab gels containing 1xTBE. Following electrophoresis, gels were soaked two times for 15 min in 0.25 M HCl followed by successive soaks of 30 min each in 0.5 M NaOH, 1 M Tris-HCl-1.5 M NaCl, and finally in 20X SSC. When this work was initiated gels were then transferred to nitrocellulose by

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Coomassie Brilliant Blue R-250 in 50% methanol-10% acetic acid. The gel was then destained for 12 to 16 h in 20% methanol-10% acetic acid. For visualization of ³⁵S-methionine labeled proteins by the method of Bonner and Laskey (1974) gels were dehydrated by incubation for 1 h in dimethylsulfoxide (DMSO). Subsequently the gel was incubated for 3 h in 22% diphenylpicrylhydrazole in DMSO. Gels were then rehydrated by incubation with several changes of H₂O over 30 min. Alternatively, ³⁵S-methionine labeled proteins were visualized by fixation of the gel in 50% methanol-10% acetic acid for 1 h, followed by incubation for 30 min in Enlightening (New England Nuclear). All gels were dried under vacuum at 80°C and label protein gels were exposed to XAR5 film (Kodak) with intensifier screens at -70°C.

2.23 Analysis of viral by resolution of labeled proteins on SDS-PAGE and cellular protein synthesis by TCA precipitation

35 mm dishes of Vero cells were infected and labeled with 50 μ Ci of ³⁵S-methionine (New England Nuclear 110 Ci/mmol; 10 mCi/mL) in 0.450 mL of 199 medium lacking methionine supplemented with 50 μ L of α -MEM. After labeling cells for 1 h, extracts were prepared by lysing the cells in 200 μ L of RIPA (50 mM Tris-HCl (pH 7.2)-150 mM NaCl-0.1% SDS-1% Triton X-100-1% sodium deoxycholate). For the analysis of viral protein synthesis, the extract was mixed with an equal volume 2x protein gel sample buffer, boiled for 5 min, and proteins were resolved on a 12% SDS-PAGE. For analysis of cellular protein synthesis, 20 μ L of cell extract was added to 1 mL of ice cold 10% TCA and the resulting precipitate was suctioned onto nitrocellulose filters and

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capillary action as described in Sambrook et al. (1989). Subsequently, gels were transferred to nitrocellulose using a Stratagene Pressure Blotter as per the manufacturer's instructions. DNA was cross-linked to the nitrocellulose by baking for two hours at 80°C, or by UV crosslinking using a Stratalinker 2400 (Stratagene) as per the manufacturer's instructions. Blots were pre-hybridized in prehybridization solution (2xSSC-0.02% Ficoll, 0.02% polyvinylpyrrolidone-0.02% BSA-250 μ g/mL salmon sperm DNA) at 60°C for 1 to 5 h. Nick translated or random primed probes were then hybridized to the filters in the prehybridization buffer supplemented with 10% dextran sulphate for 8-16 h at 60°C. Filters were subsequently washed 2 times with 2xSSC-0.1%SDS at 60°C and then 2 times with 0.1xSSC at 60°C. After drying, the filters were exposed to XAR5 film at -70°C with intensifier screens.

2.22 Electrophoresis of proteins

Electrophoretic separation of proteins through SDS-polyacrylamide gels was performed using the techniques described by Laemmli (1970). These gels were made using a stock solution consisting of 30% acrylamide and 1.2% N,N'-Diallyltartardiamide. Separating gels consisted of 375 mM Tris-HCl (pH 8.8)-0.1% SDS and contained various concentrations of polyacrylamide. The stacking gel consisted of 125 mM Tris-HCl (pH 6.8)-0.1% SDS-5% polyacrylamide. Protein gel sample buffer was composed of 2% SDS-10% glycerol-150 mM Tris-HCl (pH 6.8)-2% 2-mercaptoethanol-0.00125% bromophenol blue. For visualization of unlabeled proteins, gels were fixed for 1 h in 20% methanol-10% acetic acid and then stained for 2 h with 0.05%

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washed extensively with 10% TCA. The filters were then dried, placed in a scintillation vial, and 6 mL of Omniflor in toluene (prepared as per the manufacturer's instructions) was added to the vial. The sample was then counted in a Beckman scintillation counter.

2.24 Western blot analysis

Extracts from confluent 60 mm dishes of Vero cells were prepared by scraping the cells into PBS and pelleting the cells by centrifugation in an IEC table top centrifuge at 2800 rpm for 10 min at 4°C. The cell pellet was resuspended in 100 μ L of 1x protein gel sample buffer and boiled for 5 min. Twenty μ L of the extract was then resolved on 10% SDS-PAGE gel and after electrophoresis the gel was soaked in transfer buffer (48 mM Tris-HCl-39 mM glycine-20% methanol) for 30 min. For Western blot analysis of purified virions the indicated amount of virus was diluted in an equal volume of 2x protein gel sample buffer, boiled for 5 min, and resolved on 10% SDS-PAGE. Proteins were then transferred to nitrocellulose using a Trans-Blot semi-dry transfer cell (Bio-Rad) at 15 V for 10 min as per the manufacturer's instructions. After transfer, the filters were incubated for 1 h in blocking buffer (25 mM Tris-HCl (pH 7.5)-150 mM NaCl-0.5% Carnation instant skim milk powder-0.05% NP40-0.1% SDS). This and all subsequent incubations were performed at room temperature. Filters were then incubated for 2 h with rabbit serum diluted 1/500 with blocking buffer. Unreacted antibody was removed by washing twice with blocking buffer followed by three washes with blocking buffer containing 0.5% NP40 to remove unreacted antibodies. Bound antibodies were detected by

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incubating the filter for 1.5 h in blocking buffer containing ^{125}I -Protein A (30 mCi/mg, Amersham). Unbound ^{125}I -Protein A was subsequently removed by washing the filters twice with blocking buffer followed by 3 washes in blocking buffer containing 0.5% NP40. After the final wash, the filters were dried and exposed to XAR5 film (Kodak) with intensifier screens at -70°C .

2.25 Purification of HSV virions

The procedure used for the purification of HSV virions was based on the protocol described by Spear and Roizman (1972) and used a continuous dextran gradient prepared with a Biocomp gradient maker. A heavy dextran solution was prepared by dissolving 65 g of dextran T-10 in 200 mL of 1 mM sodium phosphate buffer (pH 7.4), and the light dextran solution was prepared by diluting 45 mL of the heavy dextran solution with 50 mL of 1 mM sodium phosphate buffer (pH 7.4). The gradient was prepared by layering 17.5 mL of light dextran over 17.5 mL of heavy dextran in an ultraclear SW28 Beckman centrifuge tube. This tube was then loaded in a Biocomp gradient maker and spun at 20 rpm for 1.3 min at an angle of 81.5° . The gradient was then placed at 4°C for 30 min prior to use. Ten 150 cm^2 flasks of confluent Vero cells were infected at a MOI of 5 pfu/cell. After total CPE was observed, infected cells were pelleted by centrifugation in an IEC table top centrifuge at 2800 rpm for 10 min at 4°C . All remaining steps were performed on ice or at 4°C . The cell pellet was resuspended in PBS and repelleted. Cells were then resuspended in 2 mL of 1 mM sodium phosphate buffer (pH 7.4) and lysed with 25 strokes of pestle A in a Dounce homogenizer. The lysate was then spun at 3000 rpm in an IEC

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protein-protein interactions using either affinity resins or co-immunoprecipitation were prepared as described above with the exception that SDS and sodium deoxycholate were omitted from the RIPA buffer.

2.27 Immunoprecipitation assays

The following rabbit antisera were used in immunoprecipitation reactions during the course of this work: a vhs antipeptide antiserum (prepared by David Johnson), a vhs serum raised against the vhs Protein A fusion purified from *E. coli* (prepared by Jennifer Newton), and a tk serum (provided by William Summers). The following mouse monoclonal antibodies were used in immunoprecipitation reactions: LP1 (directed against HSV VP16; McLean et al., 1982), LP2 (directed against HSV glycoprotein D; Minson et al., 1986), and a β -galactosidase monoclonal (purchased from BRL). Where indicated labeled extracts were first treated to remove the HSV-encoded Fc receptor as follows. Extracts were mixed end over end with 4 mg of rabbit IgG (Sigma-I-5006) and 200 μL of protein A Sepharose (Pharmacia) for 1 h at 4°C . The protein A beads were removed by centrifugation and the treatment was repeated twice more using 2 mg of rabbit IgG and 100 μL of protein A Sepharose. Residual IgG was then removed by incubation with 100 μL of Protein A Sepharose alone for 1 h at 4°C . The treated extracts were then used for immunoprecipitation assays.

Immunoprecipitation assays involved mixing the cell extract with 30 μL of protein A Sepharose (Pharmacia) and the appropriate antibody end over end for 3 h at 4°C . Subsequently, the beads were washed extensively with RIPA (or RIPA lacking SDS and sodium deoxycholate where appropriate) and bound

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table top centrifuge. The supernatant was removed and the cell pellet was dounced as above and re-centrifuged. The supernatants were pooled and loaded onto the dextran gradient which was then spun at 20,000 rpm for 65 min in a SW28 rotor. The viral band was removed from the gradient and diluted in 3 volumes of PBS. The purified virus was pelleted by centrifugation at 25,000 rpm in a 50.2 Ti rotor for 2 h and the resulting virus pellet was resuspended in 100 μL of PBS and stored at -70°C . The protein content of the purified virions was determined using the Bio-Rad Protein Assay Reagent as per the manufacturer's instructions.

2.26 Preparation of labeled cell extracts for immunoprecipitations

For immunoprecipitation of labeled proteins or solid phase capture assays, 100 mm dishes of Vero cells were infected and labeled with either 0.5 mCi of ^{35}S -methionine (New England Nuclear 110 Ci/mmol; 10 mCi/mL) in 3 mL of 199 medium lacking methionine or 1 mCi of ^{32}P in 3 mL of 199 medium lacking phosphate. When cells were transiently transfected prior to infection, 35 mm dishes of Vero cells were labeled with 0.1 mCi of ^{35}S -methionine in 0.45 mL of 199 medium lacking methionine supplemented with 50 mL of α -MEM. All subsequent steps were performed at 4°C or on ice. After labeling, the cells were washed with PBS and 100 mm dishes and 35 mm dishes were lysed with 1 mL and 0.4 mL of RIPA respectively containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% BSA, and 0.11 units/mL of aprotinin. The extract was then spun at 10,000 rpm in a JA20 rotor for 20 min. Extracts were either used directly or stored at -70°C before use. Extracts used to study

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material was eluted by boiling in 50 μL of 2x protein sample buffer. When using the vhs sera, the tk serum, or the β -galactosidase monoclonal 300 to 500 μL of extract, was mixed with 30 μL of protein A Sepharose and either 20 μL of vhs rabbit serum, or 2 μL of tk serum or β -galactosidase antibody. When immunoprecipitating VP16, 50 μL of extract was mixed with 400 μL of RIPA lacking SDS and sodium deoxycholate, and 2 μL of LP1. In some experiments, material precipitated by one antibody was subsequently tested for reactivity with a second antibody. In this case, bound material was eluted by boiling in 70 μL of 2x sample buffer lacking glycerol and bromophenol blue and then diluted in 700 μL of RIPA. The diluted material was then re-immunoprecipitated as described above.

2.28 V8 protease analysis

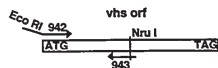
V8 protease analysis of immunoprecipitated proteins was accomplished as follows. Immunoprecipitated proteins were resolved on a 10% SDS polyacrylamide gel and the gel was subsequently dried under vacuum at 50°C (note the gel was not infused with fluor). The desired band was then cut out of the gel and the gel slice was rehydrated in 100 μL 0.1 M Tris-HCl (pH 6.8)-0.1% SDS-1 mM EDTA-10% glycerol-50 mg/mL of BSA and either 0, 5, 20, and 100 $\mu\text{g}/\text{mL}$ of V8 protease. The gel slice and the solution used to rehydrate the gel slice was then loaded onto a 18% SDS-PAGE gel to resolve the proteolytic fragments.

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2.29 The polymerase chain reaction

In order to facilitate linkage of the vhs open reading frame to various promoters, I used the polymerase chain reaction to introduce Eco RI and Nco I cleavage sites just upstream of the vhs initiator codon. To this end, the oligonucleotides AB 942 5'-

GGGGAATTCCATGGGTTTGTTCGGGATGATGAAGT-3' and AB 943 5'-TATATCGCGACAGTTGATGGTGGGA-3' (purchased from the Central Facility of the Institute for Molecular Biology, McMaster University) were used to generate a 720 bp polymerase chain reaction product that represented the 5' half of the vhs open reading frame as follows (not to scale):



This fragment was designed to begin with an Eco RI site immediately 5' to the vhs translation initiation codon and end at the unique Nru I site in the vhs gene. To perform the PCR reaction 50 ng of pvhs (which contains the vhs open reading frame of the HSV-1 strain KOS PAA'5 as a 3.6 kbp Hind III to Hpa I fragment between the Hind III and Sma I sites of pUC19) was mixed with 2 µg of AB942, 1.4 µg of AB943, 1 µL each of 100 mM dATP, dTTP, dCTP, and dGTP, and 5 units of Amplitaq polymerase (Perkin-Cetus) in 100 µL of 10 mM Tris-HCl (pH 8.3)-50 mM KCl-1.5 mM MgCl₂-0.01% gelatin. The reaction was overlaid with mineral oil and subjected to 30 PCR cycles with one cycle consisting of 2 minutes at 93°C, 2 minutes at 60°C, and 3 minutes at 72°C. After the 30 cycles,

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37°C for 30 min. All subsequent steps were performed at 4°C or on ice. The bacteria were pelleted by centrifugation at 5000 rpm in a JA10 rotor (Beckman) for 15 min and resuspended in 50 mM Tris-HCl (pH 8.0) and repelleted. The bacterial pellet was then resuspended in 30 mL of lysis buffer (250 mM NaCl-50 mM Tris-HCl (pH 8.0)-100 mM EDTA-100 mM EGTA-1 mM PMSF) and then 10 mL of lysis buffer (containing 100 mg/mL of lysozyme) was added. Following incubation for 15 min, the bacteria were lysed by the addition of NP40 to a final concentration of 0.1% and subsequent incubation for 15 min. Bacterial debris was removed by centrifugation at 15,000 rpm for 30 min in a JA20 rotor (Beckman). The supernatant was loaded onto a 5 mL IgG Sepharose column (Pharmacia) using a peristaltic pump. The column was washed with 100 mL of 50 mM Tris-HCl (pH 7.6)-150 mM NaCl-0.05% Tween 20, followed by 10 mL of 5 mM ammonium acetate (pH 5.0). Protein A fusion were then eluted using 10 mL of 0.5 M acetic acid (pH 3.4) and 1 mL fractions were collected. The protein content in each fraction was determined using the Bio-Rad Protein Assay Reagent as per the manufacturer's instructions. The peak protein containing fractions were pooled, dialyzed against 20 mM Hepes (pH 7.9)-100 mM KCl-10% glycerol-0.5 mM DTT-0.5 PMSF, and stored at -70°C.

2.31 Linkage of proteins to CNBr activated Sepharose beads

Freeze-dried cyanogen bromide activated Sepharose beads (Pharmacia) (0.3 g/mg of protein to be coupled) were swollen by mixing end over end in 1 mM HCl for 15 min and subsequently washed with 200 mL of 1 mM HCl in a sintered glass filter. All subsequent steps were performed at 4°C. In

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the reaction was further incubated at 72°C for 8 min. The reaction was then removed to a separate tube, extracted with chloroform, and precipitated with ethanol. Following digestion of the PCR product with Eco RI and Nru I the fragment was cloned between the Eco RI and Nru I sites of pvhs to generate pvhsRI. This resulted in the substitution of the 1.8 kbp Eco RI-Nru I fragment of pvhs (which contains the 5' half of the vhs open reading frame plus 1.1 kbp of 5' flanking sequences), with the 5' half of the vhs open reading frame linked directly to an Eco RI site. In addition, the sequence at the translation initiation codon was changed to generate an Nco I site.

2.30 Expression and purification of Protein A fusion proteins from *E. coli*

To express the vhs protein in *E. coli* I made use of the plasmid pRIT2T (Pharmacia) which allows of the expression of protein A fusions in *E. coli* which are easily purified using an IgG Sepharose column. To clone the vhs open reading frame into pRIT2T I made use of the plasmid, pvhsR1 (described in 2.29). The 1.7 kbp EcoRI to Hinc II fragment from pvhsR1 was cloned between the EcoRI and Sma I sites of pRIT2T to generate pRITvhs.

Mid-log phase one liter cultures of *E. coli* strain N4830-1 containing either pRITvhs or pRIT2T were grown at 30°C in terrific broth supplemented with ampicillin at a concentration of 40 µg/mL. The expression of protein A fusion in pRIT2T is repressed by a temperature sensitive λ repressor and as such fusion protein expression is induced by incubation at 42°C. Thus the expression of fusion proteins was induced by the addition of an equal volume of TB prewarmed to 56°C. After temperature shift-up, the cultures were incubated at

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preparation for coupling, purified Protein A fusion proteins were mixed with an equal volume of 0.2 M NaHCO₃ (pH 8.3)-1 M NaCl and this solution was mixed end over end with the Sepharose beads for 8 to 16 h. The beads were then pelleted by centrifugation in IEC table top centrifuge at 2000 rpm for 5 min. The supernatant was removed and the remaining active groups on the beads were block by incubation with 0.1 M Tris-HCl (pH 8.0) for 12-16 h. The beads were then pelleted by centrifugation and excess adsorbed protein was removed by washing the beads with three cycles of 0.1 M sodium acetate (pH 4.0)-0.5 M NaCl, followed by 0.1 M NaHCO₃ (pH 8.3)-0.5 M NaCl. Finally, the beads were washed three times in 50 mM Tris-HCl (pH 7.2)-150 mM NaCl-1% Triton X-100 and stored at 4°C.

2.32 *In vitro* transcription and translation

To express the vhs protein *in vitro*, the 1.7 kbp Eco RI to Hinc II fragment of pvhsR1 was cloned between the Eco RI and Hinc II sites of pGEM4Z, generating pGEMvhs. This places the vhs open reading frame under the control of the SP6 RNA polymerase promoter. Various mutant derivatives that express deleted or truncated portions of the vhs protein were then generated as follows. A mutant bearing a deletion of vhs residues 24 to 180 was generated by deleting the 471 bp Apa I fragment from the vhs open reading frame to generate pGEMΔApa. A mutant bearing a deletion of vhs residues 149 to 344 was generated by deleting the 588 bp Sma I fragment from the vhs open reading frame to generate pGEMΔSma. A derivative bearing an amber stop codon after residue 344 was constructed by inserting the oligonucleotide duplex created by annealing the

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oligonucleotides 5'-GATCCCTAGTCCCGGG-3' and 5'-GATCCCGGGACTAGG-3' (purchased from the Central Facility of the Institute for Molecular Biology, McMaster University) into the unique Bam HI site in the vhs open reading frame to generate pGEMBam amb. A construct containing an amber stop codon after residue 238 was constructed by inserting the oligonucleotide duplex created by annealing the oligonucleotides 5'-CTAGCCATGGGTCG-3' and 5'-CGACCCATGGTCAG-3' (purchased from the Central Facility of the Institute for Molecular Biology, McMaster University) into unique Nru I site in the vhs open reading frame to generate pGEMNru amb. The structures of pGEMBam amb and pGEMNru amb was confirmed by DNA sequencing which was performed by Jennifer Newton. Amino acids 179 to 344 of the vhs protein were expressed by cloning the 492 bp Apa I to Sma I fragment of the vhs open reading frame between the Apa I and Sma I sites of the SP6 expression vector pSPUtk (Falcone and Andrews, 1991) to generate pSPA/S. To express amino acids 238 to 344 of the vhs protein *in vitro* the oligonucleotide duplex used to make pGEMNru amb was cloned into the unique Nru I site in pSPA/S to generate pSPASN. This introduces an Nco I site adjacent to codon 238 and removal of the 190 bp Nco I fragment from pSPASN, to generate pSPN/S, allows for the expression of amino acids 238 to 344 *in vitro*. The VP16 *in vitro* transcription/translation plasmids, which were provided by John Capone and Robert Wheatly, contained the VP16 open reading frame under the control of the SP6 promoter in pSPUtk (Falcone and Andrews, 1991).

RNA for *in vitro* translation was produced using a Riboprobe kit (Promega) as per the manufacturer's instructions. Briefly, 10 µg of plasmid template was incubated in a 100 µL of 40 mM Tris-HCl (pH 7.5)-6 mM MgCl₂-2 mM

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then diluted in 700 µL of RIPA. The dilute material was then immunoprecipitated as described above.

2.34 Transient transfections

To prepare cells for transfection experiments, 2 x 10⁴ Vero cells were seeded per 35 mm dish approximately 18 h prior to transfection. Cells were transfected using the Calcium Phosphate Transfection System (BRL) as per the manufacturer's instructions. For each transfection, 221.25 µL of H₂O was combined with 25 µL of 10x Hepes buffer, 3.75 µL of 1 M NaOH, and 5 µL of phosphate solution in a polypropylene tube. A second polypropylene tube was prepared containing a total of 20 µg of plasmid DNA and 30 µL of CaCl₂ solution. The calcium phosphate precipitate was formed by combining the contents of the two tubes by gently bubbling air through the first tube while slowly adding the contents of the second tube dropwise of the first tube. The resulting precipitate was incubated at room temperature for 20 min and then added to the cells. After returning the cells to an incubator for 24 h, the precipitate was removed by washing the cells several times with α-MEM. Forty-eight hours post-transfection cells were either infected with virus or processed to assay for β-galactosidase activity. For transfections assays using pRSVβgal (supplied by Robert Rosenberger), 5 µg of pRSVβgal was transfected per 35 mm dish. All transient transfection were brought to a total of 20 µg of plasmid DNA using pUC18.

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spemidine-10 mM NaCl-10 mM DTT- 500 u/mL of RNasin-0.5 mM each of ATP, CTP, and UTP-0.05 mM GTP-0.5 mM GpppG and 80 u of SP6 polymerase for 2 h at 37°C. Following phenol-chloroform extraction, the resulting RNA was precipitated with ethanol and the RNA pellet was resuspended in 50 µL of H₂O.

Two µL of RNA generated *in vitro* or 2 µL of Bromo mosaic virus RNA (Promega) was translated at 30°C for 1 h in a 50 µL reaction consisting of 35 µL of rabbit reticulocyte lysate (Promega), 1 µL of 1 mM amino acids lacking methionine (supplied with the lysate), 10 µL of ³⁵S-methionine (New England Nuclear 110 Ci/mmol; 10 mCi/mL), and 80 u of RNasin (Promega).

2.33 Solid phase capture assay for binding of the labeled proteins to protein A fusions

These capture assays employed affinity resins generated by linking protein A fusions to CNBr activated Sepharose beads. Proteins in RIPA lacking SDS and sodium deoxycholate were mixed end over end with 50 µL of Sepharose resin for 3 h at 4°C. The beads were then washed extensively with RIPA lacking SDS and sodium deoxycholate. When using proteins translated *in vitro*, 5 µL of the translation reaction was diluted in 400 µL of RIPA lacking SDS and sodium deoxycholate before addition to the beads. When using proteins extracts prepared from labeled cells 400 µL of extract was mixed directly with the Sepharose resin. For detection of bound material proteins were eluted by boiling in 70 µL of 2x protein sample buffer for 5 min and resolved on SDS-PAGE. For immunoprecipitation of bound material, proteins were eluted by boiling in 70 µL of 2x sample buffer lacking glycerol and bromophenol blue and

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2.35 β-galactosidase assays

Extracts from cells transfected with pRSVβgal were prepared 48 h post-transfection. Thirty-five mm dishes of Vero cells were washed with 1 mL of 40 mM Tris-HCl (pH 7.5)-1 mM EDTA-150 mM NaCl and lysed by the addition 100 µL of 250 mM Tris-HCl (pH 7.8)-0.5% NP40-1 mM PMSF. Forty µL of this extract was mixed with 400 µL of 10 mM KCl-1 mM MgSO₄-100 mM sodium phosphate (pH7.5)-50 mM 2-mercaptoethanol and 150 µL of 13 mM O-nitrophenyl β-D-galactopyranoside. The reaction was incubated at 37°C for 15 to 30 min and subsequently terminated by the addition of 200 µL of Na₂CO₃. The absorbance was then read at 420 nm in an Ultraspec.

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Results

3.1 Expression of endogenous and virally transduced β -globin genes in MEL cells infected with HSV

3.1.1 Expression of a virally transduced rabbit β -globin gene in MEL cells infected with an HSV-1 recombinant.

Mouse erythroleukemia (MEL) cells undergo terminal erythroid differentiation after treatment with various chemical inducers, including HMBA (reviewed by Marks and Rifkin, 1978). This results in a large increase in mouse β -globin mRNA levels through transcriptional activation of the mouse β -globin gene and stabilization of β -globin transcripts (Volloch and Housman, 1981; Hoefler *et al.*, 1983). As discussed in the introduction, HSV infection of MEL cells dramatically inhibits endogenous mouse β -globin gene expression (Nishioka and Silverstein, 1977, 1978). In contrast, HSV gene products activate the rabbit β -globin gene resident in the HSV-1 PAA'5 recombinant L7/14, in L7/14 infected Vero cells (Smiley *et al.*, 1987). This recombinant (diagrammed in figure 1) contains the intact rabbit β -globin gene and 1.2 kbp of 5' flanking sequences inserted at the tk locus, in the tk antisense orientation.

To examine this apparent contradiction, L7/14 was used to infect both HMBA induced and uninduced MEL cells and rabbit β -globin mRNA levels were assayed by S-1 nuclease mapping (figure 3), using the probe detailed in figure 2. Similar to the results previously obtained in L7/14 infected Vero cells, the rabbit β -globin gene was expressed from its own promoter in L7/14 infected

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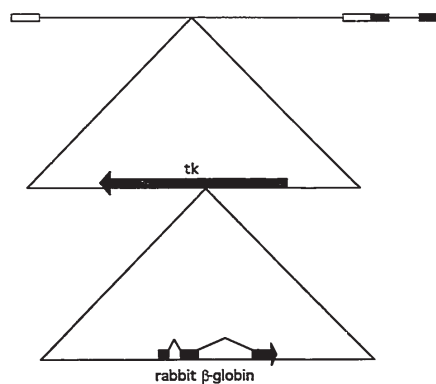


Figure 1. Structure of the L7/14 recombinant. L7/14 (Smiley, *et al.*, 1987) is an HSV-1 strain KOS PAA'5 recombinant that carries a 3.7 kbp fragment of the rabbit β -globin gene with 1.2 kbp of 5' flanking sequences. This fragment was inserted at the tk locus, in the tk antisense orientation. The HSV-1 genome is displayed here in the prototype arrangement.

69

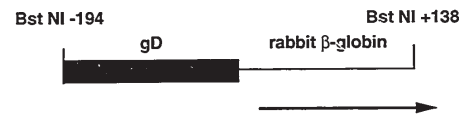
MEL cells, with the levels of rabbit β -globin mRNA peaking at 6 hours post-infection and declining somewhat thereafter. In addition, the differentiated state of the MEL cells had no significant effect on the expression of the transduced rabbit β -globin gene. These kinetics, typical of an HSV early gene, are similar to the kinetics of rabbit β -globin gene in L7/14 infected Vero cells (Smiley *et al.*, 1987). Furthermore, the intensity of the rabbit β -globin signal in 10 μ g of infected cell RNA was comparable to the signal derived from 0.5 ng of commercially available globin mRNA (40% β -globin) (figure 3). Therefore, rabbit β -globin mRNA constitutes approximately 2×10^{-5} of total cytoplasmic RNA in L7/14 infected MEL cells. Since the same result was previously obtained in L7/14 infected Vero cells (Smiley *et al.*, 1987), this suggests that the amount of rabbit β -globin mRNA expressed in L7/14 infected Vero cells and MEL cells is similar. This level of mRNA is also similar to the levels of viral tk mRNA detected in HSV-1 infected Vero cells (McKnight *et al.*, 1981). Therefore the rabbit β -globin gene transduced by L7/14 is efficiently expressed in MEL cells.

RNA from L7/14 infected MEL cells contains two aberrant globin-related transcripts, designated AT1 and AT2 (figure 3). The AT1 and AT2 transcripts were previously detected in L7/14 infected Vero cells, and arise from aberrant splicing and read-through transcription, respectively (Smiley *et al.*, 1987).

Expression of the rabbit β -globin gene in L7/14 infected Vero cells requires HSV IE gene products (Smiley *et al.*, 1987; Smiley and Duncan, 1992); thus mimicking the regulation of bonafide HSV early genes. To determine if IE gene products are required for rabbit β -globin expression in terminally differentiated MEL cells, infections were carried out in the presence of the translation inhibitor cycloheximide. The levels of rabbit β -globin mRNA were then determined using

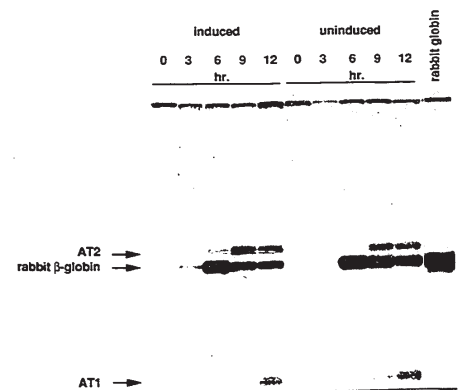
71

Figure 2. The rabbit β -globin S-1 nuclease probe. This 320 bp Bst NI probe (previously described by Everett, 1983) is derived from the gD-rabbit β -globin fusion plasmid pRED4. This fragment spans the bonafide rabbit β -globin cap site allowing for the detection of correctly initiated rabbit β -globin mRNA in an S-1 nuclease protection analysis.



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Figure 3. Expression of the rabbit β -globin gene in L7/14 infected MEL cells. Terminally differentiated (induced) and proliferating (uninduced) MEL cells were infected with L7/14 at a MOI of 10 PFU/cell, and cytoplasmic RNA was harvested at the times indicated post-infection. Ten μ g of RNA was hybridized to the 5' end-labeled single-strand probe described in figure 2 and the hybrids were digested with S-1 nuclease. The protected fragments were then resolved through an 8% sequencing gel. 0.5 ng of rabbit globin mRNA (40% β -globin mRNA; BRL) was also hybridized to the probe to provide a size marker for correctly initiated rabbit β -globin mRNA. The arrows indicate the position of correctly initiated rabbit β -globin mRNA and the previously described aberrant transcripts, AT1 and AT2 (Smiley et al., 1987)



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a primer extension assay that employed an oligonucleotide complementary to nucleotides +63 to +87 of the rabbit β -globin transcript (figure 4). Cycloheximide strongly inhibited accumulation of rabbit β -globin transcripts, suggesting a requirement for HSV IE gene products. In summary, the data in figures 3 and 4 demonstrate that the rabbit β -globin gene in L7/14 infected MEL cells was efficiently expressed from its own promoter as a viral early gene.

3.1.2 Inhibition of mouse β -globin gene expression by HSV-1 infection.

Previous work had demonstrated that HSV inhibits mouse β -globin gene expression in MEL cells (Nishioka and Silverstein, 1977, 1978). To determine if the same is true in L7/14 infected MEL cells, I utilized the RNA samples from figure 3. The levels of mouse β -globin mRNA were quantified (figure 6) using the S-1 nuclease protection probe detailed in figure 5. Consistent with previous work (Nishioka and Silverstein, 1977, 1978), mouse β -globin mRNA levels rapidly and irreversibly declined upon infection of both induced and uninduced cells, thereby confirming that HSV induces a decrease in mouse β -globin mRNA levels in MEL cells. Thus, although expression of the transduced rabbit β -globin gene borne by L7/14 is activated by viral regulatory proteins during infection of MEL cells, expression of the homologous endogenous gene is strongly suppressed.

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Figure 4. The effect of cycloheximide on expression of the rabbit β -globin gene in L7/14 infected MEL cells. Terminally differentiated MEL cells were infected with L7/14 at a MOI of 10 PFU/cell in the presence or absence of 100 μ g/mL of cycloheximide (Cx.). Cytoplasmic RNA was harvested at 6 h post infection and 10 μ g of RNA was subjected to primer extension analysis to detect correctly initiated rabbit β -globin mRNA. The extension products were then resolved through an 8% sequencing gel. The arrow indicates the position of correctly initiated rabbit β -globin mRNA at 87 nucleotides. The markers (lane M) were 3'-end-labeled Hpa II fragments of pBR322, and the size of the marker fragments (in nucleotides) is indicated.

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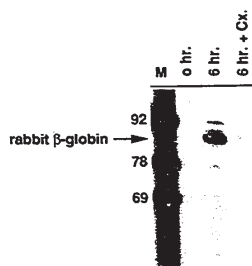


Figure 5. The mouse β -globin S-1 nuclease probe. This 152 nucleotide Hae III to Dde I fragment spans the bonafide mouse β -globin transcription start site allowing for the detection of correctly initiated mouse β -globin mRNA.

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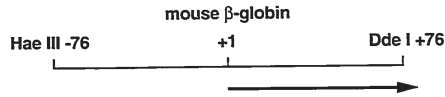
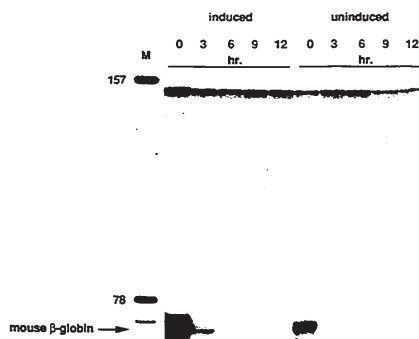


Figure 6. The expression of the mouse β -globin gene in L7/14 infected MEL cells. One μg aliquots of the RNA samples harvested in figure 2 were hybridized to the 5' end-labeled single-strand probe described in figure 5, and the hybrids were digested with S-1 nuclease. The protected fragments were then resolved through an 8% sequencing gel. The arrow indicates the position of properly initiated mouse β -globin mRNA at 76 nucleotides. The markers (lane M) were 3'-end labeled Hinf I fragments of pTK173 and the size of the marker fragments (in nucleotides) is indicated.

8 1

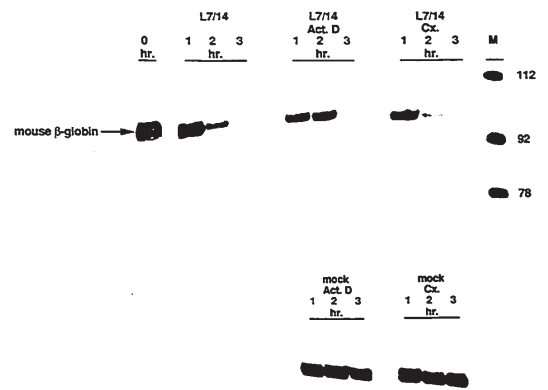


3.1.3 The effects of inhibitors on the L7/14 induced decrease in mouse β -globin mRNA.

As reviewed in the introduction, previous work had indicated that the HSV-1 induced degradation of mouse β -globin mRNA in MEL cells requires *de novo* viral gene expression (Nishioka and Silverstein, 1978). This result conflicts with more recent data suggesting that the degradation of cellular mRNA during infection of Vero cells is induced by a virion component (Schek and Bachenheimer, 1985). To reexamine this issue, I determined the effects of cycloheximide and actinomycin D on the ability of L7/14 to inhibit mouse β -globin gene expression. In this experiment, a synthetic primer complementary to nucleotides +63 to +87 of mouse β -globin transcript was used to monitor mouse β -globin mRNA levels in a primer extension assay (figure 7). Contrary to previous reports, L7/14 induced a decrease in mouse β -globin mRNA levels in the presence of either actinomycin D or cycloheximide. Therefore, this decrease does not require viral RNA or protein synthesis suggesting that a virion component is responsible. The results in figure 7 also demonstrate that actinomycin D treatment alone did not effect mouse β -globin mRNA levels. Taken in combination, these data suggest that HSV-1 infection decreases mouse β -globin mRNA levels by inducing the degradation of pre-existing cytoplasmic mouse β -globin mRNA rather than by inhibiting transcription or mRNA transport. The failure of cycloheximide treatment to effect HSV-induced mRNA degradation also demonstrates that this process does not require on-going translation of the target mRNA.

8 3

Figure 7. The effects of inhibitors on the L7/14 induced decrease in mouse β -globin mRNA levels. Terminally differentiated MEL cells were infected at a MOI of 10 PFU/cell with L7/14, and cytoplasmic RNA was harvested at the times indicated post-infection. Where indicated, infections were carried out in the presence of 10 μ g/mL of actinomycin D (Act. D) or 100 μ g/mL of cycloheximide (Cx.). The level of mouse β -globin mRNA in 10 μ g of RNA was scored by primer extension analysis and the extension products were resolved through an 8% sequencing gel. The arrow indicates the position of properly initiated mouse β -globin mRNA at 92 nucleotides. The markers (lane M) were 3'-end labeled Hpa II fragments of pBR322, and the size of the marker fragments (in nucleotides) is indicated.



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3.1.4 The HSV-1 mutant vhs1 does not induce degradation of mouse β -globin mRNA.

The data described in section 3.1.3 demonstrated that a virion component induces mouse β -globin mRNA degradation, suggesting the vhs function might be involved. As reviewed in the introduction, the vhs1 mutant was isolated based on its inability to induce shutoff of host protein synthesis in the absence of viral gene expression (Read and Frenkel, 1983). To determine if the vhs function is required to induce mouse β -globin mRNA degradation, terminally differentiated MEL cells were infected with the vhs1 mutant, and mouse β -globin mRNA levels were scored by primer extension (figure 8). The vhs1 mutant did not induce mouse β -globin mRNA degradation over a three hour time course; however, β -globin mRNA levels declined after six hours post-infection (figure 9A). These results might suggest that the life cycle of the vhs1 mutant is, in general, slow and hence host shutoff would occur at a later time post-infection. However, expression of the viral glycoprotein D mRNA, as assayed by primer extension, occurs with a normal time course during a vhs1 infection (figure 9B), indicating that the infectious cycle is similar to that of wild type virus. More likely, vhs1 induces mouse β -globin mRNA degradation late in infection through the secondary host shutoff function described by Read and Frenkel (1983). These data demonstrate that the rapid degradation of mouse β -globin mRNA induced by L7/14 relies on the vhs function, and are consistent with other work suggesting that the vhs function induces cellular mRNA degradation (Kwong and Frenkel, 1987; Strom and Frenkel, 1987).

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Figure 8. The effect of vhs1 infection on the levels of mouse β -globin mRNA in MEL cells. Terminally differentiated MEL cells were infected at a MOI of 10 PFU/cell with vhs1 or L7/14, and cytoplasmic RNA was harvested at the times indicated post-infection. The level of mouse β -globin mRNA in 10 μ g of RNA was scored using the primer extension assay. The arrow indicates the position of properly initiated mouse β -globin mRNA at 92 nucleotides. The markers (lane M) were 3'-end labeled Hpa II fragments of pBR322, and the size of the marker fragments (in nucleotides) is indicated.

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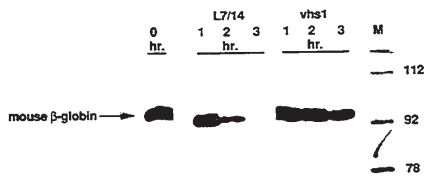
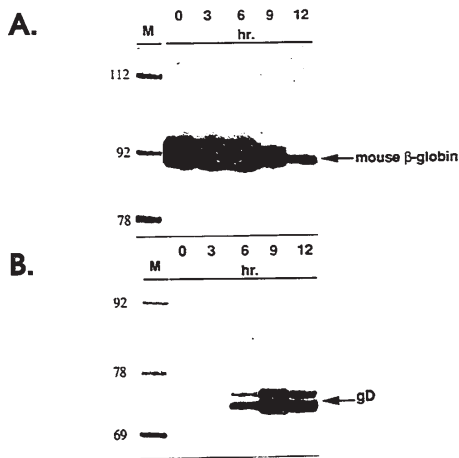


Figure 9. The effect of vhs1 infection on the levels of mouse β -globin mRNA over a 12 h time course. Terminally differentiated MEL cells were infected at a MOI of 10 PFU/cell with vhs1 and cytoplasmic RNA was harvested at the times indicated post-infection. 10 μ g of RNA was scored for the levels of mouse β -globin mRNA (A.) and HSV-1 glycoprotein D mRNA (B.) using primer extension analysis. The arrows indicates the position of properly initiated mouse β -globin mRNA and glycoprotein D (gD) mRNA. The markers (lane M) were 3'-end labeled Hpa II fragments of pBR322, and the size of the marker fragments (in nucleotides) is indicated.

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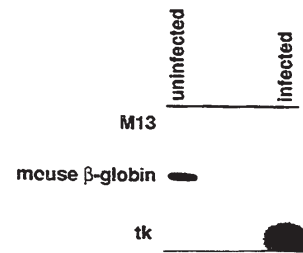
3.1.5 HSV-1 infection inhibits transcription of the endogenous mouse β -globin gene in infected MEL cells.

The data presented in figures 3 and 4 demonstrate that the rabbit β -globin gene is transactivated by HSV IE gene products when it is delivered into MEL cells as part of the HSV genome. Therefore, I determined what effect, if any, HSV-1 infection had on transcription of the endogenous mouse β -globin gene, using the nuclear run-on technique. This technique involves the isolation of nuclei and their subsequent incubation in the presence of ribonucleotides plus α^{32} P-UTP. Under these conditions only transcripts that were initiated at the time of isolation continue synthesis, and therefore the transcription rate of a particular gene is proportional to the amount of radioactivity incorporated into its RNA. After incubation, RNA is isolated and hybridized to mRNA complementary probes, and thus one can compare relative transcription rates of a gene under different conditions. Figure 10 shows the results of a run-on assay performed on nuclei isolated from PAA'5 infected and uninfected terminally differentiated MEL cells. The resulting run-on RNA was hybridized to single stranded probes complementary to mouse β -globin mRNA and the HSV-1 tk mRNA. These data indicate that the levels of mouse β -globin gene transcription are greatly reduced by 5 hours post-infection. Thus, despite the ability of HSV products to activate transcription of the virally transduced rabbit β -globin gene, HSV-1 infection inhibits mouse β -globin transcription in MEL cells.

To determine which HSV gene products are required to inhibit mouse β -globin transcription, nuclei were harvested from MEL cells infected with the HSV-1 mutant d120 (DeLuca *et al.*, 1985). d120 is deleted for both of the

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Figure 10. The effect of HSV-1 infection on the transcription of the mouse β -globin gene resident in MEL cells. Terminally differentiated MEL cells were infected at a MOI of 10 PFU/cell with HSV-1 strain PAA'5. Nuclei were harvested at five h post-infection and used to generate run-on RNA as described in the materials and methods. The RNA was then hybridized to nitrocellulose filters containing M13 single stranded probes specific the mouse β -globin and the HSV-1 tk mRNAs.



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copies of the gene encoding ICP4 and since ICP4 is required to activate both E and L gene expression, cells infected with d120 only express IE gene products. Figure 11 shows that d120 inhibited transcription of the mouse β -globin gene as assayed by nuclear run-on. As expected, d120 infected nuclei gave only low level transcription of the HSV tk gene (an E gene) while d120 overexpressed the HSV ICP0 gene (an IE gene). These results demonstrate that one or more of the HSV gene products present in d120 infected cells are required to inhibit mouse β -globin transcription suggesting that this process is mediated by an IE protein (excluding ICP4) and/or a virion component.

3.1.6 Construction of an HSV-1 recombinant containing the mouse β -globin gene.

The data presented above demonstrate that in L7/14 infected MEL cells, transcripts from the HSV-transduced rabbit β -globin gene accumulated, while expression of the endogenous mouse β -globin gene was inhibited. These results suggest that preferential expression of viral genes in HSV infected cells does not rely solely on sequence specific differentiation between viral and cellular mRNAs or promoters. This hypothesis predicts that the mouse β -globin gene would also be expressed after insertion into the viral genome. I tested this prediction by constructing an HSV-1 recombinant containing the mouse β -globin gene under the control of its own promoter. The mouse β -globin gene was inserted into the dispensable viral gene encoding glycoprotein I (gI), using the novel rescue strategy diagrammed in figure 12. This system uses an HSV-1 strain F mutant, FgD β , where the coding sequences for gD and part of the

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Figure 11. The effect of d120 infection on the transcription of the mouse β -globin gene resident in MEL cells. Terminally differentiated MEL cells were infected at a MOI of 10 PFU/cell with either HSV-1 strain d120 (which is deleted for both copies of the gene encoding ICP4) or HSV-1 strain PAA'5. Nuclei were harvested at five h post-infection and used to generate run-on RNA. The RNA was hybridized to nitrocellulose filters containing M13 single stranded probes specific for mouse β -globin and the HSV-1 tk mRNAs. The filters also contained denatured pIGA15, which served as a probe for the HSV-1 ICP0 gene.

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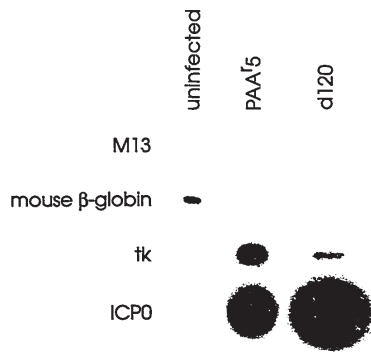


Figure 12. Construction of an HSV-1 recombinant carrying the mouse β -globin gene. The HSV-1 mutant FgD β contains the bacterial β -galactosidase gene in place of the gD open reading frame and part of the gI open reading frame. This mutant will only grow on cells that supply gD in trans. The gD/gI plasmid pSS17L contains the mouse β -globin gene inserted into the gI open reading frame within the sequences deleted from FgD β . Co-transfection of FgD β infectious DNA with pSS17 β M will only give a viable virus through a double crossover event between homologous sequences flanking the deletion in FgD β . This crossover will therefore incorporate the mouse β -globin gene into the HSV-1 genome. Note this figure is not to scale.

adjacent gI gene have been replaced by the bacterial β -galactosidase gene (Ligas and Johnson, 1988). FgD β will only grow on cells that supply gD in trans since gD is essential for viral replication in tissue culture. This virus is rescued to a viable phenotype by co-transfecting a plasmid encoding gD with infectious FgD β DNA. Since the deletion in FgD β includes part of the gI gene, homologous recombination of a plasmid borne gD gene will always incorporate the deleted gI sequences into the genome (see figure 12). Therefore, this recombination event will also rescue alterations to the gI gene that are within the deleted sequences. Since gI is not essential for replication in tissue culture, alterations which inactivate the gI gene will still produce viable recombinants. This system offers an efficient technique for the construction of recombinant viruses since infectious FgD β DNA will only give plaques on non-complementing cells if a wild type gD gene is rescued into the viral genome.

To make use of this technique I employed a 2951 bp Eco RI to Sau 3A fragment containing the mouse β -globin gene and 1.0 kbp of 5' flanking sequences. This fragment was cloned in the gI sense orientation into the gI portion of the gDgI plasmid pSS17L, giving pSS17L β M (see figure 12). Infectious FgD β DNA was co-transfected with pSS17L β M and the desired recombinant, designated β M (diagrammed in figure 13), was plaque purified. The structure of this recombinant was confirmed by Southern blot analysis on viral DNA cleaved with Bam HI and probed with pSS17L β M (figure 14). Wild-type strain F DNA gave one Bam HI fragment of 6.5 kbp while FgD β DNA gave two Bam HI fragments of 2.5 kbp and 2.2 kbp (as predicted by Ligas and Johnson, 1988). Insertion of the mouse β -globin gene into the gI coding

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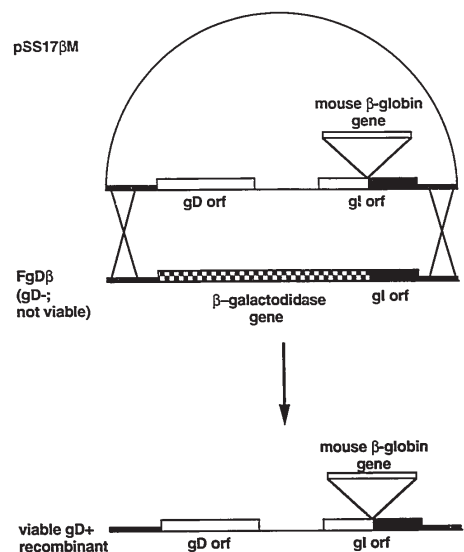
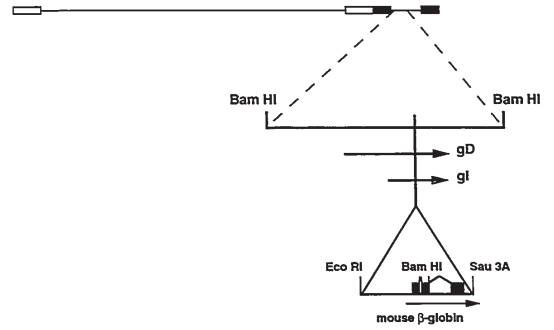
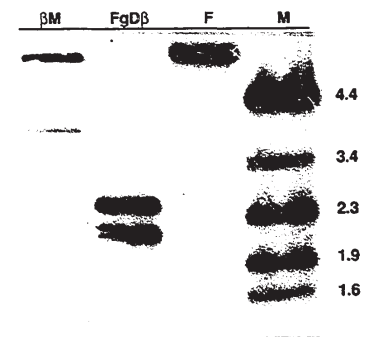


Figure 13. Structure of the β M recombinant. The mouse β -globin gene was cloned into HSV-1 strain F as a 2951 bp Eco RI to Sau 3A fragment containing 1.0 kbp of 5' flanking sequences. This fragment was inserted in the coding sequences of the HSV-1 glycoprotein I (gI) gene in the gI sense orientation. The HSV-1 genome displayed here is in the prototype arrangement.



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Figure 14. Southern blot analysis of the β M recombinant. The structure of the β M recombinant was confirmed by Southern blot analysis of viral DNA cut with Bam HI, resolved through a 1% agarose gel and transferred to nitrocellulose. FgD β and strain F DNA were also analyzed on this blot. The filter was probed with the gD-gI plasmid pSS17. The marker (lane M) is a mixture fragments resulting from digestion of pSS17L with Sac I and pSS17 with Nco I and Sph I. The sizes of the resulting marker fragments (in kbp) are indicated.



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sequences resulted in two novel Bam HI β -globin fusion fragments of 5.8 kbp and 3.7 kbp.

3.1.7 Mouse β -globin is expressed when inserted into the HSV-1 genome.

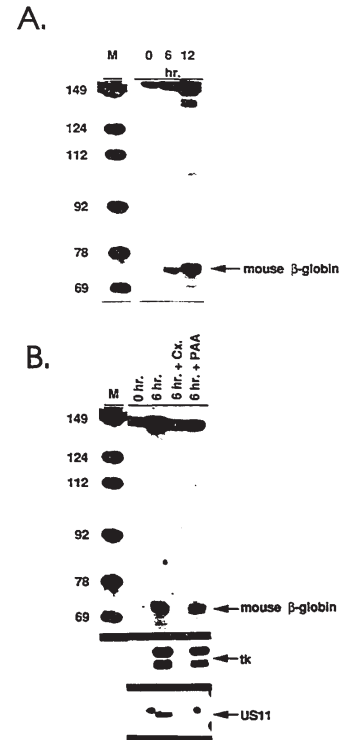
Expression of the mouse β -globin gene resident in the β M recombinant was monitored using the S-1 nuclease analysis employed in section 3.1.2 (figure 15A). Properly initiated mouse β -globin mRNA was detected at 6 hours post-infection, with an increase in levels observed at the 12 hour time point. To further investigate expression of the transduced mouse β -globin gene, infections were carried out in the presence of cycloheximide (to inhibit protein synthesis) or PAA (to inhibit viral DNA replication) (figure 15B). This experiment demonstrated that mouse β -globin mRNA accumulated in the absence of viral DNA replication but did not accumulate in the absence of viral protein synthesis. As with the rabbit β -globin gene (Smiley, *et al.*, 1987), these results suggest that expression of an HSV-transduced mouse β -globin gene requires HSV IE proteins but does not require viral DNA synthesis. As controls in this experiment, the same RNA samples were assayed for viral tk mRNA (an HSV E gene) and US11 mRNA (an HSV true L gene) using a primer extension analysis. As expected, expression of both the tk and US11 genes required viral protein synthesis, while DNA replication was only required for expression of the US11 gene. These data indicate that both the mouse and rabbit β -globin genes are expressed to high levels when inserted into the viral genome. However, the details of their control may differ, in that rabbit β -globin mRNA levels peak at 6 hours post-infection while mouse β -globin mRNA continues to accumulate

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between 6 and 12 hours post-infection. Thus, the mouse β -globin gene is expressed when inserted into the HSV-1 genome, while HSV-1 infection inhibits expression of the mouse β -globin gene resident in MEL cells. These observations further support the hypothesis that preferential expression of viral genes versus cellular genes in HSV infected cells does not rely on sequence specific differentiation between viral and cellular mRNAs or promoters.

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Figure 15. Expression of the mouse β -globin gene in β M infected Vero cells. Vero cells were infected with β M at a MOI of 10 PFU/cell and cytoplasmic RNA was harvested at the times indicated post-infection in the absence of drug (A) or in the presence of 100 μ g/mL of cycloheximide or 200 μ g/mL of phosphonoacetic acid (B). The level of mouse β -globin mRNA in 10 μ g of RNA was scored using the S-1 nuclease assay detailed in figure 6. The RNA in panel B was also assayed for the presence of HSV-1 tk and US11 mRNA by primer extension. The arrows indicate the position of properly initiated mouse β -globin, tk, and US11 mRNA. The markers (lane M) were 3'-end labeled Hpa II fragments of pBR322, and the size of the marker fragments (in nucleotides) is indicated.



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3.2 Construction and characterization of vhs mutants

3.2.1 Construction of UL41 mutants

The data in the previous sections raise three interesting questions: how are heterologous promoters present in the viral genome activated; why is the transcription of some cellular genes located in the cellular genome repressed; and how do mRNAs arising from the viral genome escape vhs-mediated degradation? I chose to focus on the latter issue by investigating the vhs function in more detail. Using the vhs1 mutant, Kwong *et al.* (1988) have suggested that an intact UL41 open reading frame is required for vhs function. I confirmed this requirement by constructing two mutants in the UL41 open reading frame using a cassette that contains the lacZ gene driven by the HSV-1 ICP6 promoter (Goldstein and Weller, 1988b). Rescue of this construct into the HSV-1 genome results in viral progeny whose plaques stain blue in the presence of X-gal, allowing for the isolation of viral insertion mutants. To facilitate the construction of UL41 mutants, the UL41 open reading frame of HSV-1 strain KOS PAA'5 was cloned as a 3.6 kbp Hind III to Hpa I fragment between the Hind III and Sma I sites of pUC19, giving pvhs. The 4276 bp Bam HI lacZ cassette was then inserted into the unique Bam HI site in pvhs in the UL41 sense orientation, generating pvhsB (figure 16). This insertion disrupts the UL41 open reading frame after sequences predicted to encode amino acid 342. pvhsA (figure 16) was constructed by blunting the lacZ cassette with T4 DNA polymerase and cloning it, in the UL41 sense orientation, between the two blunted Apa I sites found in the UL41 open reading frame. These

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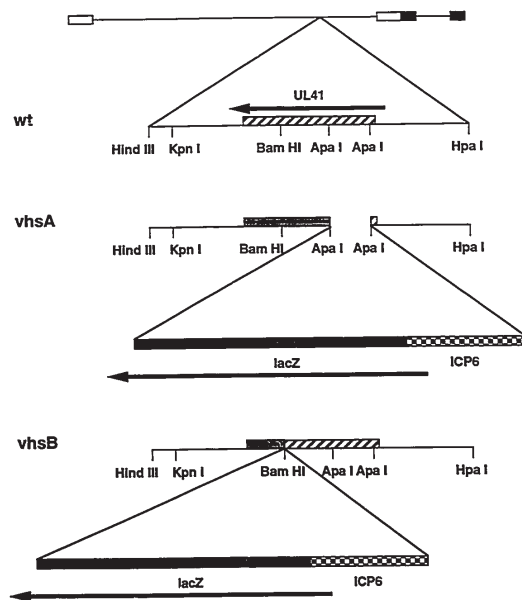


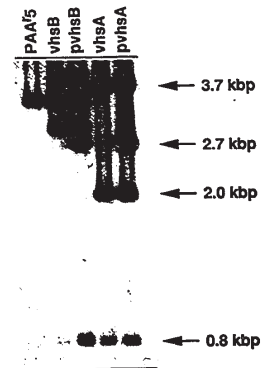
Figure 16. Structure of the vhsA and vhsB mutants. The HSV-1 genome displayed here is the prototype arrangement and the structure of the wild type HSV-1 UL41 gene is diagrammed in the top panel (wt) with the open reading frame (hatched) and the direction of transcription (arrow) indicated. The ICP6-lacZ cassette contains the lacZ coding sequences (dotted) under the control of the ICP6 promoter region (dotted) and the direction of transcription of the ICP6-lacZ cassette is indicated (arrow). vhsA contains the lacZ cassette inserted between the two Apa I sites in the UL41 open reading frame. This deletes the sequences predicted to encode amino acids 22-179 of the UL41 protein and inserts stop codons in all three reading frames between the UL41 promoter and downstream UL41 sequences. vhsB contains the lacZ cassette inserted in the Bam HI site in the UL41 open reading frame. This insertion disrupts the UL41 open reading frame after sequences predicted to encode amino acid 342.

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manipulations replaced codons 22 through 179 with the lacZ expression cassette. The mutations carried by pvhsA and pvhsB were then rescued into virus by co-transfection with HSV-1 strain KOS PAA'5 infectious DNA. The desired recombinants were isolated and plaque purified based on the blue plaque phenotype in the presence of X-gal. The structure of both vhsA and vhsB (diagrammed in figure 16), was confirmed by Southern blot analysis on viral DNA cut with a mixture of Hind III, Hpa I, and Kpn I and probed with pvhsB (figure 17). pvhsA and pvhsB were also included in this analysis to provide convenient marker fragments. Replacement of the UL41 Apa I fragment with the lacZ cassette in the vhsA mutant disrupted the 3354 bp Kpn I-Hpa I fragment of PAA'5 and generated one 3472 bp Kpn I-Hpa I UL41-lacZ fusion fragment and a 1950 bp Kpn I-Hpa I UL41-ICP6 fusion fragment. Insertion of the lacZ cassette at the Bam HI site of the UL41 open reading frame generated one 3255 bp Kpn I-Hpa I UL41-lacZ fusion fragment and a 2968 bp Kpn I-Hpa I UL41-ICP6 fusion fragment. Both vhs mutants also contained 2 internal lacZ fragments of 834 bp and 624 bp (the latter fragment was detected in the original blot but is not visible in figure 17). The 235 bp Hind III-Kpn I fragment found in PAA'5 was not detected due to its small size. In addition, both plasmids gave an additional band at 2.7 kbp which represents the pUC19 vector used to make these clones.

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Figure 17. Southern blot analysis of the vhs mutants. The structure of each vhs mutant was confirmed by cutting vhsA and vhsB viral DNA with a mixture of Hind III, Kpn I, and Hpa I and resolving the digested DNA through a 1% agarose gel. pvhsA, pvhsB, which were the plasmids used to make the corresponding mutants, and parental strain PAA'5 viral DNA were also included in this analysis. After transferring the DNA to nitrocellulose, the filter was probed with labeled pvhsB. The fragment sizes, indicated in kbp, are for the digest of pvhsB.



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3.2.2 UL41 mutants do not inhibit host cell translation in the absence of viral gene expression.

The vhsA and vhsB mutants were first tested for their ability to inhibit cellular translation in the absence of viral gene expression. Vero cells were infected in the presence of actinomycin D and labeled from 8-9 hours post-infection with ³⁵S-methionine in an excess of unlabeled methionine. After labeling, cell lysates were prepared and the levels of cellular translation were quantified by TCA precipitation (figure 18). The parental strain, PAA'5, inhibited cellular translation by, on average, 62% while vhs1, vhsA, and vhsB gave levels similar to mock infected cells. These results confirm that the UL41 open reading frame is required for virion induced inhibition of host cell translation.

3.2.3 UL41 mutants do not destabilize mouse β-globin mRNA.

To determine if vhsA and vhsB induce mRNA degradation, MEL cells were infected in the presence and absence of actinomycin D, and mouse β-globin mRNA levels were scored using the primer extension assay (figure 19). At 6 hours post-infection, the parental PAA'5 strain induced a significant reduction in mouse β-globin mRNA levels, while vhsA and vhsB gave levels similar to vhs1. At 12 hours post-infection the vhs mutants displayed consistent differences in mouse β-globin mRNA levels with vhs1 showing the most profound decrease. This result might suggest that vhs1 encodes a partially active UL41 protein and that high level *de novo* expression of this protein may contribute to the late decline in mouse β-globin mRNA. Alternatively, another HSV protein could

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mediate late degradation of cellular mRNAs. If individual vhs mutants were differentially retarded in their progress through the infectious cycle, the expression of this late shutoff factor might not be uniform. Thus, the strength of late shutoff would not be consistent in the different vhs mutants. In summary, these results confirm that an intact UL41 open reading frame is required for HSV virions to inhibit cellular translation and degrade cellular mRNA.

3.2.4 Growth properties of vhs mutants.

During this work, vhs mutant stocks consistently displayed reduced titers compared to the parent strain. This suggests that the UL41 open reading frame is required to optimize viral replication in tissue culture. To document this requirement, I performed a single step growth curve experiment using vhsA, vhsB, vhs1 and PAA'5. Vero cells were infected at a MOI of 10 PFU/cell and virus was harvested at 6, 12, 18, 24, and 30 hours post-infection. Viral yields were subsequently quantified by plaque assay and expressed as the number of PFU produced per infected cell (figure 20). All three vhs mutants displayed a reduction in the peak amount of virus produced, with vhs1, vhsA, and vhsB producing 8.4 fold, 5.1 fold, and 2.6 fold less virus, respectively, than PAA'5. These results confirm that although the UL41 open reading frame is not required for virus growth in tissue culture, it is required to maximize virus production. All three vhs mutants also displayed a lag in the production of virus; For example, PAA'5 showed a 12.2 fold increase in the amount of virus detected at 6 hours and 12 hours post-infection, while over the same period

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vhsA showed only a 1.5 fold increase. This lag suggests that the UL41 open reading frame functions to initiate a productive infection.

3.2.5 Synthesis of viral proteins during infection with the vhsA mutant.

To further investigate the growth impairment of UL41 mutants, the protein synthesis profile of vhsA was compared to the parental virus, PAA'5. Vero cells were infected with vhsA or PAA'5, then labeled at various times post-infection for 1 hour with ³⁵S-methionine in an excess of cold methionine. Protein extracts were then prepared and resolved through a 12% SDS polyacrylamide gel. Figure 21 demonstrates that vhsA displayed a lag in viral protein synthesis; for example, PAA'5 protein synthesis was easily detected at 3 hours post-infection, whereas vhsA protein synthesis was not. These results suggest that the vhs function is required early in infection to initiate viral protein synthesis.

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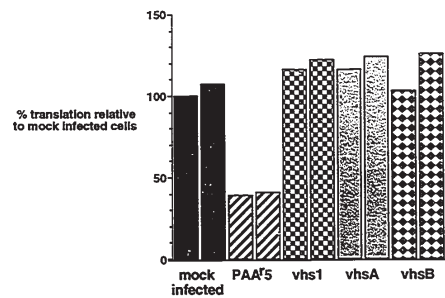


Figure 18. The effect of UL41 mutations on the ability of HSV-1 to inhibit cellular translation. Duplicate wells of Vero cells were infected with PAA'5, vhs1, vhsA, or vhsB in the presence of 10 µg/mL of actinomycin D at a MOI of 10 PFU/cell. Cells were then labeled from 8-9 h post-infection with ³⁵S-methionine in an excess of unlabeled methionine. The levels of cellular translation were quantified by measuring TCA precipitable counts in extracts prepared from infected cells. The levels of translation were expressed as a percentage of the average level of translation in mock infected cells.

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Figure 19. The effect of UL41 mutations on the ability of HSV-1 to induce degradation of cellular mRNA. Terminally differentiated MEL cells were infected at a MOI of 10 PFU/cell and cytoplasmic RNA was harvested at 6 and 12 h post-infection. Where indicated infections were performed in the presence of 10 µg/mL of actinomycin D. The level of mouse β-globin mRNA in 10 µg of RNA was determined using the primer extension assay detailed above.

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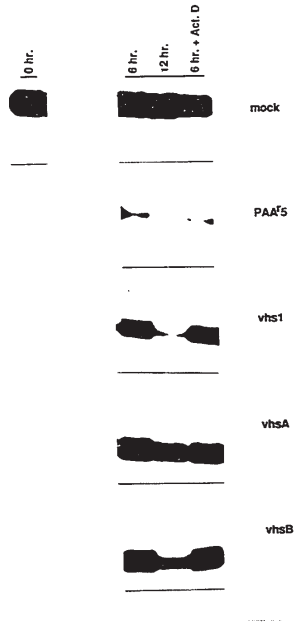


Figure 20. Single step growth curve analysis of the vhs mutants. Duplicate 35 mm dishes of Vero cells were infected at a MOI of 10 PFU/cell. Virus was harvested at the times indicated post-infection and virus yields were determined by the plaque assay described in the materials and methods. The results were expressed as an average number of PFU produced per infected cell.

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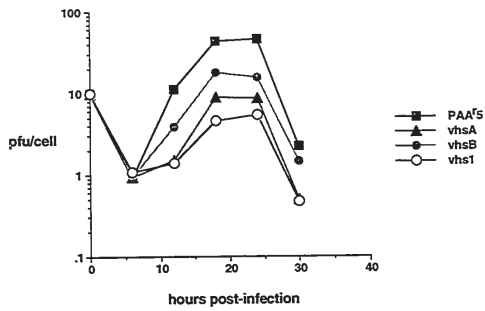
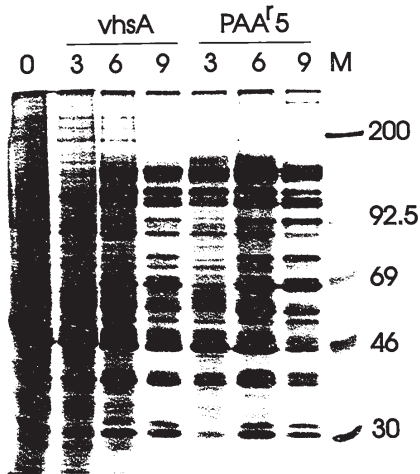


Figure 21. Viral protein synthesis in cells infected with the vhsA mutant. Cells were infected at a MOI of 10 PFU/cell with either vhsA or the parent virus PAA'5. At the times indicated post infection cells were labeled with ³⁵S-methionine in an excess of cold methionine for 1 h. Protein extracts were prepared and resolved through a 10% SDS-polyacrylamide gel. The marker proteins (lane M) are ¹⁴C-labeled proteins (Amersham) with sizes indicated in kDa.

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3.2.6 Construction of an HSV-2 vhs mutant.

As reviewed in the introduction, some strains of HSV-2 encode a more potent vhs function than strains of HSV-1. In addition, Fenwick and Everett (1990b) have shown that the HSV-2 vhs gene maps to the same location as the HSV-1 gene, and that it is responsible for the potent shutoff function of HSV-2 strain G (Fenwick and Everett, 1990a). In combination, these results imply that some investigations into UL41 function might be more successful if the HSV-2 UL41 gene is utilized. Therefore an HSV-2 vhs mutant was constructed using the ICP6-lacZ cassette detailed in section 3.2.1. The HSV-2 strain 333 vhs gene was first cloned into pUC18 as a 3 kbp Bam HI fragment, generating pBamHlvhs333. To generate pvhs333B, the Bam HI lacZ cassette was blunted with T4 DNA polymerase and cloned, in the vhs sense orientation, into the blunted Bst XI site found in the HSV-2 vhs gene. This insertion, which disrupts the vhs open reading frame after codon 30, was rescued into virus by co-transfection of pvhs333B with infectious HSV-2 strain 333 DNA. The structure of the mutant, designated 333vhsB (diagrammed in figure 22), was verified by Southern blot analysis of viral DNA cut with Bam HI and probed with pvhs333B (figure 23). Wild type 333 DNA gave one band that, as predicted, comigrated with the 3 kbp band from pvhs333. The 333vhsB mutant also gave one band that, as predicted, comigrated with the 7.3 kbp band from pvhs333B. Both plasmids gave an additional band at 2.7 kbp representing the pUC18 vector used to make these clones.

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3.2.7 The 333vhsB mutant does not inhibit host cell translation in the absence of viral gene expression.

The 333vhsB mutant was tested for its ability to inhibit cellular translation in the absence of viral gene expression. Vero cells were infected in the presence of actinomycin D and then labeled from 3-4 hours post-infection with ³⁵S-methionine in an excess of unlabeled methionine. After labeling, cell lysates were prepared and the levels of cellular translation were quantified by TCA precipitation (figure 24). The wild type 333 parental strain inhibited cellular translation by, on average, 97%. In contrast, the 333vhsB mutant reproducibly stimulated cellular translation, in this case by 1.7 fold. These results confirm that, as with HSV-1, an intact UL41 gene is required for HSV-2 to inhibit cellular translation. These results also suggest that HSV-2 virions carry a previously undetected function that stimulates cellular translation. This function was not detected prior to the construction of an HSV-2 vhs mutant as a functional vhs effect would mask this modest stimulation.

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Figure 22. Structure of the 333vhsB mutant. The HSV-2 genome displayed here is in the prototype arrangement and the structure of the wild type HSV-2 vhs gene is diagrammed with the open reading frame (▨) and the direction of transcription (←) indicated. The ICP6-lacZ cassette was inserted into the unique Bst XI site of the vhs gene. This insertion disrupts the vhs open reading frame after sequences predicted to encode amino acid 30.

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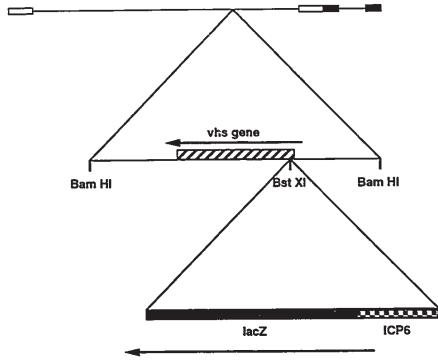


Figure 23. Southern blot analysis of the 333vhsB mutant. The structure of the 333vhsB mutant was confirmed by cutting 333vhsB viral DNA with Bam HI and resolving the digested DNA through a 1% agarose gel. After transferring the DNA to nitrocellulose, the blot was probed with labeled p333vhsB DNA, which was the plasmid used to make this mutant. p333vhsB and pBamHlvhs333 (which contains the wild-type 333 Bam HI vhs fragment) as well as the parental strain DNA 333 were also included in this analysis. The restriction fragment sizes indicated are in kbp.

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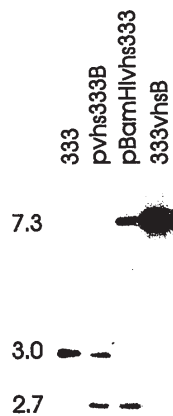


Figure 24. The effect of vhs mutations on the ability of HSV-2 to inhibit cellular translation. Duplicate wells of Vero cells were infected in the presence of 10 µg/mL of actinomycin D at a MOI of 10 PFU/cell. Cells were labeled from 3-4 h post-infection with ³⁵S-methionine in an excess of unlabeled methionine. The levels of cellular translation were quantified by measuring TCA precipitable counts in extracts prepared from infected cells. The levels of translation were expressed as a percentage of the average level of translation in mock infected cells.

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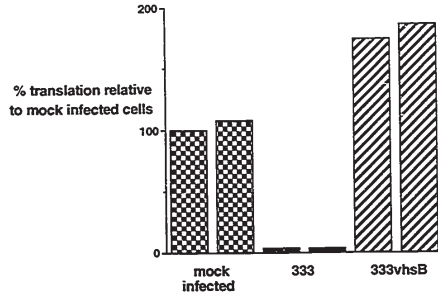
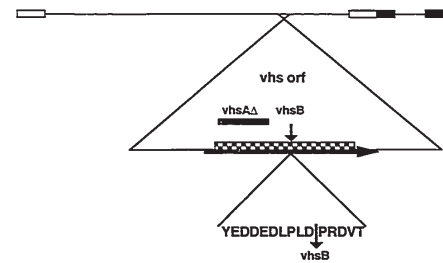


Figure 25. Sequence of the peptide used to make the vhs antipeptide antiserum. The location of the peptide used to make the vhs antipeptide antiserum, representing amino acids 333 to 347, is displayed on the vhs open reading frame (▨▨▨) and the direction of transcription is also indicated (→). The vhs open reading frame displayed in the figure is inverted with respect to its orientation in the prototype genome arrangement. The vhsA mutant is deleted for the sequences predicted to encode amino acids 22-179 of the vhs protein (■) as the result of insertion of the ICP6-lacZ cassette. The insertion site of the ICP6-lacZ cassette in the vhsB mutant occurs after codon 342 (↓) and as such the vhsB mutant contains 10 of the 15 amino acids present in the immunogenic peptide.



3.3 Identification and characterization of the vhs protein

3.3.1 Identification of the vhs protein.

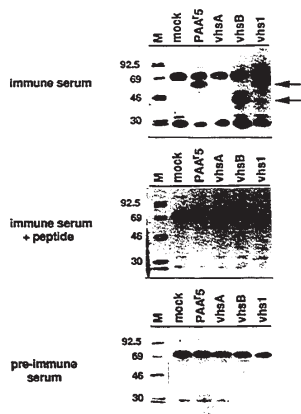
The construction of mutants in the UL41 open reading frame confirmed that UL41 is required for vhs function. To identify the protein encoded by the UL41 open reading frame (the vhs protein), I utilized a rabbit antipeptide antiserum generated by David Johnson. This antiserum was raised against a peptide corresponding to amino acids 333 to 347 of the predicted vhs amino acid sequence (figure 25). The antiserum was initially tested using proteins harvested from PAA'5, vhs1, vhsA, and vhsB infected cells. As some of these viruses bear mutations that should alter the electrophoretic mobility of the vhs protein, they were used to characterize the antiserum. PAA'5 contains a wild type vhs gene which is predicted to encode a 55kDa protein (Frink *et al.*, 1981; McGeoch *et al.*, 1988). vhs1 contains a single nucleotide substitution in the UL41 open reading frame that changes threonine 214 to an isoleucine (Frank Jones, personal communication). As this nucleotide substitution does not affect the sequences encoding the immunogenic peptide, the vhs1 protein was also predicted to react with the antipeptide antiserum. vhsA contains the lacZ cassette in place of sequences predicted to encode amino acids 22 to 179. Since this insertion occurs between the vhs initiator codon and the sequences encoding the immunogenic peptide, vhsA should not encode a protein capable of interacting with the antipeptide antiserum. vhsB contains the lacZ cassette inserted after codon 342. Based on the sequence of the ICP6 promoter region (McGeoch *et al.*, 1988), vhsB should encode a 43kDa vhs-related protein. The

amino terminus of this protein would consist of the first 342 amino acids of the UL41 open reading frame fused to 21 amino acids encoded by the ICP6 promoter region. Since this protein contains 10 of the 15 amino acids present in the immunogenic peptide, it might react with the antipeptide antiserum. Therefore, to characterize the antipeptide antiserum, Vero cells were infected with PAA'5, vhs1, vhsA, and vhsB and total protein extracts were prepared at 10 hours post-infection. These extracts were then resolved through a 10% SDS-polyacrylamide gel and subjected to Western blot analysis with pre-immune serum, immune serum, and immune serum plus competitor peptide (figure 26). Mock infected cell extracts contained two proteins (70kDa and <30kDa) that were detected by the immune serum. The 70kDa protein was also recognized by the pre-immune serum and did not compete with peptide; thus, this host protein appears to non-specifically interact with the rabbit serum. The second host protein of <30kDa was not detected by the pre-immune serum, and was competed by the peptide. Therefore, it likely represents a cross-reacting cellular protein. PAA'5 and vhs1 infected cell extracts contained a single virus-specific protein of 58kDa that was detected by the immune serum, competed with peptide, and was not recognized by the pre-immune serum. This 58kDa protein was not present in extracts from vhsB infected cells, which instead contained a 43kDa protein that was specifically detected by the immune serum. In contrast, the vhsA extract contained no virus specific proteins detectable with the immune serum. These data suggest that the 58kDa protein is the UL41 encoded vhs protein since its mobility was predictably altered by mutations in the UL41 gene.

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Figure 26. Western blot detection of the vhs protein in extracts from infected cells. Vero cells were infected at a MOI of 10 PFU/cell with the indicated viruses. At 10 h post-infection protein extracts were prepared and resolved through a 10% SDS-polyacrylamide gel. Proteins were then transferred to nitrocellulose and subjected to Western blot analysis using rabbit pre-immune serum, the rabbit antipeptide antiserum, and the antipeptide antiserum in the presence of 10 µg/mL of peptide competitor. The positions of the 58kDa vhs protein encoded by PAA'5 and the 43 kDa vhs related protein encoded by vhsB are indicated by the arrows. The marker proteins (lane M) are ¹⁴C-labeled proteins (Amersham) with sizes indicated in kDa.

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Using the same antipeptide antiserum, I next characterized the expression of the vhs protein in PAA'5 infected Vero cells as a function of time post-infection (figure 27). The vhs protein accumulated with late kinetics, in that appreciable levels were not detected until 8 hours post-infection and subsequently increased between 8 and 12 hours post-infection.

3.3.2 The vhs protein is packaged into virus particles.

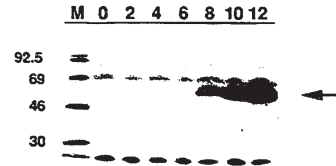
Since the vhs effect occurs in the absence of viral gene expression it is therefore mediated by a virion component. This suggests that the vhs protein might be packaged into virus particles. To test this possibility, the amount of vhs protein in a partially purified preparation of PAA'5 virions was compared to the starting crude lysate. Virions were purified on dextran T-10 gradients and vhs protein levels were quantified using the antipeptide antiserum in a Western blot analysis (figure 28). This purification resulted in a 8 to 16 fold enrichment of the vhs protein, thereby demonstrating that the vhs protein co-purifies with virions on a dextran T-10 gradient. This result therefore suggests that the vhs protein is packaged into virions.

3.3.3 Immunoprecipitation of the vhs protein.

To further characterize the vhs protein, the antipeptide antiserum was employed in immunoprecipitation assays on metabolically labeled cell extracts. Initially, cells were infected with PAA'5, or vhsB, or mock infected, and then labeled from 8 to 10 hours post-infection with ³⁵S-methionine. After labeling,

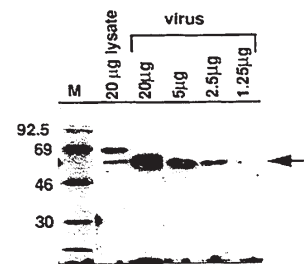
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Figure 27. The time course of vhs protein expression. Vero cells were infected at a MOI of 10 PFU/cell with HSV-1 PAA'5 and at the indicated times post-infection protein extracts were prepared and the levels of vhs protein were assayed using the antipeptide antiserum in a Western blot. The position of the 58kDa vhs protein is indicated by the arrow. The marker proteins (lane M) are ¹⁴C-labeled proteins (Amersham) with sizes indicated in kDa.



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Figure 28. Detection of the vhs protein in a partially purified preparation of HSV-1 virions. PAA'5 virions were purified as described in the materials and methods and the indicate amount of purified material was resolved through a 10% SDS-polyacrylamide gel. Twenty μ g of the starting crude lysate was also loaded on the gel. After electrophoresis, the levels of vhs protein were assayed using the antipeptide antiserum in a Western blot. The position of the 58kDa vhs protein is indicated by the arrow. The marker proteins (lane M) are ¹⁴C-labeled proteins (Amersham) with sizes indicated in kDa.



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cell extracts were immunoprecipitated with pre-immune serum, immune serum, or immune serum plus peptide competitor (figure 29). No proteins were immunoprecipitated from mock-infected cells. Both PAA'5 and vhsB infected cell extracts contained three proteins in the range of 70kDa that were immunoprecipitated by both the pre-immune serum and the immune serum. These proteins are the HSV-1 Fc receptor, which is a complex of the mature and immature forms glycoproteins gE and gI (Johnson *et al.*, 1988). Thus, the Fc receptor consists of four proteins which did not completely resolve in this experiment. In addition, PAA'5 and vhsB infected cell extracts contained a 58kDa protein and a 43kDa protein respectively, that were immunoprecipitated by the immune serum, competed with peptide and were not detected by the pre-immune serum. Therefore, the vhs protein is immunoprecipitated by the antipeptide antiserum.

Extracts from PAA'5, vhsA, and vhsB infected cells also contained a 140kDa protein (p140) that was only detected by the immune serum and was strongly competed by peptide (figure 29 and 30). As the mobility of p140 was not affected by mutations in the vhs gene, it is not encoded by the UL41 open reading frame. Also, since p140 was detected in a vhsA (a vhs null mutant) extract, it is not co-immunoprecipitated with the vhs protein. These results suggest that p140 cross-reacts with the antipeptide antiserum through a shared epitope. The large subunit of the viral ribonucleotide reductase, ICP6, is 140kDa and therefore p140 might be ICP6. I tested this possibility by infecting Vero cells with PAA'5 or hrR3 (Goldstein and Weller, 1988a). hrR3 is a HSV-1 KOS ICP6 mutant that expresses a 155kDa ICP6-related protein which consists of the amino terminal 434 residues of ICP6 fused to the β -galactosidase protein.

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Figure 29. Immunoprecipitation of the vhs protein. Vero cells were infected with PAA'5 or vhsB at a MOI of 10 PFU/cell. Infected cells, as well as mock infected cells, were labeled from 8-10 h post-infection with ³⁵S-methionine. Cell extracts were prepared and immunoprecipitated with rabbit pre-immune serum, the rabbit antipeptide antiserum, and the antipeptide antiserum in the presence of 10 μ g of peptide competitor. Immunoprecipitated proteins were then resolved through a 10% SDS-polyacrylamide gel. The positions of the 58kDa vhs protein encoded by PAA'5 and the 43 kDa vhs related protein encoded by vhsB are indicated by the arrows. The marker proteins (lane M) are ¹⁴C-labeled proteins (Amersham) with sizes indicated in kDa.

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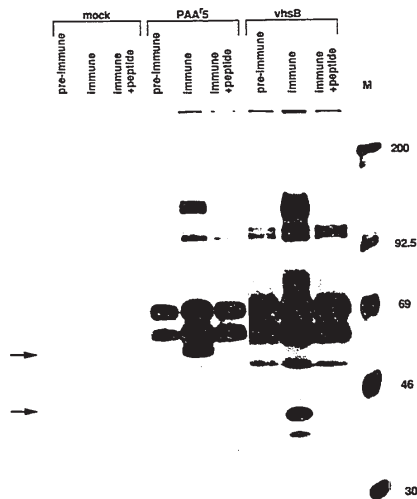


Figure 30. Immunoprecipitation of p140kDa from extracts infected with vhsA. Vero cells were infected with vhsA at a MOI of 10 PFU/cell and labeled from 8-10 h post-infection with ³⁵S-methionine. A cell extract was then prepared and immunoprecipitated with rabbit pre-immune serum, the rabbit antipeptide antiserum, and the antipeptide antiserum in the presence of 10 μ g of peptide competitor. Immunoprecipitated proteins were then resolved through a 10% SDS-polyacrylamide gel. The marker proteins (lane M) are ¹⁴C-labeled proteins (Amersham) with sizes indicated in kDa.

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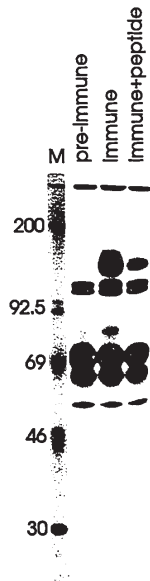


Figure 31. Immunoprecipitation of the vhs protein from hrR3 infected cell extracts. Vero cells were infected with PAA'5 or hrR3 at a MOI of 10 PFU/cell and labeled with ^{35}S -methionine from 8-10 h post-infection. Cell extracts were prepared and immunoprecipitated with rabbit pre-immune serum, the rabbit anti-peptide antiserum, and the anti-peptide antiserum in the presence of 10 μg of peptide competitor. Immunoprecipitated proteins were then resolved through an 8% SDS-polyacrylamide gel. The arrows indicate the position of: the wild type 140kDa ICP6 protein encoded by PAA'5, the 155kDa ICP6 related protein encoded by hrR3, and the vhs protein. The marker proteins (lane M) are ^{14}C -labeled proteins (Amersham) with sizes indicated in kDa.

After labeling the infected cells from 8-10 hours post-infection with ^{35}S -methionine, cell extracts were immunoprecipitated with the pre-immune serum, immune serum, or immune serum plus peptide competitor (figure 31). As expected, both the PAA'5 and hrR3 extracts contained the 58kDa vhs protein. Also, in place of p140, the hrR3 extract contained a 155kDa protein that was specifically recognized by the immune serum. This experiment demonstrated that p140 was ICP6. Despite these results, no obvious sequence similarities between the immunogenic peptide and the ICP6 protein have been detected.

To test if the vhs protein is phosphorylated, Vero cells were infected with PAA'5, or vhsB, or mock infected, and labeled from 6 to 10 hours post-infection with $^{32}\text{PO}_4$. Cell extracts were then immunoprecipitated with pre-immune serum, immune serum or immune serum plus peptide competitor (figure 32). Since ICP6, gE, and gI are phosphoproteins (C. Brunetti, S. Bacchetti, personal communication), all of these proteins were detected in $^{32}\text{PO}_4$ -labeled infected cell extracts. The immune serum also detected a 58kDa protein in PAA'5 extracts, that was competed by peptide and was not recognized by the pre-immune serum. This 58kDa protein is the vhs protein as it was not detected in vhsB infected cells. Thus the vhs protein is a phosphoprotein. In addition, the 43kDa vhsB protein was not convincingly detected in $^{32}\text{PO}_4$ -labeled extracts. Since the vhsB protein lacks the carboxy terminal 147 residues of intact protein, these results suggest that efficient phosphorylation requires the carboxy terminal residues of vhs.

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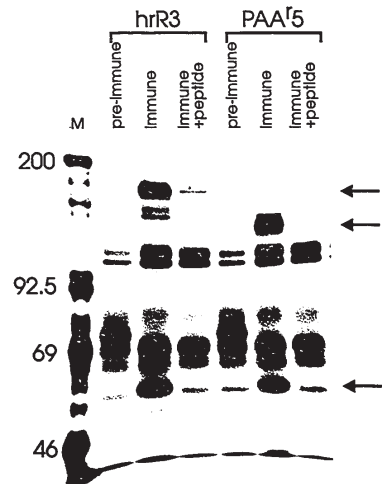


Figure 32. The immunoprecipitation of the vhs protein from cells labeled with $^{32}\text{PO}_4$. Vero cells were infected with PAA⁵ or vhsB at an MOI of 10 PFU/cell. Infected cells, as well as mock infected cells, were labeled from 6-10 h post-infection with $^{32}\text{PO}_4$. Cell extracts were prepared and immunoprecipitated with rabbit pre-immune serum, the rabbit antipeptide antiserum, and the antipeptide antiserum in the presence of 10 μg of peptide competitor. Immunoprecipitated proteins were resolved through a 10% SDS-polyacrylamide gel. The positions of the 58kDa vhs protein is indicated by the arrow. The marker proteins (lane M) are ^{14}C -labeled proteins (Amersham) with sizes indicated in kDa.



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3.4 Identification and characterization of vhs binding proteins

3.4.1 Expression of the vhs protein in *E. coli*

In an effort to understand how the vhs protein inhibits cellular translation and degrades mRNA, I searched for proteins that interact with the vhs protein. Potential vhs binding proteins were identified using an affinity resin prepared by coupling the vhs protein to CNBr activated Sepharose beads. This approach required large quantities of vhs protein which were generated using the *E. coli* expression vector pRIT2T (Pharmacia). This vector allows for inducible expression of protein A fusions which are then purified on IgG Sepharose columns. To facilitate the construction of a pRIT2T plasmid containing the vhs open reading frame, the polymerase chain reaction was used to engineer an Eco RI site immediately upstream of the UL41 initiator codon, generating pvhsR1. This plasmid contains a 1.7 kbp EcoRI to Hinc II fragment which was cloned between the EcoRI and Sma I sites of pRIT2T, generating pRITvhs. Lysates from *E. coli* containing either pRITvhs, (expressing the vhs/protein A fusion), or pRIT2T, (expressing protein A), were purified on IgG Sepharose and the purified proteins were resolved through a 12% SDS-polyacrylamide gel and stained with Coomassie blue (figure 33). Both preparations contained a 78kDa *E. coli* protein that bound to the IgG column; this protein is also present in *E. coli* lacking plasmid DNA (John Capone, personal communication). The protein A sample contained a 31kDa protein; the size predicted for the protein A encoded by pRIT2T, whereas the vhs/protein A sample contained a 65kDa protein; considerably smaller than the size predicted for the intact vhs/protein A.

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Figure 33. Purification of the vhs protein expressed in *E. coli*. A mid-log phase culture of *E. coli* containing pRITvhs was shifted from 30°C to 42°C to induce expression of vhs/protein A fusion. A cell extract was then prepared and loaded onto an IgG Sepharose column. After washing the column, the vhs/protein A fusion was eluted and dialyzed as described in the materials and methods. Ten μg of the purified vhs/protein A fusion and ten μg of similarly purified protein A were resolved through a 12% SDS-polyacrylamide gel and stained with Coomassie blue. The sizes of the marker proteins (lane M) (Sigma) are indicated in kDa.

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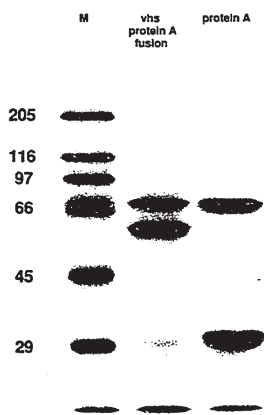


Figure 34. Detection of cellular and viral vhs binding proteins. Vero cells were infected with PAA'5 at a MOI of 10 PFU/cell or mock infected and subsequently labeled from 2-4 h post-infection with ³⁵S-methionine. Cell extracts were then prepared and mixed with either vhs Sepharose (prepared using the vhs/protein A fusion purified from *E. coli*) or protein A Sepharose. After mixing, the beads were washed and bound protein was eluted by boiling in SDS. Eluted material was then resolved through a 12% SDS-polyacrylamide gel. The marker proteins (lane M) are ¹⁴C-labeled proteins (Amersham) with sizes indicated in kDa.

fusion (81kDa). Assuming that the 65kDa protein contains intact protein A sequences, this fusion carries the amino terminal two-thirds of the vhs protein.

3.4.2 Detection of cellular and viral proteins that interact with the vhs protein.

To identify viral and cellular proteins that interact with the vhs protein, an affinity resin and a control resin were generated by linkage of the purified vhs/protein A fusion and protein A, respectively (figure 33) to CNBr activated Sepharose beads. PAA'5 and mock infected cells were labeled from 2 to 4 hours post-infection with ³⁵S-methionine and cell extracts were mixed with either vhs Sepharose or protein A Sepharose. After mixing, the beads were washed extensively and bound material was eluted by boiling in SDS and resolved through a 12% SDS-polyacrylamide gel (figure 34). The PAA'5 infected cell extract contained a 65kDa protein that was retained by the vhs Sepharose but not the protein A Sepharose. In mock infected cells, four proteins of 63kDa, 57kDa, 47kDa, and 43kDa appeared to specifically interact with the vhs Sepharose. Repetition of this experiment however demonstrated that only the 57kDa and 47kDa proteins were consistently and selectively retained by the vhs Sepharose, whereas the 43kDa protein was normally retained by both resins and the 63kDa protein was not routinely detected.

To determine if any cellular phosphoproteins interact with the vhs protein, ³²PO₄-labeled uninfected cell extracts were mixed with either the vhs Sepharose or the protein A Sepharose and bound material was resolved through a 12% SDS-polyacrylamide gel (figure 35). I found that the vhs

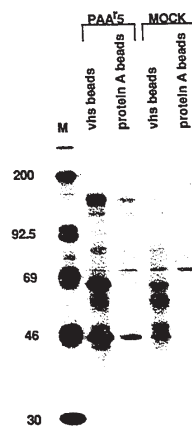
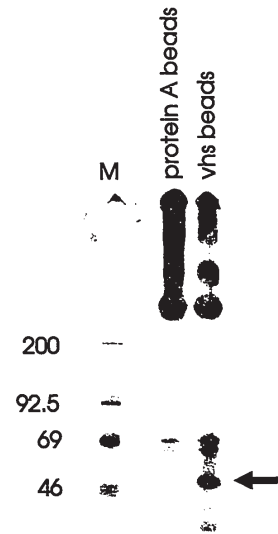


Figure 35. Detection of a phosphorylated cellular vhs binding protein.

Vero cells were labeled for 4 h with $^{32}\text{P}\text{O}_4$ and a cell extract was prepared and mixed with either protein A Sepharose or vhs Sepharose. After mixing, the beads were washed and bound protein was eluted by boiling in SDS and resolved through a 12% SDS-polyacrylamide gel. The arrow indicates the position of the 47kDa cellular phosphoprotein that interacts specially with the vhs Sepharose. The marker proteins (lane M) are ^{14}C -labeled proteins (Amersham) with sizes indicated in kDa.



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Sepharose specifically retained one cellular phosphoprotein of 47kDa, suggesting that the 47kDa ^{35}S -methionine labeled vhs binding protein might be a phosphoprotein.

3.4.3 Identification of the 65kDa virus-induced vhs binding protein as VP16.

I next set out to identify the 65kDa virus-specific vhs binding protein. Figure 36 shows that this protein comigrated with the 65kDa HSV transcription factor, VP16, that was immunoprecipitated from infected cell extracts with LP1 (a VP16 specific monoclonal antibody). To test the possibility that these two proteins were identical, protein bound to the vhs Sepharose was eluted by boiling in SDS, diluted, and then immunoprecipitated with LP1. Figure 36 shows that LP1 detected VP16 in protein eluted from either the vhs Sepharose or the LP1 immunoprecipitation, while no protein was detected in these eluates using either LP2 (a HSV glycoprotein D monoclonal antibody) or immunoprecipitation in the absence of antibody. In addition, no protein was detected in material eluted from the protein A Sepharose by any treatment. These results therefore demonstrate that the vhs protein produced in *E. coli* binds to VP16 from infected cell extracts.

3.4.4 A vhs antiserum immunoprecipitates vhs in a complex with VP16

To determine if VP16 is co-immunoprecipitated with the vhs protein, a rabbit antiserum raised against the vhs/protein A fusion was used (this antiserum was generated by Jennifer Newton). Extracts, prepared from cells infected with

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Figure 36. Identification of the 65kDa vhs binding protein as VP16.

Vero cells were infected at a MOI of 10 PFU/cell with PAA75 and labeled from 2-4 h post-infection with ^{35}S -methionine. A cell extract was prepared and mixed with either vhs Sepharose or protein A Sepharose, or immunoprecipitated with the VP16 specific monoclonal antibody LP1. The protein bound to the vhs beads, the protein A beads or the LP1 immunoprecipitation beads was eluted by boiling in SDS. A fraction of the eluate was resolved through a 10% SDS-polyacrylamide gel while the remainder was diluted and immunoprecipitated with LP1, or LP2 (a monoclonal antibody against HSV glycoprotein D) or immunoprecipitated in the absence of antibody. The marker proteins (lane M) are ^{14}C -labeled proteins (Amersham) with sizes indicated in kDa.

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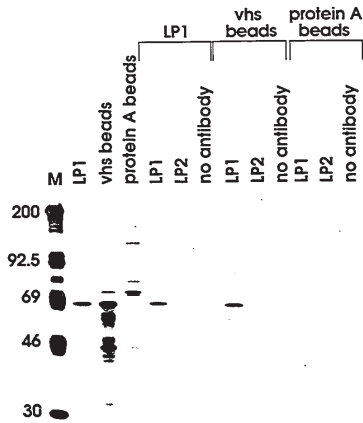


Figure 37. A polyclonal vhs serum immunoprecipitates the vhs protein and VP16. Vero cells were infected with PAA'5 and cells were labeled from 4-6 h post-infection with ³⁵S-methionine. An extract was prepared and immunoprecipitated with either the rabbit polyclonal vhs serum (raised against the vhs protein expressed in *E. coli*), rabbit pre-immune serum, or LP1. The proteins immunoprecipitated were eluted by boiling in SDS and some of this material was resolved through a 10% SDS-polyacrylamide gel while the remaining material was diluted and immunoprecipitated with LP1 or LP2. The marker proteins (lane M) are ¹⁴C-labeled proteins (Amersham) with sizes indicated in kDa.

PAA'5 and labeled from 4 to 6 hours post-infection with ³⁵S-methionine, were pre-treated with rabbit IgG and protein A Sepharose to remove the HSV Fc receptor. These pre-treated extracts were then immunoprecipitated with the vhs serum, pre-immune serum, or LP1 (figure 37). The immune serum and pre-immune serum reacted non-specifically with several proteins. In addition, the vhs antiserum specifically immunoprecipitated two proteins, the 58kDa vhs protein and a 65kDa protein that co-migrates with VP16.

To test if this 65kDa protein was VP16, LP1 was used to assay for VP16 in protein eluted from the vhs serum immunoprecipitations. Figure 37 shows that LP1 detected VP16 from the protein initially immunoprecipitated with either the vhs serum or LP1, but detected no protein in the eluate from the pre-immune serum immunoprecipitation. Also, the LP2 monoclonal antibody detected no protein in any of the eluted samples. These results demonstrate that the vhs serum specifically immunoprecipitated both the vhs protein and VP16 from infected cell extracts.

To confirm that the vhs serum immunoprecipitates VP16, a partial V8 protease analysis was performed on VP16 immunoprecipitated by either the vhs antiserum or LP1 (figure 38). Both proteins displayed similar V8 digestion patterns, thereby confirming that the 65kDa protein immunoprecipitated by the vhs serum was VP16.

There are two possible explanations for the immunoprecipitation of VP16 by the vhs serum: either VP16 and vhs form a complex, or the vhs serum contains VP16 specific antibodies. If VP16 and the vhs protein form a complex, the vhs serum would not immunoprecipitate VP16 from extracts that are devoid of the vhs protein. To examine this issue, vhsA infected cell extracts were

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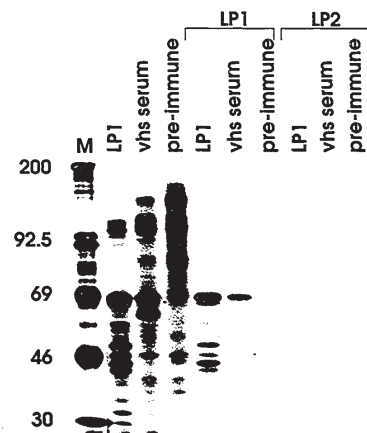
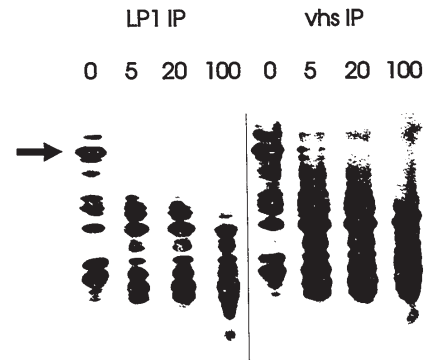


Figure 38. Comparison of the Partial V8 protease map of VP16 immunoprecipitated by LP1 and VP16 immunoprecipitated by the vhs polyclonal antiserum. Vero cells were infected with PAA'5 at a MOI of 10 PFU/cell and labeled with ³⁵S-methionine from 4-6 h post-infection. VP16 was immunoprecipitated from this extract with either the polyclonal vhs antiserum or with LP1 and the results of this immunoprecipitation were resolved through a 10% SDS-polyacrylamide gel. The VP16 immunoprecipitated by both antibodies was cut from the gel, rehydrated, and treated with 0, 5, 20, and 100 µg/mL of V8 protease. The resulting digestion products were resolved through an 18% SDS-polyacrylamide gel. The arrow indicates the position of intact VP16.



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immunoprecipitated with the vhs serum (figure 39). None of the treatments employed detected any proteins from mock infected cell extracts. The vhs serum detected both the vhs protein and VP16 in PAA'5 infected cell extracts. In contrast, the vhs serum failed to immunoprecipitate VP16 from the vhsA mutant despite the detection of VP16 using the LP1 monoclonal antibody. These results demonstrate that immunoprecipitation of VP16 by the vhs serum requires the vhs protein and suggests that the vhs protein and VP16 form a complex in infected cell extracts.

3.4.5 The vhs protein translated *in vitro* interacts with VP16 produced in *E. coli*

To determine which parts of the vhs protein are required to interact with VP16, various mutant forms of vhs were expressed using an *in vitro* transcription/translation system. These proteins were then assayed for their ability to bind to a VP16 affinity resin. To determine the feasibility of this approach, I initially tested if the intact vhs protein, translated *in vitro*, would interact with a VP16 affinity resin. This resin was prepared by linking a previously described VP16/protein A fusion (Wers'uck and Capone, 1989a), to CNBr activated Sepharose beads. To express the vhs protein *in vitro* the 1.7 kbp EcoRI to Hinc II fragment of pvhsR1 (see section 3.4.1) was cloned between the Eco RI and Hinc II sites of pGEM4Z (Promega). This construction places the vhs open reading frame under the control of the SP6 promoter, allowing for the production of vhs RNA *in vitro* using SP6 polymerase. The vhs RNA generated was then translated in rabbit reticulocyte lysate in the presence of

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Figure 39. VP16 is immunoprecipitated in a complex with the vhs protein. Vero cells were infected at a MOI of 10 PFU/cell with PAA'5 or vhsA. Infected cells, as well as mock infected cells, were labeled with ³⁵S-methionine from 4-6 h post-infection. Extracts were then immunoprecipitated with the polyclonal vhs serum, pre-immune serum, or LP1 and the immunoprecipitated proteins were resolved through a 10% SDS-polyacrylamide gel. The marker proteins (lane M) are ¹⁴C-labeled proteins (Amersham) with sizes indicated in kDa.

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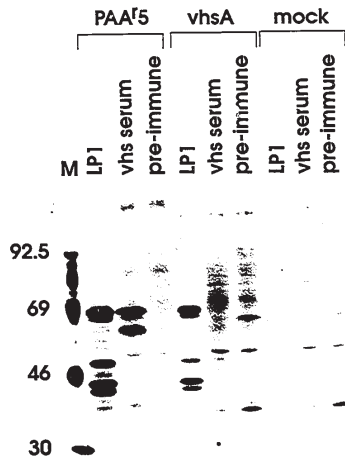


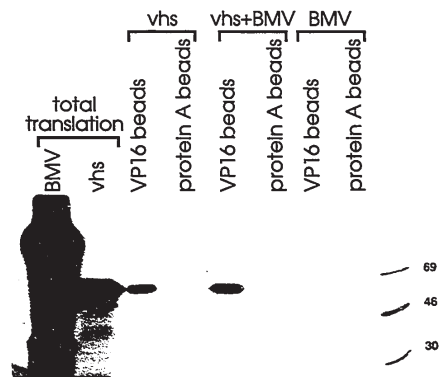
Figure 40. The vhs protein translated *in vitro* binds to a VP16 affinity resin. vhs RNA generated *in vitro* and BMV RNA (Promega) were translated in rabbit reticulocyte lysate (total translation) and the results of the translations were mixed separately or together with VP16 Sepharose or protein A Sepharose. After mixing, the unbound material was removed by washing and the bound material was eluted by boiling in SDS. The eluted material was then resolved through a 10% SDS-polyacrylamide gel. The marker proteins are ¹⁴C-labeled proteins (Amersham) with sizes indicated in kDa.

³⁵S-methionine and the resulting labeled protein was mixed with either VP16 Sepharose or protein A Sepharose. The beads were then washed and bound material was eluted and resolved through a 10% SDS-polyacrylamide gel (figure 40). The translation of the vhs protein *in vitro* resulted in a single major protein of 58kDa which was retained by VP16 Sepharose and not protein A Sepharose. As a control in this experiment, Brome mosaic virus (BMV) RNA (Promega) was translated in rabbit reticulocyte lysate giving five proteins ranging in size from 15 to 110kDa. Figure 40 shows that two of the BMV proteins were retained, to a small extent, by both the VP16 Sepharose and the protein A Sepharose. Mixture of the BMV and vhs translations together with the Sepharose beads demonstrated that the vhs protein is selectively retained by the VP16 Sepharose. These results not only confirm that the vhs protein and VP16 form a complex, they also demonstrate that no other HSV proteins are required.

3.4.6 A small portion of the vhs protein is necessary and sufficient for the interaction with VP16

To determine which portions of the vhs protein are required for binding to VP16 I employed the assay described above. First, several plasmids were constructed to express truncated and in frame deleted vhs proteins *in vitro*. Figure 41 shows that RNA transcribed *in vitro* from these plasmids directed the expression of the appropriately sized proteins in rabbit reticulocyte lysate. These proteins were then tested for their ability interact with VP16 Sepharose (figure 41 with data summary on figure 42). vhs proteins deleted for amino

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acids 24 to 180 (Δ Apa) or truncated beyond amino acid 344 (Bam amb) were still able to interact with VP16 Sepharose. In contrast, the deletion of amino acids 149 to 344 (Δ Sma) or truncation of the protein beyond amino acid 237 (Nru amb) resulted in proteins that were not retained by VP16 Sepharose. Taken in combination, these results suggest that amino acids 181 to 344 are required for the vhs protein to complex with VP16. Consistent with these results, proteins containing only amino acids 179 to 344 (Apa/Sma) or amino acids 238 to 344 (Nru/Sma) interacted with VP16 Sepharose. These results suggest that the amino acids between 238 and 344 are necessary and sufficient for the vhs protein to bind to VP16.

3.4.7 The VP16 acidic activation domain is not required for complex formation with vhs

In an effort to identify the VP16 residues required to interact with the vhs protein, several truncated and in frame deleted VP16 proteins were tested for their ability to bind to the vhs protein. These proteins were generated by *in vitro* translation of SP6-transcribed mRNAs (these RNAs were produced using plasmids provided by J. Capone and R. Wheatly, Dept. of Biochemistry, McMaster University). Figures 43 and 44 demonstrates that these mRNAs direct expression of the appropriately sized proteins. Each protein was tested for its ability to interact with vhs Sepharose and protein A Sepharose (figure 43 and 44, with results summarized in figure 45). None of the VP16 proteins translated *in vitro* were retained by the protein A Sepharose. In contrast, VP16 proteins truncated at residues 424, 403, 379, and 369 were retained by vhs Sepharose;

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Figure 41. Mapping of the VP16 binding domain on the vhs protein. Several mRNAs transcribed *in vitro*, which encode in frame deleted or truncated vhs proteins, were translated in rabbit reticulocyte lysate (total translation). Aliquots of these translations were mixed with either protein A Sepharose or vhs Sepharose. After unbound material was removed by washing, the bound protein was eluted by boiling in SDS and resolved through an 18% SDS-polyacrylamide gel. The marker proteins (lane M) are 14 C-labeled proteins (Amersham) with sizes indicated in kDa.

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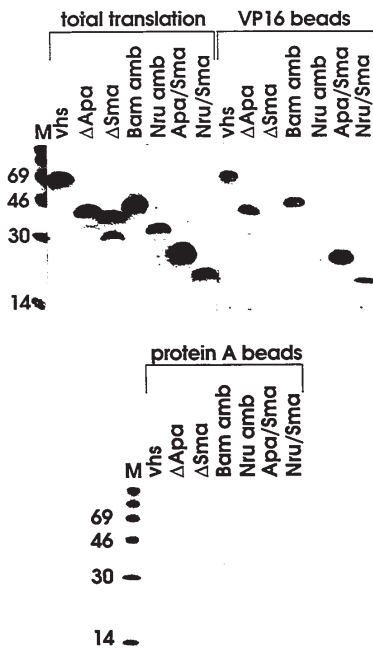


Figure 42. Regions of the vhs protein required to interact with VP16. Summary of data from figure 40. The various vhs constructs used in figure 40 are diagrammed with the deleted sequences or the amino acid at which the protein terminates indicated as well as their ability to bind to VP16.

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		binding to VP16
vhs	—————	+
ΔApa (24-180)	- —————	+
ΔSma (149-344)	————— —————	-
Bam amb (344)	—————	+
Nru amb (237)	—————	-
Apa/Sma (179-344)	—————	+
Nru/Sma (238-344)	—————	+

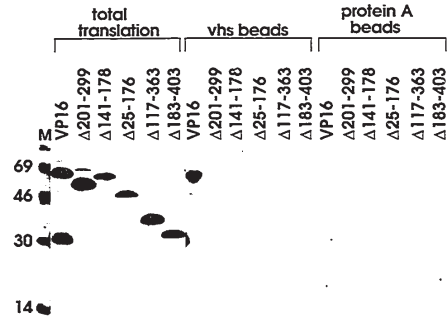
however the truncations at amino acids 379 and 369 appeared to bind with reduced affinity. Furthermore, proteins truncated beyond amino acid 335, were not retained by the vhs Sepharose. In addition, all in frame deletions prevented binding of VP16 to the vhs Sepharose. Although these results do not readily delineate a single vhs binding domain on VP16, they clearly demonstrate that the carboxy terminal acidic activation domain of VP16, located between amino acids 411 and 490, (Sadowski *et al.*, 1988; Cousens *et al.*, 1989), is not required for this interaction.

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Figure 43. Mapping of the vhs binding domain on the VP16 protein using truncated VP16 proteins translated in a rabbit reticulocyte lysate. Several mRNAs transcribed *in vitro*, which encode truncated VP16 proteins, were translated in rabbit reticulocyte lysate (total translation). Aliquots of these translations were mixed with either protein A Sepharose or VP16 Sepharose. After unbound material was removed by washing, the bound protein was eluted by boiling in SDS and resolved through an 18% SDS-polyacrylamide gel. The marker proteins (lane M) are ¹⁴C-labeled proteins (Amersham) with sizes indicated in kDa.

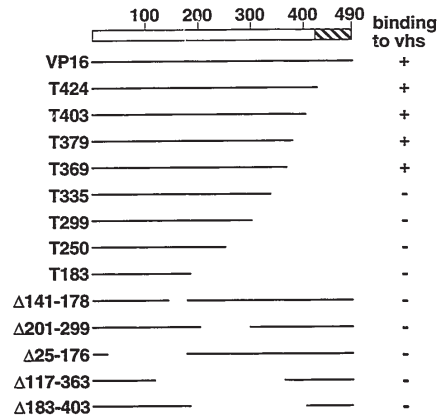


Figure 44. Mapping of the vhs binding domain on the VP16 protein using inframe deleted VP16 proteins translated in a rabbit reticulocyte lysate. Several mRNAs transcribed *in vitro*, which encode in frame deleted VP16 proteins, were translated in rabbit reticulocyte lysate (total translation). Aliquots of these translations were mixed with either protein A Sepharose or VP16 Sepharose. After unbound material was removed by washing, the bound protein was eluted by boiling in SDS and resolved through an 18% SDS-polyacrylamide gel. The marker proteins (lane M) are ¹⁴C-labeled proteins (Amersham) with sizes indicated in kDa.



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Figure 45. Regions of the VP16 protein required to interact with the vhs protein. Summary of data from figures 43 and 44. The various VP16 constructs used in figures 42 and 43 are diagrammed with the deleted sequences or the amino acid at which the protein terminates indicated as well as their ability to bind to the vhs protein.



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3.5 Functional significance of the VP16-vhs interaction

3.5.1 Description of the phenotype of the VP16 deletion mutant 8MA

What is the functional significance of the interaction between VP16 and the vhs protein? Perhaps this interaction downregulates the vhs function. This hypothesis was initially inferred from the phenotype of 8MA (Weinheimer *et al.*, 1992), an HSV-1 mutant deleted for the VP16 gene (the structure of the 8MA mutant is diagrammed in figure 46). This mutant virus will only replicate on cells that supply VP16 in trans, demonstrating that VP16 is required for virus growth in tissue culture. Non-complementing cells infected with 8MA show a striking inability to synthesize viral proteins late in infection (Steve Weinheimer, personal communication). To confirm this observation Vero cells were infected with either 8MA, 8MAR (a wild type rescue product of 8MA), or HSV-1 KOS (the wild type parent of 8MA) (figure 47). Infected cells were then labeled with ³⁵S-methionine for 1 hour at 2, 5, 8, and 11 hours post-infection and total protein was resolved through a 12% SDS-polyacrylamide gel. Infection with all three viruses led to the synthesis of virus specific proteins at 2 hours post-infection. At 8 and 11 hours post-infection, viral protein synthesis was detected in KOS and 8MAR infected cells, while little, if any, protein synthesis was detected in 8MA infected cells. In addition, this protein synthesis defect is reversed if 8MA infects 16-8 cells, which supply VP16 in trans (figure 47). This defect might be explained if VP16 is required to downregulate the vhs function. According to this hypothesis, in a wild type infection, newly synthesized vhs protein is downregulated by VP16. Therefore, in the absence of VP16, the vhs

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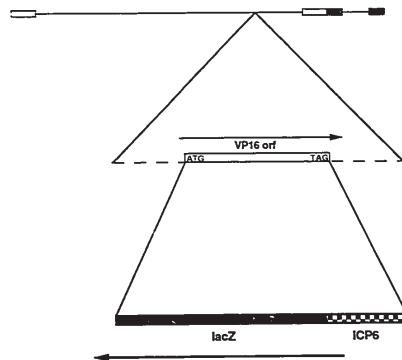
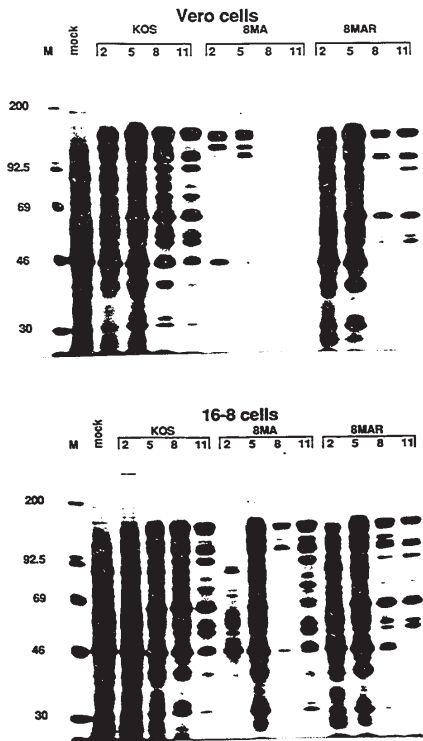


Figure 46. Structure of the 8MA mutant. The structure of the wild type VP16 open reading frame with the initiator ATG, the stop codon and the direction of transcription are indicated (→). In the 8MA mutant (Weinheimer *et al.*, 1992) the entire VP16 open reading frame is deleted and replaced with the ICP6-lacZ cassette used in the construction of the vhs mutants described above. The HSV-1 genome displayed here is in the prototype arrangement.

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Figure 47. Viral protein synthesis in cells infected with the 8MA mutant. In the upper panel Vero cells were infected at a MOI of 10 PFU/cell with 8MA, KOS (the wild type parent of 8MA), or 8MAR (a wild type rescue product of the 8MA mutant) and at the times indicated post-infection cells were labeled for 1 h with ³⁵S-methionine in an excess of unlabeled methionine. Cells extracts were then harvested and proteins were resolved through a 12% SDS-polyacrylamide gel. In the lower panel the experiment was repeated on 16-8 cells which express VP16 upon infection. The marker proteins (lane M) are ¹⁴C-labeled proteins (Amersham) with sizes indicated in kDa.

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protein, which accumulates late in infection (see section 3.3.1), degrades viral mRNA thereby inhibiting viral protein synthesis. This hypothesis predicts that inactivation of the vhs function in 8MA would reverse this defect. However, despite repeated attempts, I have been unable to generate such a mutant. I have therefore resorted to more indirect tests to examine this hypothesis.

If unregulated vhs activity underlies the 8MA protein synthesis defect, then 8MA should also display a defect in the accumulation of HSV mRNAs. To test this, Vero cells were infected with 8MA or 8MAR, and the levels of HSV encoded glycoprotein B (gB) mRNA were assayed by Northern blot analysis (figure 48). These results were quantified using a Phosphorimager and Imagequant software. At 3 hours post-infection the levels of gB mRNA were similar in 8MA and 8MAR infected cells. However, at nine hours post-infection the levels of gB mRNA in 8MA infected cells were 8 fold less than in 8MAR. Thus the 8MA protein synthesis defect is reflected at the level of mRNA, consistent with the possibility that VP16 downregulates the vhs function.

3.5.2 The vhs protein functions in a transient transfection

To determine if the vhs function is downregulated by VP16, a functional assay for the vhs effect was required. Therefore, I investigated the possibility that vhs would inhibit the expression of a co-transfected reporter gene in the absence of other viral factors. pRSVβgal, which contains the lacZ gene under the control of the RSV LTR, was selected as the reporter plasmid in these experiments. A vhs expression plasmid was constructed by inserting the vhs open reading frame into pRC/CMV, which contains the human cytomegalovirus

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Figure 48. Accumulation of glycoprotein B mRNA in cells infected with the 8MA mutant. Vero cells were infected with 8MA or 8MAR and at the times indicated post-infection cytoplasmic mRNA was harvest. Ten μg of RNA was resolved through a 1% agarose-formaldehyde gel, transferred to nitrocellulose, and probed with a oligonucleotide complementary to HSV-1 glycoprotein B mRNA.

8MAR				8MA					
0	3	6	9	12	3	6	9	12	
hr.									

gB mRNA



(HCMV) IE promoter/enhancer sequences. This plasmid, designated pCMVvhs, was constructed by Jennifer Newton. Following co-transfection of pRSVβgal with various amounts of pCMVvhs, cell extracts were assayed for β-galactosidase activity (figure 49). Co-transfection of 100 ng or 250 ng of pCMVvhs resulted in, on average, a 77% and 95% inhibition of β-galactosidase activity respectively. To ensure that this inhibition did not result from competition between the HCMV IE promoter/enhancer and the RSV LTR for transcription factors, the level of HCMV promoter in each transfection was held constant by transfection of an appropriate amount of pRC/CMV. These results suggest that the vhs gene, on its own, inhibits the accumulation of β-galactosidase activity. However, this assay may not accurately reflect the vhs function. To address this issue, Frank Jones has tested if the vhs1 mutant protein could inhibit β-galactosidase activity. The vhs1 protein contains an amino acid substitution at residue 214 (threonine to isoleucine) that renders it inactive for the vhs effect when delivered into cells by the virion (Read and Frenkel, 1983; G.S. Read and Frank Jones, personal communication). Frank has shown that the vhs1 protein does not inhibit β-galactosidase activity, suggesting that this assay accurately reflects vhs function.

3.5.3 VP16 does not inhibit vhs function in a transient assay

To test whether VP16 inhibits the vhs function I utilized the transient assay developed above. If VP16 downregulates vhs activity, then expression of VP16 in cells transfected with pCMVvhs and pRSVβgal might rescue expression of the lacZ reporter gene. The VP16 expression plasmid, pEVRF65, where the

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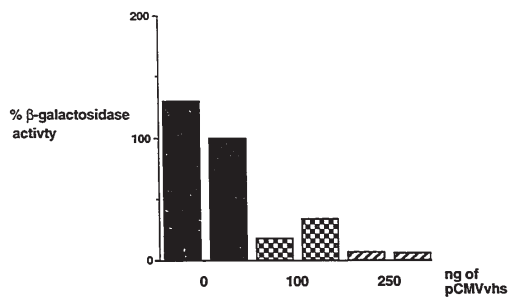


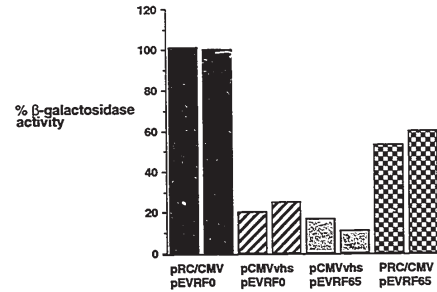
Figure 49. The vhs protein inhibits the expression of a co-transfected reporter gene in a transient transfection. Duplicate wells of Vero cells were transfected with 5 of pRSVβgal and 0, 100, and 250 ng of pCMVvhs, which expresses the vhs open reading frame under the control of the HCMV IE promoter/enhancer. Note that the amount of HCMV promoter/enhancer in each transfection was held constant by transfection of an appropriate amount of empty HCMV expression vector, pRC/CMV. Also, the amount of DNA in each transfection was held constant by the addition of an appropriate amount of pUC18 DNA. Cells extracts were prepared 48 h post-transfection and β-galactosidase activity in these extracts was determined as described in the materials and methods. The levels of β-galactosidase activity were expressed as a percentage of the β-galactosidase activity expressed in cells transfected with pRSVβgal and no pCMVvhs.

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VP16 gene is under the control of the HCMV IE promoter/enhancer was employed in these experiments. pEVRF65, supplied by John Capone, was constructed by inserting the VP16 open reading frame into the HCMV expression vector pEVRF0. Two-hundred and fifty ng of pCMVvhs was co-transfected with 1.28 mg of pEVRF65 and pRSVβgal, and β-galactosidase activity was determined (figure 50). In this experiment the amount of HCMV promoter was held constant by transfection of appropriate quantities of pRC/CMV and pEVRF0. Therefore, the base line β-galactosidase activity was determined in a transfection containing pRC/CMV and pEVRF0. pEVRF65 inhibited β-galactosidase activity by 44%, while pCMVvhs inhibited β-galactosidase activity by 78%. When pCMVvhs and pEVRF65 were co-transfected with the lacZ reporter β-galactosidase activity was inhibited by 86%. Given that pEVRF65 inhibited β-galactosidase by only 44%, and that a 6 fold molar excess of pEVRF65 over pCMVvhs was used, it seems likely that this experiment could have detected a significant reversal of vhs function by VP16. Therefore, these results demonstrate that VP16 did not inhibit vhs function in this transient assay. Assuming that the transient assay mimics vhs function faithfully, there are at least three possible interpretations of these results. For example, these results might suggest that VP16 does not downregulate the vhs function. Alternatively, VP16 might require other HSV products to downregulate the vhs function. Thus, when VP16 and the vhs protein are expressed on their own in this transient assay, no affect on vhs activity would be expected. Finally, VP16 may have to accumulate prior to the expression of the vhs protein to downregulate the vhs function. Thus, in the transient assay where both the vhs protein and VP16 are expressed under the control of the HCMV IE

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Figure 50. VP16 has little effect on the ability of the vhs protein to inhibit accumulation of β -galactosidase activity in a transient assay. Duplicate wells of Vero cells were transfected with pRSV β gal, in the presence or absence of the vhs expression vector pCMVvhs and the VP16 expression vector pEVRF65. The VP16 gene in pEVRF65 was under the control of the HCMV IE promoter/enhancer sequences found in the plasmid pEVRF0. In these experiments the levels of HCMV promoter/enhancer were held constant by the transfection of an appropriate amount of pEVRF0 and pRC/CMV and the total amount of DNA in each transfection was held constant by transfection of pUC18 DNA. Cells extracts were prepared 48 h post-transfection and β -galactosidase activity in these extracts was determined as described in the materials and methods. The levels of β -galactosidase activity were expressed as a percentage of the β -galactosidase activity in cells transfected with pRSV β gal and no pCMVvhs or pEVRF65.



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promoter/enhancer sequences (and presumably accumulate in parallel) downregulation of the vhs function would not occur.

3.5.4 VP16 inhibits the vhs function associated with HSV virions

As another approach to investigate the hypothesis that VP16 downregulates the vhs function, I tested the effects of overexpressing VP16 on the vhs function delivered into cells as part of an infecting virion. To overexpress VP16, I transfected cells with pEVRF65 and pRSV β gal. Following transfection, cells were superinfected with HSV-2 strain 333 and labeled from 3 to 4 hours post-infection cells with 35 S-methionine. Cell extracts were then immunoprecipitated with a β -galactosidase monoclonal antibody to determine the rate of β -galactosidase translation. This experimental design therefore allowed me to assess the effects of HSV infection on the synthesis of the β -galactosidase reporter protein in the presence or absence of VP16.

The initial experiment involved transfection of pRSV β gal, alone followed by infection with HSV-2 strain 333 or the HSV-2 333vhsB mutant (figure 51). The 155kDa β -galactosidase protein was detected in cells transfected with pRSV β gal but not in cells transfected with pUC18. Infection with 333 resulted in a vhs-dependent inhibition of β -galactosidase synthesis. However, when pRSV β gal was co-transfected with either 1 or 2 mg of pEVRF65, β -galactosidase synthesis became resistant to the inhibition mediated by the vhs function (figure 52). This effect required the VP16 open reading frame, since co-transfection of pEVRF0 did not protect β -galactosidase synthesis from the vhs effect (figure 52). Taken in combination, these results suggest that prior

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Figure 51. The expression of a transiently transfected lacZ reporter gene is inhibited by the virion associated vhs effect. Vero cells were transfected with either pUC18 or pRSV β gal. Forty-eight h post-transfection cells were infected at a MOI of 10 PFU/cell with either HSV-2 strain 333, 333vhsB (the 333 vhs mutant), or mock infected in the presence of 10 μ g/mL of actinomycin D. Cells were then labeled from 3-4 h post-infection with 35 S-methionine in an excess of unlabeled methionine. Cell extracts were then prepared and immunoprecipitated with an anti- β -galactosidase monoclonal antibody. The immunoprecipitates were resolved through a 10% SDS-polyacrylamide gel and the arrow indicates the position of the 155kDa β -galactosidase protein. The marker proteins (lane M) are 14 C-labeled proteins (Amersham) with sizes indicated in kDa.

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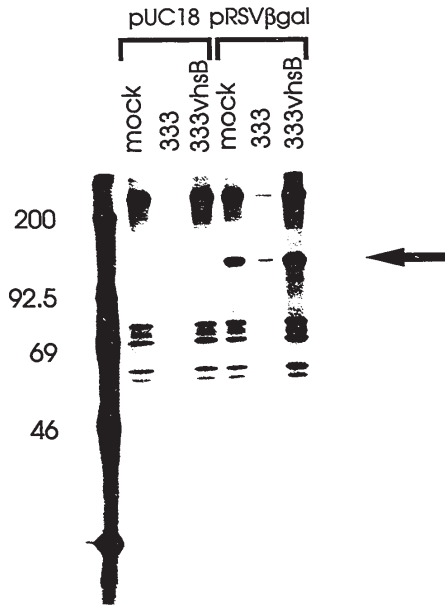
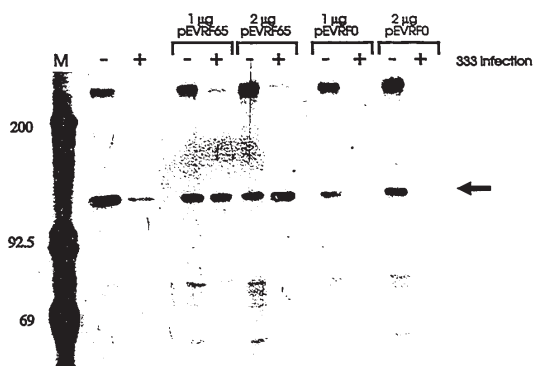


Figure 52. Transfection of cells with a VP16 expression plasmid renders them resistant to the vhs effect delivered by HSV-2 infection. Duplicate wells of Vero cells were transfected with 5 μ g of pRSV β gal and either 0, 1 or 2 μ g of either the VP16 expression plasmid pEVRF65 or the parental plasmid pEVRF0. One well of each pair was then infected with HSV-2 strain 333 in the presence of 10 μ g/mL of actinomycin D, while the other well was mocked infected under similar conditions. Cells were then labeled from 3–4 h post-infection with 35 S-methionine in an excess of unlabeled methionine and cell extracts were prepared and immunoprecipitated with an anti- β -galactosidase monoclonal antibody. The immunoprecipitates were resolved through a 10% SDS-polyacrylamide gel and the arrow indicates the position of the 155kDa β -galactosidase protein. The marker proteins (lane M) are 14 C-labeled proteins (Amersham) with sizes indicated in kDa.

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expression of VP16 inhibits the vhs function, however they do not reveal the mechanism underlying this inhibition. One possibility is that VP16 binds to the incoming vhs protein, thereby inhibiting the vhs effect. Alternatively, overexpression of VP16 might inhibit uncoating of the incoming virion, preventing release of active vhs protein into the cell. Given this ambiguity, these results provide only indirect support for the hypothesis that vhs function is directly downregulated by VP16.

3.5.5 Cells expressing a truncated form of VP16 are resistant to the vhs effect

The data described above suggest that cells transiently transfected with VP16 are resistant to host shutoff mediated by superinfecting HSV. If so, then one predicts that stably transformed cell lines which constitutively express high levels of VP16 should also be resistant to the vhs effect. I tested this prediction using a previously constructed mouse L cell line, designated line 4, that constitutively expresses the first 412 amino acids of VP16 (Friedman *et al.*, 1988). This cell line was constructed by unlinked co-transfection of the HSV-1 tk gene with the VP16 gene under the control of the Moloney murine sarcoma virus LTR. Given the *in vitro* mapping data presented in section 3.4.7, the truncated form of VP16 expressed by this cell line should bind to the vhs protein. To test if these cells were resistant to host shutoff, the susceptibility of cell line 4 to the vhs effect was determined as a function of input MOI. As a control, cell line 1A, which was constructed by transfection of only the tk selectable marker, was also used in this experiment. These cell lines were infected with HSV-2 strain 333 at various MOIs in the presence of actinomycin D

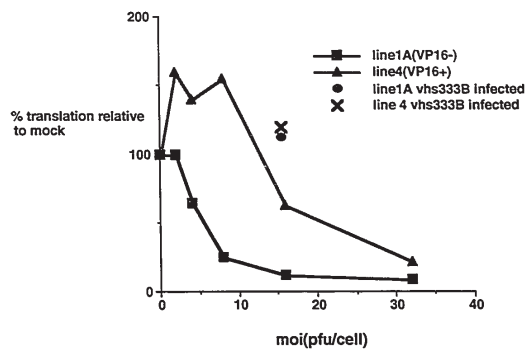
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and then labeled with ^{35}S -methionine from 3 to 4 hours post-infection. The levels of translation were then quantified by TCA precipitation. Figure 53 shows that at a MOI of 4 PFU/cell, line 4 showed a slight increase in the level of translation compared to mock infected cells, while line 1A displayed a 59% inhibition of protein synthesis. The slight increase in translation seen in cell line 4 is consistent with the ability of vhs-deficient 333 virions to boost cellular translation (see section 3.2.7). As the input MOI increased, cell line 4 showed a decrease in the levels of translation, but at all multiplicities translation was elevated compared to line 1A. Figure 53 also shows that the translation inhibition in both cell lines is vhs-dependent, as the 333vhsB mutant was defective for shutoff. Overall, these data indicate that cells which express VP16 are resistant to host shutoff. However, interpretation of these findings is subject to the same caveats described in the previous section.

3.5.6 Rescue of the 8MA phenotype in a transient transfection

In an effort to explore the 8MA protein synthesis defect in more detail, I developed a convenient transient assay to determine which regions of VP16 are required to sustain viral protein synthesis. This assay exploited the fact that transfected viral genes come under the control of HSV products when the transfected cells are superinfected with HSV. Thus, the expression of the transfected gene often mimics the expression of the gene located in the viral genome. To develop this assay, I employed ptkSB, which carries an in frame deleted HSV-1 tk gene under control of the tk promoter, and tested if a

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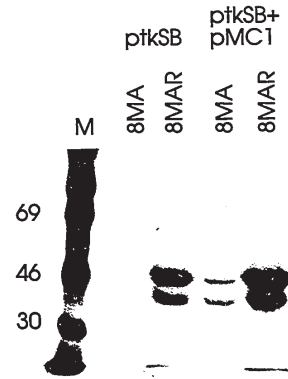
Figure 53. Cells that constitutively express VP16 are resistant to the vhs effect delivered by HSV-2 virions. Mouse L cell line, 4 which expresses the first 412 amino acids of VP16, and cell line 1A, which does not express VP16, were infected with HSV-2 strain 333 at various input multiplicities in the presence of 10 $\mu\text{g}/\text{mL}$ of actinomycin D. Each cell line was also infected at a MOI of 16 PFU/cell with the 333 vhs mutant 333vhsB. Three to four h post-infection cells were labeled with ^{35}S -methionine in an excess of unlabeled methionine and cell extracts were prepared. The levels of cellular translation were then determined by measuring TCA precipitable counts in these extracts. The amount of translation was expressed as a percentage of translation in mock infected cells.

transiently transfected VP16 gene would rescue expression of this tk reporter gene in 8MA infected cells. This approach therefore required that the tk reporter gene be subject to the protein synthesis defect in 8MA infected cells. To address this issue, cells were transfected with 5 μg ptkSB and superinfected with either 8MA or 8MAR. Cells were then labeled with ^{35}S -methionine from 9 to 10 hours post-infection, and extracts were immunoprecipitated with a rabbit antiserum against HSV-1 tk (figure 54). This approach allowed me to evaluate the synthesis of both the virally encoded tk protein and the plasmid encoded tk protein, due to the deletion in ptkSB. In cells transfected with ptkSB and superinfected with 8MAR the 46kDa virally encoded tk protein and the 38kDa in frame deleted tk protein were detected. However, in cells superinfected with 8MA, neither tk protein was detected, suggesting that expression of the transfected gene was subject to the 8MA protein synthesis defect.

I next tested if co-transfection of a VP16 gene under the control of its own promoter would rescue the synthesis of the transfected tk gene. In this experiment, the VP16 gene was supplied by pMC1 and cells were co-transfected with 5 mg of ptkSB and 5 mg pMC1 and then superinfected with 8MA. Figure 54 shows that expression of both the transfected tk gene and the virally transduced tk gene were detected, demonstrating that a transfected VP16 gene can rescue the 8MA protein synthesis defect. Therefore, using various mutant VP16 molecules in this assay, one could define which regions of VP16 are required to rescue the 8MA phenotype. Such analysis might demonstrate that the regions required to sustain viral protein synthesis are also required to bind to the vhs protein. This would suggest that VP16 must bind to the vhs

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Figure 54. Transfection of a VP16 gene under the control of the native VP16 promoter reverses the 8MA mutants protein synthesis defect. The plasmid ptkSB, which contains the HSV-1 tk gene under the control of its own promoter, was transfected into Vero cells along or in the presence of pMC1, which carries the VP16 gene under the control of its own promoter. Forty-eight h post-transfection cells were infected with the 8MA mutant or 8MAR. Cells were then labeled from 9-10 h post-infection with ³⁵S-methionine in an excess of unlabeled methionine and tk proteins were immunoprecipitated using a tk antiserum. The marker proteins (lane M) are ¹⁴C-labeled proteins (Amersham) with sizes indicated in kDa.



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protein to rescue the 8MA phenotype and therefore lend support to the hypothesis that VP16 downregulates the vhs function.

Discussion

When HSV infects a cell, viral genes are expressed to high levels, while cellular gene expression is dramatically inhibited. The major goal of the work described in this thesis was to understand the molecular basis for the differential expression of viral and cellular genes in HSV infected cells. A two part-approach was taken to examine this problem. First, I compared the expression of virally-transduced and endogenous β -globin genes in HSV infected cells. These experiments were prompted by previous work which demonstrated that an HSV transduced rabbit β -globin gene is efficiently expressed in infected Vero cells (Smiley *et al.*, 1987), a result that was in apparent conflict with previous experiments demonstrating that HSV infection inhibits the expression of the mouse β -globin gene resident in MEL cells (Nishioka and Silverstein, 1977, 1978). This approach demonstrated that HSV products inhibit the expression of an endogenous β -globin gene while transactivating the expression of a virally transduced β -globin gene. These results illustrate that the selective inhibition of cellular gene expression plays an important role in the preferential expression of genes associated with the viral genome. Therefore, the second approach involved a detailed investigation of the herpes simplex virus vhs gene product. This protein is one of the viral functions that inhibits cellular gene expression.

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4.1 The expression of virally-transduced and endogenous β -globin genes in HSV infected MEL cells

To monitor the effect of HSV regulatory proteins on the expression of transduced and endogenous β -globin genes, the HSV-1 recombinant L7/14, which bears the rabbit β -globin gene, was used to infect MEL cells. I found that the rabbit β -globin gene was efficiently expressed, inasmuch as the levels of mRNA were similar to those reported for both rabbit β -globin in L7/14 infected Vero cells (Smiley *et al.*, 1987) and HSV-1 tk mRNA in infected Vero cells (McKnight *et al.*, 1981). The expression pattern of the rabbit β -globin gene was typical of an HSV early gene in two ways: mRNA levels peaked at 6 hours post-infection, declining thereafter, and the accumulation of β -globin transcript required prior synthesis of HSV proteins. This kinetic pattern and the requirement for protein synthesis was previously seen in L7/14 infected Vero cells (Smiley *et al.*, 1987). It was possible that the requirement for IE gene products in terminally differentiated MEL cells might have been negated; indeed, previous studies reported that the erythroid specific expression of β -globin genes transfected into MEL requires only the β -globin promoter region (Rutherford and Nienhuis, 1987; Cowie and Myers, 1988). However, my data demonstrate that the transduced β -globin gene was not activated by the erythroid specific factors present in MEL cells. These results suggest that rabbit β -globin gene expression in L7/14 infected MEL cells is completely controlled by HSV regulatory proteins.

While expression of the rabbit β -globin gene borne by L7/14 was induced by HSV polypeptides, expression of the endogenous mouse β -globin gene was

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endogenous genes, and rapid degradation of cellular mRNAs. Moreover, my results argue that these processes do not rely on sequence-specific differentiation between viral and cellular promoters or mRNAs. Below I will consider possible explanations for these findings.

4.2 Mechanisms underlying the preferential transactivation of virus borne β -globin genes

The activation of HSV-transduced β -globin genes requires IE proteins, and work by Smiley and Duncan (1992) has shown that activation of the rabbit β -globin gene in L7/14 infected Vero cells is dependent on ICP4. How does ICP4 selectively transactivate the expression of virus associated β -globin genes? A growing body of evidence suggests that ICP4 transactivates transcription through the TATA box element and the TATA binding factor, TBP (as reviewed in the introduction). The data discussed above suggests that the preferential expression of viral genes does not rely on sequence specific differentiation between viral and cellular promoters. Therefore, it seems unlikely that the selective activation mediated by ICP4 stems from interactions with TBP.

A more likely explanation for the selective activation of genes located in the viral genome involves site-specific DNA binding by ICP4. Mutational analysis has demonstrated a strong correlation between the ICP4 DNA binding function and the ICP4 transactivation function. Therefore, it seems likely that DNA binding is important for transactivation. One possibility is that ICP4 binding sites, like promoter elements, act over relatively short distances to activate the transcription of linked genes. Thus, the rabbit and mouse β -globin genes might

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inhibited. This inhibition was due, at least in part, to an inhibition of transcription and a vhs-dependent degradation of pre-existing cytoplasmic mRNA. The possibility that this differential behavior was due to sequence differences between the rabbit and mouse β -globin genes was addressed by constructing an HSV recombinant (β M) containing the mouse β -globin gene. Using this recombinant, I have shown that the mouse β -globin gene was expressed in infected Vero cells and that expression required viral protein synthesis, suggesting a requirement for IE gene products. Mouse β -globin transcripts were detected at 6 hours post-infection, and the levels increased between 6 and 12 hours post-infection. These kinetics differed somewhat from those obtained with the rabbit β -globin gene borne by L7/14, where the levels of rabbit β -globin mRNA declined between 6 and 12 hours post-infection. These kinetic differences might result from any combination of the three following factors: sequence differences between the mouse and rabbit β -globin genes, the different sites of insertion into the viral genome, or differences between the HSV strains used to generate the two recombinants (the rabbit β -globin gene was cloned into the tk gene of HSV-1 KOS PAA'5 and the mouse β -globin gene was cloned into the gl gene HSV-1 strain F). Notwithstanding the slightly different expression pattern observed with the transduced mouse and rabbit genes, the mouse β -globin gene is transactivated by HSV products. Thus, it is unlikely that the radically different behavior of the rabbit and mouse β -globin genes in L7/14 infected MEL cells is due to sequence differences between the two genes. Taken in combination, these results suggest that preferential expression of viral genes in HSV infected cells involves selective transcription of genes associated with the viral genome, transcriptional repression of

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have fortuitous ICP4 binding sites in their promoters that render them sensitive to activation by ICP4. This model, however, does not explain the failure of ICP4 to transactivate the endogenous mouse β -globin gene. In addition, other heterologous genes that are activated by ICP4, such as the human α -globin gene (Panning and Smiley, 1989; Smiley and Duncan, 1992), would also have to contain fortuitous ICP4 binding sites. Although ICP4 binding sites have been found in some heterologous DNAs (Faber and Wilcox, 1986b), it seems unlikely that both the α and β globin genes contain binding sites for ICP4. Also, recent experiments suggest that the tk and gD promoters, in the context of the viral genome, do not require proximal ICP4 binding sites (Imbalzano *et al.*, 1990; Smiley *et al.*, 1992). Therefore, ICP4 binding sites in the β -globin promoter might not be required for ICP4-mediated activation.

At first glance, the finding that transactivation by ICP4 requires sequence-specific DNA binding seems in conflict with evidence that transactivation does not require proximal ICP4 binding sites. However, it is possible that the loss of ICP4 binding sites within a promoter can be compensated for by other ICP4 binding sites in the HSV genome. In this model, ICP4 binding sites act as transcriptional enhancer elements whose activity is relatively independent of distance and orientation. This model predicts that ICP4 would activate a cellular gene inserted into the viral genome through binding sites in the adjacent viral sequences. The same gene, when located in the cellular genome, would not be activated if the surrounding DNA did not contain ICP4 binding sites. Thus, the preferential transactivation of viral genes versus cellular genes might occur if the HSV genome contains a high density of ICP4 binding sites relative to the cellular genome.

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If activation of heterologous gene expression is mediated by ICP4 binding sites in adjacent viral DNA, then ICP4 must function over long distances. The recent results of Smith *et al.* (1993) are consistent with this possibility. In these experiments, when ICP4 and TBP are bound to their respective DNA binding sites, they cooperatively interact with TFIIB to form a tripartite complex. These results led to speculation that ICP4 activates transcription through this complex. In this model, ICP4 could act over large distances if the DNA between the ICP4 binding site and the TATA box were looped out thereby allowing this tripartite complex to form.

Another potential explanation for the preferential activation of transduced β -globin genes involves the organization of viral and cellular DNA within the nucleus of HSV infected cells. For example, cellular genes might not be accessible for activation by ICP4 because of their higher order packaging into chromatin. In this context, HSV DNA is not associated with nucleosomes in productively infected cells, while in latently infected cells, where viral gene expression is severely restricted, the viral genome is associated with nucleosomes (Moultet *et al.*, 1979; Leinbach and Summers, 1980; Deshmane and Fraser, 1989). Preferential activation of viral genes might also be achieved if viral transcription factors and the viral genome assume a subcellular localization that is distinct from most cellular DNA. For example, the margination of cellular chromatin that occurs in HSV infected nuclei (Roizman and Sears, 1990) suggests that the cellular genome and the viral genome take up distinct nuclear locations. This possibility is also supported by the identification of large globular structures (replication compartments) in infected cell nuclei. These structures appear to be the active sites of HSV DNA

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uridine in cellular mRNA (Flanagan, 1967). These data however is only suggestive of a global transcriptional inhibition.

The inhibition of mouse β -globin transcription might also be due to the recruitment of the cellular transcription machinery from the cellular genome to the viral genome. This recruitment might underlie the ability of HSV transcription factors to activate viral gene expression and therefore, transcription inhibition could be viewed as a secondary effect of this process. Consistent with this model, the host RNA polymerase II is localized to virus replication compartments in HSV infected cells (Rice *et al.*, 1994). In addition, cellular transcription factors might also be modified to favor the transcription of viral genes over cellular genes. This idea is not unprecedented as an HSV gene product, expressed *de novo* in the infected cell, induces a uniquely phosphorylated form of RNA polymerase II (Rice *et al.*, 1994). This phosphorylation might modulate RNA polymerase II function to favor the transcription of viral genes. The recruitment of cellular transcription factors or their modification to favor viral transcription would result in a general inhibition of cellular gene transcription in HSV infected cells. Either of these models would also link transcriptional repression of cellular genes with preferential transcriptional activation of viral genes.

My data suggests that an IE protein other than ICP4 and/or a virion protein are involved in inhibiting mouse β -globin transcription. A similar conclusion was reached in a study demonstrating that HSV inhibits the transcription of adenovirus sequences in 293 cells (Stenberg and Pizer, 1982). In addition, Kemp and Latchman (1988) demonstrated that ICP22 (an IE protein) is required to inhibit the transcription of several cellular genes. Together these studies and

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replication and transcription (Quinlan *et al.*, 1984; Randall and Dinwoodie, 1986; Knipe *et al.*, 1987; de Bruyn Kops and Knipe, 1988; Rice *et al.*, 1994). Furthermore, since ICP4 localizes to viral replication compartments, the ICP4 DNA binding function may simply serve to localize ICP4 to the HSV genome (Randall and Dinwoodie, 1986; Knipe *et al.*, 1987). In this model, ICP4 need not be bound to DNA to activate transcription and thus the DNA binding activity of ICP4 is not directly required to transactivate viral genes. Instead, the DNA binding function is required to recruit large quantities of ICP4 to the viral genome thereby facilitating efficient transactivation. Again, preferential activation of viral genes would be achieved if the viral genome contains a high density of ICP4 binding sites relative to the cellular genome.

4.3 Mechanisms underlying the inhibition of mouse β -globin transcription

What mechanism underlies the selective transcriptional inhibition of the mouse β -globin gene resident in MEL cells? In these cells, mouse β -globin expression is dependent on tissue specific enhancers and other far upstream sequences (reviewed in Evan *et al.*, 1990). These sequences, or the proteins that bind to them, might be the targets of an HSV gene product that directly inhibits transcription. Thus, the number of genes inhibited by this mechanism is limited to the genes that utilize the affected targets. At this time, it is not known if HSV induces a general inhibition of cellular transcription, or if a limited number of cellular genes are affected. As mentioned in the introduction, HSV infection inhibits the transcription of several cellular genes (Stenberg and Pizer, 1982; Kemp and Latchman, 1988) and also inhibits the incorporation of labeled

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my work suggest that transcriptional inhibition might be a global event that is mediated by a single HSV IE gene product. One might identify the HSV gene product involved in inhibiting mouse β -globin transcription using HSV mutants that are defective for the expression of various IE proteins. One could then test the ability of this HSV gene product to induce either the altered phosphorylation or localization of RNA polymerase II.

Any model that explains the contrasting transcriptional fates of viral and endogenous β -globin genes must also consider why several viral genes inserted into the cell genome are transactivated by HSV infection (for examples see, Smiley *et al.*, 1983; Goldstein and Weller, 1988a; Ligas and Johnson, 1988). The model must also consider that all cell lines containing a given HSV gene will not necessarily express that gene upon infection (Lloyd Hutchinson, personal communication). This suggests that insertion site might affect the ability of HSV transactivators to access the inserted gene. For example, some regions of the cellular genome might possess a specific chromatin structure that renders them sensitive to HSV transactivators. It is also possible that certain regions of the cellular genome might assume a specific location and this location represents a preferred site for HSV transcription. Either of these explanations could also explain why the human α -globin gene, resident at its normal chromosomal locus, is induced by HSV infection (Panning, Cheung, and Smiley, unpublished). Finally, one must consider why many viral and cellular genes, which are transiently transfected into cells, are transactivated by HSV infection (as discussed in the introduction). Perhaps the large number of potential templates in a transfected cell would guarantee that some plasmid DNA is localized to sites of viral transcription. It is also possible that newly

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introduced plasmid DNA assumes a chromatin conformation that renders it susceptible to HSV trans-acting factors.

4.4 Degradation of mouse β -globin mRNA in MEL cells infected with HSV

I have demonstrated that mouse β -globin gene expression is inhibited by two processes: the degradation of mouse β -globin mRNA (consistent with previous reports Nishioka and Silverstein, 1977; Nishioka and Silverstein, 1978) and the inhibition of mouse β -globin transcription. In addition, the degradation of mouse β -globin mRNA did not require viral gene expression, suggesting that this process is mediated by a virion component. This result is contrary to the work of Nishioka and Silverstein (1978), who reported that HSV-induced degradation of mouse globin mRNA in MEL cells requires viral gene expression. This discrepancy may stem from the different detection techniques used in these two studies. For example, Nishioka and Silverstein quantified globin mRNA using oligo dT primed cDNAs that were generated by reverse transcription of globin mRNAs. As reverse transcriptase does not necessarily generate complete cDNAs *in vitro*, the probe generated would likely be biased towards detection of the globin mRNAs 3' end. On the other hand, both the primer extension analysis and S-1 nuclease mapping employed in the present study detected the 5' end of mouse β -globin mRNA. If the degradative function initiates at the 5' end of the target transcript, then the primer extension assay and S-1 nuclease analysis would be more sensitive to this effect than the analysis employed by Nishioka and Silverstein. Alternatively, these different results might be

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Does the preferential expression of viral genes during infection imply that viral mRNAs are spared the vhs effect? A substantial body of evidence suggests that the vhs function is involved in both degrading viral mRNAs and inhibiting their translation. Initial work by Read and Frenkel (1983) demonstrated the following: the vhs1 mutant overproduces IE proteins, IE mRNAs are functionally more stable in vhs1 infected cells, and superinfection of vhs1-infected cells with wild type virus results in a more profound shutoff of IE protein synthesis than does superinfection with the vhs1 mutant. Subsequent studies have shown that viral mRNAs in vhs1 infected cells are overexpressed and their functional half-lives increased (Kwong and Frenkel, 1987; Strom and Frenkel, 1987; Kwong *et al.*, 1988; Oroskar and Read, 1989). In addition, Oroskar and Read (1987, 1989) have shown that representative transcripts from each kinetic class are stabilized in vhs1 infected cells when compared to wild type infected cells. Together these results suggest that the vhs function is involved in degrading both viral and cellular mRNA. However, this hypothesis does not exclude the possibility that viral mRNAs might, to some extent, be spared the full force of the vhs function. This possibility is especially relevant considering the very short half-life imposed on cellular mRNAs by the vhs function. Fenwick and co-workers have suggested that viral mRNAs might escape the full force of the vhs effect through the downregulation of the vhs function (Fenwick and Owen, 1988; Fenwick and Everett, 1990 a,b). These studies demonstrated that the vhs function prevents the accumulation of HSV-2 IE mRNAs in the presence of cycloheximide, however if viral translation is allowed to occur, IE mRNAs are stabilized. Based on these findings, Fenwick *et al.* propose that a newly synthesized viral protein downregulates the vhs

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attributed to the different HSV-1 strains used, since Nishioka and Silverstein employed HSV-1 strain F while I used HSV-1 strain KOS.

I have shown that the HSV-1 mutant, vhs1, does not induce degradation of mouse β -globin mRNA. This mutant was originally isolated based on its inability to inhibit host cell translation in the absence of viral gene expression (Read and Frenkel, 1983), and more recent reports have shown that this mutant does not induce degradation of several cellular mRNAs in infected Vero cells (Kwong and Frenkel, 1987; Strom and Frenkel, 1987). Work by Kwong *et al.* (1988) has mapped the mutation in vhs1 to a 265 bp fragment of the HSV genome located at coordinates 0.604 to 0.606. This 265 bp fragment is located within the UL41 open reading frame of the HSV-1 genome, which is predicted to encode a 55kDa protein (Frink *et al.*, 1981; McGeoch *et al.*, 1988). Rescue of the vhs1 mutant with this fragment rescues the ability of virions to both inhibit cellular translation and decrease the functional half-life of cellular mRNA (Kwong *et al.*, 1988). These results demonstrate that the UL41 gene product is required to inhibit cellular translation and induce cellular mRNA degradation, and suggest that these two processes might be mechanistically linked.

How does the vhs protein function? Perhaps the UL41 protein induces mRNA degradation, thereby inhibiting cellular translation. However, this model is inconsistent with experiments suggesting that virion-mediated inhibition of actin translation precedes actin mRNA degradation (Schek and Bachenheimer, 1985). If one assumes that both the inhibition of actin translation and the degradation of actin mRNA are vhs dependent, this observation implies that mRNA degradation is a secondary effect, and that the primary function of the UL41 gene product is to inhibit translation.

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Collectively, these data suggest the following model: the vhs protein is initially delivered into the cell as part of the virus particle, thereby destabilizing pre-existing cellular mRNA; the vhs downregulator then accumulates, leading to partial stabilization of all mRNAs. The resulting temporal regulation of vhs activity would initially destabilize cellular mRNAs, thus clearing the cell translation machinery to facilitate the translation viral mRNAs. The residual vhs activity would impose a relatively short half-life on viral mRNAs, thereby closely coupling mRNA levels to transcription rates. In other words, the vhs function would permit tight control of viral gene expression at the transcriptional level. At first glance, this model might seem to imply that cellular mRNAs would reaccumulate after reversal of the vhs effect. However, this would not occur if the production of cellular mRNAs was inhibited. For example, the HSV-mediated inhibition of cellular transcription that I and others have observed would prevent reaccumulation of cellular mRNAs. In addition, the ICP27-mediated inhibition of cellular pre-mRNA processing (reviewed in the introduction) might also prevent the reaccumulation of cellular transcripts.

4.5 Investigation of the vhs function

The model described above suggests viral mRNAs would have a very short half-life in the absence of the hypothetical vhs downregulator. Therefore, the interaction between the vhs function and the vhs downregulator would play a critical role in the preferential expression of viral genes in HSV infected cells. Taking this into consideration, I studied the vhs effect in more detail. First, I confirmed the role of the UL41 open reading frame in the vhs effect by

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constructing two UL41 mutants in HSV-1 and a single UL41 mutant in HSV-2. These mutants confirmed that the UL41 gene product is required for virions to accelerate cellular mRNA degradation and inhibit cellular translation. These data are consistent with recent work by Fenwick and Everett (1990) who have also constructed mutants in the UL41 open reading frame.

These HSV-1 vhs mutants also demonstrated that the UL41 gene product is important to the viral lytic cycle, since UL41 mutants displayed both a lag in the production of infectious virus and a reduced virus yield in a single step growth curve experiment. This lag correlated with a delayed production of viral proteins in vhsA infected cells. One interpretation of these results is that the vhs function serves to clear the cellular translation machinery, thereby favoring translation of viral mRNAs. Thus, in cells infected with a vhs mutant, viral mRNA would have to compete with cellular mRNAs for access to the translation machinery. This model predicts that the rate of IE protein synthesis in a vhs mutant would be reduced while the levels of IE mRNAs would not be affected. This model has been tested by Jennifer Newton (personal communication), who found that the levels of ICP0 mRNA produced by the vhsA mutant and PAA'5 (the parent strain) are comparable. If the rate of ICP0 translation were reduced in the vhsA mutant this would provide direct evidence that a functional vhs effect is required for the efficient translation of viral mRNA. To date however, we have not determined the rate of ICP0 translation.

The construction of an HSV-2 vhs mutant has demonstrated that HSV-2 virions boost cellular translation in the absence of a vhs effect. This observation suggests that HSV-2 virions contain one or more factors that increase the translational capacity of the infected cell. This function has not been previously

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by Read *et al.* (1993) has demonstrated that two forms of the vhs protein are detected in HSV infected cells, only one of which is packaged into virus particles. However, I was able to detect only one vhs protein using the rabbit antipeptide antiserum. Perhaps my failure to detect both forms of the vhs protein indicates that the epitope recognized by this antiserum is present on only one form of the vhs protein.

4.7 A functional assay for the vhs effect

Many investigations into the vhs function would benefit from a convenient functional assay for the vhs effect. If the vhs protein functions in the absence of other viral proteins, one predicts that expression of a co-transfected reporter gene would be inhibited in a transient transfection assay. Consistent with this possibility, I have shown that a co-transfected vhs expression vector dramatically inhibits the accumulation of β -galactosidase activity expressed from a lacZ reporter gene. This experiment does not, however, definitively establish that this inhibition results from an activity related to the vhs function present in virus particles. This issue was further investigated by Frank Jones, who made use of the vhs protein expressed by the HSV-1 vhs1 mutant (Read and Frenkel, 1983). The vhs1 mutant is defective for the vhs effect and work by Kwong *et al.* (1988) has shown that this maps to a small region of the UL41 gene. Frank sequenced this region, and found that the vhs1 protein contains a single amino acid substitution which changes threonine 214 to an isoleucine. In addition, G.S. Read (personal communication) has shown that this mutant protein is packaged into virus particles; therefore, the inactivity of the vhs1

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identified because it is masked by the vhs effect. A virion function that boosts translation rates might be particularly important in the HSV-2 lytic cycle as the HSV-2 vhs effect is very potent (reviewed in the introduction). Thus, the translation boost might serve to maximize translation of HSV-2 mRNAs before they are inactivated by vhs, an effect that might be especially important early in the infectious cycle before the accumulation of a vhs downregulator. Perhaps the factor responsible for this translational boost could be identified by constructing HSV-2 vhs mutants that are also defective for the expression of known virion proteins.

4.6 Characterizations of the vhs protein

Having confirmed the role of the UL41 gene product in the vhs effect, I next identified the protein encoded by the UL41 open reading frame. This open reading frame is predicted to encode a 489 amino acid protein of 55kDa (Frink *et al.*, 1981; McGeoch *et al.*, 1988). Using a rabbit antipeptide antiserum generated by David Johnson, I demonstrated that the vhs protein migrates with an apparent molecular weight of 58kDa on SDS polyacrylamide gels and that it is phosphorylated. In addition the vhs protein is expressed late in the infectious cycle which parallels the expression of the vhs transcript reported by Frink *et al.* (1981). Also, the vhs protein was detected in partially purified preparations of HSV virions, suggesting that the vhs protein is packaged into virus particles. This observation is consistent with the role of the UL41 gene product in virion induced host shutoff, and agrees with the results of McLauchlan *et al.* (1992) who showed that the vhs protein is found in the virus tegument. A recent report

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protein is almost certainly due to a defect which directly inhibits its function. For this reason, the vhs1 protein served as an ideal control in the transient assay for vhs activity. Frank has shown that the vhs1 protein is unable to inhibit the accumulation β -galactosidase activity, suggesting that this transient assay mimics the vhs function. This assay will facilitate an in depth mutational analysis of the vhs protein. Mutational analysis of the vhs protein in virus would be time consuming as it necessitates the rescue of each mutant vhs gene into virus. In addition, the results of such an analysis would be difficult to interpret as this approach would score both the ability of the mutant proteins to induce the vhs effect and their incorporation into virus particles.

4.8 Identification of vhs binding proteins

How does the vhs protein inhibit translation and induce mRNA degradation? Perhaps the vhs protein is an RNase that directly degrades mRNA. Alternatively, it may interact with the cellular translation machinery or the mRNA degradative machinery, thereby activating a latent cellular pathway that inhibits translation and/or degrades mRNA. In an attempt to understand how the vhs protein functions, I searched for viral and cellular proteins that interact with the vhs protein. Using a vhs affinity resin, two cellular vhs binding proteins, of 57kDa and 47kDa, were identified. To date, no data are available on what role, if any, these cellular proteins play in the vhs effect. This approach also demonstrated that the HSV transcription factor, VP16, binds to the vhs protein. Initially, VP16 from HSV infected cell extracts was shown to interact with the vhs protein expressed in *E. coli*. In addition, a vhs antiserum was used to show that

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VP16 is immunoprecipitated in a complex with the vhs protein. Finally, VP16 and the vhs protein translated *in vitro* bound to vhs and VP16 affinity resins, respectively. Using *in vitro* translated proteins, I have shown that amino acids 238 to 344 of the vhs protein are necessary and sufficient for this interaction, and that the carboxy terminal 121 amino acids of VP16 are dispensable.

4.9 The functional significance of the vhs-VP16 interaction

What is the functional significance of the interaction between vhs and VP16? When contemplating the possibilities, one must consider that VP16 is essential for virus replication in tissue culture and that one of its essential roles involves virus assembly. In contrast, the vhs protein is not required for viral growth in tissue culture. It is also worth noting that VP16 is more abundant than the vhs protein in both the virus particle and the infected cell.

Inasmuch as vhs and VP16 are both virion proteins, this interaction might be involved in targeting these proteins to the virus particle. Given that VP16 has an essential role in virion assembly and that it is much more abundant than vhs, perhaps the interaction with VP16 serves to package vhs into virions. This hypothesis is consistent with observations that a vhs protein deleted for amino acids 149 to 344, (and which consequently lacks the VP16 binding domain), is not packaged into virus particles (Read *et al.*, 1993). Further experiments to more precisely define the vhs residues required to incorporate vhs in virions and bind to VP16 might show that the same amino acids are required for both of these processes. This type of result would provide strong evidence that the vhs protein is packaged into virions through its interaction with VP16.

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as the basis for a viral treatment aimed at inhibiting the VP16 transactivation function.

A final possibility is that VP16 regulates vhs function, either positively or negatively through its interaction with vhs. This possibility seems plausible, given the abundance of VP16 relative to the vhs protein. Since VP16 is a virion protein, the vhs-VP16 complex might represent the unit that induces host shutoff. However, vhs does not require VP16 to inhibit β -galactosidase activity in the transient co-transfection assay, which argues against this hypothesis. Alternatively, VP16 might inhibit vhs, a possibility that is supported by several results described in this thesis. First, the VP16 mutant, 8MA, demonstrates a protein synthesis defect that is paralleled by a defect in the accumulation of gB mRNA. This phenotype is consistent with a mutation in the vhs downregulator. This model strongly predicts that inactivation of the vhs gene in the 8MA mutant would reverse the protein synthesis defect; however despite considerable effort I have been unable to construct a VP16/vhs double mutant. To further test the hypothesis that VP16 downregulates the vhs function, VP16 was over-expressed in either transiently transfected cells or in a stably transformed cell line. Overexpression of VP16 by either method rendered cells resistant to the vhs effect mediated by superinfecting virions, a result that is consistent with downregulation of the vhs function by VP16. However, since I could not demonstrate that the prior expression of VP16 blocks the vhs function directly, I was not able to exclude several alternative explanations. For example, overexpression of VP16 might inhibit either entry or uncoating of the infecting virus. Also, at first glance the failure of VP16 to reverse the vhs effect in the transient transfection assays appears to argue that VP16 does not

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The interaction between vhs and VP16 might also serve to regulate the VP16 transactivation function. However, transient transfection experiments have demonstrated that VP16 is the only viral protein required to transactivate TAATGARATTC linked promoters (as reviewed in the introduction), suggesting that vhs is not required for this process. In addition, Peter Xiao and John Capone (personal communication) have shown that vhs blocks the assembly of the VP16 complex over the TAATGARATTC sequence, suggesting that vhs could downregulate VP16 transactivation. However, these results probably have little relevance to the viral lytic cycle, because a large molar excess of vhs was required to inhibit VIC formation. Perhaps of more interest is the precise way in which vhs blocks VIC formation. For example, vhs and the cellular components of VIC, might interact with identical surfaces on VP16, perhaps through common structural elements shared with either Oct-1 or HCF. However, the failure to detect any sequence similarities between vhs and Oct-1 or HCF may argue that vhs blocks VIC assembly through steric hindrance. Recent work has suggested that amino acids 360 to 390 of VP16 are involved in binding to Oct-1 in the presence of DNA (Stern and Herr, 1991; Hayes and O'Hare, 1993). Also, amino acids 360 to 367 of VP16 represent one of the major exposed portions of VP16, and have been shown to interact with HCF (Stern and Herr, 1991; Hayes and O'Hare, 1993). Given that the interaction between vhs and VP16 requires VP16 residues 335-369, the exposed portion of VP16 may contain coincident binding sites for HCF, Oct1, and the vhs protein. Since the vhs protein blocks VIC assembly, it is possible that artificially high levels of vhs would block VP16-mediated transactivation of HSV IE genes. These considerations suggest that the VP16 binding domain of vhs might serve

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downregulate the vhs function. However, it is entirely possible that VP16 must accumulate prior to the expression of the vhs protein for this downregulation to occur. Thus, VP16 would not reverse the vhs effect in the transient assay since both proteins are expressed from the HCMV IE promoter/enhancer and presumably accumulate together.

If VP16 is in fact the vhs downregulator proposed by Fenwick and colleagues, one must explain the ability of HSV virions to induce the vhs effect in the presence of a large excess of virion-associated VP16. This apparent contradiction might be explained if the formation of the vhs/VP16 complex is regulated during the infectious cycle. For example, phosphorylation of either the vhs protein or VP16 might regulate their ability to complex. In this model, differential phosphorylation of either the vhs protein or VP16 would account for both the release of active vhs protein from virus particles and the subsequent downregulation of the vhs function by *de novo* expressed VP16. Another possibility is that VP16 is bound to the vhs protein in the virus particle and upon infection this complex is transported to the nucleus. In the nucleus a large portion of VP16 is assembled in the transcription complex over the TAATGARATTC element. This would release the vhs protein and allow it to function since VP16 cannot bind to the vhs protein while participating in VIC. The subsequent high level *de novo* expression of VP16 would leave a substantial quantity of VP16 free to complex with the vhs protein and therefore it would downregulate the vhs effect. This model predicts that an HSV mutant which expresses a VP16 protein defective for VIC assembly might be negative for the vhs effect. As more is learned about VP16 complex assembly, this model could be tested. Alternatively, the VP16/vhs complex may have a high

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disassociation constant and therefore, upon infection, the complex falls apart, releasing active vhs protein. Subsequent high level *de novo* expression of VP16 would then favor the formation of the VP16/vhs complex thereby reversing the vhs effect.

If VP16 downregulates the vhs function, what mechanism underlies this process? If the vhs protein must interact with a cellular target to function, then the binding of VP16 to vhs might block this interaction. With the identification of such a cellular target, one could then test the ability of VP16 to disrupt its interaction with vhs. It seems likely that vhs functions in the cytoplasm, because it inhibits translation and induces degradation of cytoplasmic mRNA. Thus binding of VP16 to vhs might serve to transport vhs to the nucleus, thereby removing it from the cytoplasm and inhibiting vhs function. In this light, it would be interesting to determine if the subcellular localization of the vhs protein is affected by the presence of VP16.

Elucidation of the functional significance of the interaction between the vhs protein and VP16 might provide new insights into the assembly of the virus particle, as well as the regulation of gene expression in HSV infected cells. If VP16 downregulates vhs, then VP16 would play two critical roles in selective expression of viral genes in HSV infected cells: stimulation of IE gene expression and modulation of the vhs function leading to selective degradation of cellular mRNAs. VP16 would therefore represent a unique protein that regulates gene expression at both the transcriptional and post-transcriptional levels. According to this hypothesis, VP16 allows vhs to serve two functions in the infected cell: Initially, vhs inhibits cellular gene expression; Subsequently,

it serves to tightly couple viral transcription rates to viral mRNA levels, allowing for fine control of viral gene expression at the transcriptional level.

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