SUBCELLULAR LOCALIZATION OF THE HSV–1 PROTEINS VHS AND VP16.

SUBCELLULAR LOCALIZATION OF THE HSV–1 PROTEINS VHS AND VP16.

By

JAMIE RONALD INGLIS, B.Sc. (Hons.) MB

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (1998)
(Biochemistry)

MCMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Subcellular Localization of The HSV– Proteins vhs and VP16.

AUTHOR: Jamie Ronald Inglis, B.Sc.(Hons.)MB

SUPERVISOR: Professor John P. Capone

NUMBER OF PAGES: xii; 80.

ABSTRACT

Infection of a host cell by the Herpes Simplex Virus Type 1 leads to the efficient reprogramming of the cells' synthetic machinery to replicate the viral genome ultimately producing progeny virions. Two proteins introduced upon viral fusion are thought to initiate this effect. The potent transactivator of immediate early genes (VP16) and the mRNA destabilizing virion host shutoff protein (vhs), work in concert with one another to invoke the cascade of viral gene expression, and to destroy pre-existing cellular mRNA.

Due to the non-specific nature of vhs induced mRNA degradation, its activity is downregulated at later times during infection to spare virally encoded mRNA. Recent evidence has shown that VP16 is responsible for this vhs downregulation, a process thought to occur by mutual interactions between the two proteins and a potential compartmentalization of vhs within the nucleus (Lam *et al.*, 1996; Smibert *et al.*, 1994). Furthermore, such an event is also thought to position vhs so it can be efficiently packaged, a supposition supported by the observation that vhs lacking the ability to bind VP16 is not incorporated into new virions (Read *et al.*, 1993).

To ascertain if VP16 was indeed capable of relocalizing vhs to the nucleus of a cell in the absence of any other viral factors, we created multiple

constructs consisting of various portions of vhs fused in frame to the fluorescent marker protein EGFP. In addition, various truncated forms of VP16 were also fused to EGFP for the purpose of delineating the region of VP16 that is responsible for VP16 and possibly vhs nuclear localization.

Co-transfection experiments utilizing EGFP-vhs fusions demonstrated that vhs relocalizes to perinuclear regions in the presence of VP16, an effect absolutely dependent upon its ability to interact with VP16. In addition, deletion mapping of VP16 implicated the region spanning amino acids 335 to 355 as being necessary for this localization, with a stretch of 15 amino acids (330 to 344) appearing to constitute a putative bipartite nuclear localization signal. Interestingly, our observation that the vhs/VP16 complex localizes to a region of the cell thought to ultimately encompass the tegument of new virions gives credence to the notion that this interaction and subsequent localization may indeed function to package vhs into new virions. Furthermore, it is also suggested that vhs may in fact be downregulated at intermediate times during infection through VP16 mediated compartmentalization within the nucleus. For these reasons we propose that the disruption of the vhs/VP16 interaction could severely abrogate the infectivity of HSV and as such could present a novel target for antiviral intervention.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor, Dr. John Capone for granting me not only the opportunity to work in his lab, but also for his patience and support. I would also like to acknowledge my committee members Dr. Hara Ghosh and Dr. David Andrews for their insightful advice and guidance during my stay here at McMaster.

Secondly, I would like to acknowledge all of my fellow lab members who were forced to endure my jokes and good-humored pranks. I would like to especially recognize my esteemed colleagues in 'Intensity-Ally' for enhancing my scientific growth over the years. Your fascinating, objective and astute conversations not only increased my knowledge as a whole but also ultimately contributed my future career choice.

Special mention must go to my fellow Sleeman's appreciation club partners Kenji "fall under the table" Miyata and the Croatian Sensation Jozo Knez. Beyond occasional yet congruous drooks of cream ale and intermittent field trips to the Phoenix, your advice, kind, yet incessant sarcasm and good humour throughout the past few years were especially appreciated. I must also thank Lisa, (the mistress of merry melodies) for her relatively creative compositions and renditions as well as her gentle sarcasm and polite suggestions. I won't hesitate to say that they definitely made my stay more entertaining.

To my HSV co-worker Pat (Fu-Yoo), I must admit that I will truly miss our morning coffee breaks and in depth conversations. Your patience and assistance throughout the years has been amazing and I think I can speak for many past and present grad students when I say that we would have had a much harder time without your help.

Beyond their constant support, I would like to thank my parents for giving me every opportunity that I had as well as the constant encouragement and assurances that I would indeed persevere. In addition, I would also like to express my gratitude to all the O'Doherty's (even the little one) for their support, words of encouragement and numerous recreational breaks.

Last but certainly not least I want to thank Lynda for her patience, never-ending support and love through both the best and worst of times. Even though it has been a long tumultuous journey (for both of us) you always had a way of making me smile and look on the bright side of everything. For this I am truly grateful.

"*And now for something completely different…*"

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

a.a.	amino acid(s)
amp	ampicillin
APS	ammonium persulphate
AD	activation domain
BSA	bovine serum albumin
Ci	curie
CIP	calf intestinal phosphatase
CTD	carboxyl-terminal domain
CMV	cytomegalovirus
Da	dalton
DBD	DNA binding domain
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotides
ddNTP	dideoxynucleotides
DTT	dithiothreitol
E	early
E. coli	*Escherichia coli*
EDTA	N-N'-ethylenediaminetetraacetic acid

FBS	fetal bovine serum
HBSS	hepes-buffered saline solution
HEPES	n-(2-hydroxyethyl)piperazine-M'-(2-ethanesulfonic acid)
hrs	hours
HSV	herpes simplex virus
IE	immediate early
kan	kanamycin
kDa	kilodalton
kb	kilobase
L	late
μ	micro
m	milli
M	molar
MBP	maltose binding protein
min	minutes
MOI	multiplicity of infection
mRNA	messenger RNA
n	nano
N_2	nitrogen
NP-40	Nonidet P-40
ORF	open reading frame
PBS	phosphate buffered saline

PMSF	phenylmethylsufonylfluoride
RPM	revolutions per minute
RNA	ribonucleic acid
RNAse A	ribonuclease A
sec	seconds
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TE	10mM Tris-HCl, 1mM EDTA
TEMED	N,N,N',N',-tetramethylethylenediamine
VBD	VP16 binding domain
vhs	virion host shutoff
VIC	VP16 induced complex
wt	wild type

1. INTRODUCTION

1.1. Herpes Simplex Virus: an overview

Herpesviruses are large complex double stranded DNA viruses, which can infect a wide array of different cells with equally diverse effects. To date, eight such human herpesviridae have been classified into 3 distinct sub-families based on their trophic effect, host range, *in vitro* growth rate and viral latency characteristics.

(i) The alphaherpesviruses are neurotrophic viruses since they become latent in nervous tissue. Upon reactivation, this sub-family emerges in epithelial tissues causing characteristic lesions. They tend to be highly lytic in culture, and infect a broad range of hosts.

(ii) Betaherpesvirus infections are characterized by cell enlargement, with a slow reproductive cycle in a limited range of hosts.

(iii) The lymphotrophic or gammaherpesviruses are B-cell trophic and usually remain latent.

(Chee and Barrell, 1990)

Herpes simplex virus (HSV) is a member of the alphaherpesvirus sub-family. Like all the other herpesviridae, HSV is characterized by a distinct virion architecture consisting of an electron dense core, a protein capsid

surrounding the core, an amorphous layer surrounding the capsid termed the tegument and a lipid bilayer envelope containing spiked structures thought to be glycoproteins (Ward and Roizman, 1994). The ~152 kb genome occupies the capsid of the virion, and is organized into two uneven dsDNA stretches. The long unique sequence (U_L) contains 58 genes flanked by inverted repeats $a_n b$ at the U_L termini and $b'a'_m$ at the U_L/U_S junction. The unique short (U_S) segment of the HSV genome consists of 13 genes, and analogous to the U_L sequence, is surrounded by inverted repeats *a'c'* and *ca* (Figure 1). Both the U_L/U_S junction and U_L terminus '*a*' repeats are reiterated up to ten times and play an important role in viral replication (Roizman and Sears, 1990). It is interesting to note that the coding sequences for the five HSV immediate early genes are clustered around these repeats, a position possibly contributing to their significant expression levels (Steiner and Kennedy, 1993).

HSV initially fuses with the plasma membrane of a target cell through interactions between the viral glycoprotein gB and cell surface receptors. Upon entry, the envelope remains embedded within the plasma membrane while the nucleocapsid containing the viral genome is released into the cytoplasm and subsequently localizes to the nucleus. At this point HSV can either follow a pre-programmed cascade of gene expression, termed the lytic (productive) infection, or become quiescent and establish a latent state.

Figure 1. Functional organization of the HSV-1 genome.
The genome of HSV-1 is organized into two distinct components designated as L (long) and S (short). Each segment consists of unique sequences (U_L and U_S) bounded by inverted repeats b-b' and c'-c. Located at the L-S junction and at the ends of the genome are redundant 400 bp stretches termed a and a' sequences.

(Adapted from McGeoch *et al.*, 1988)

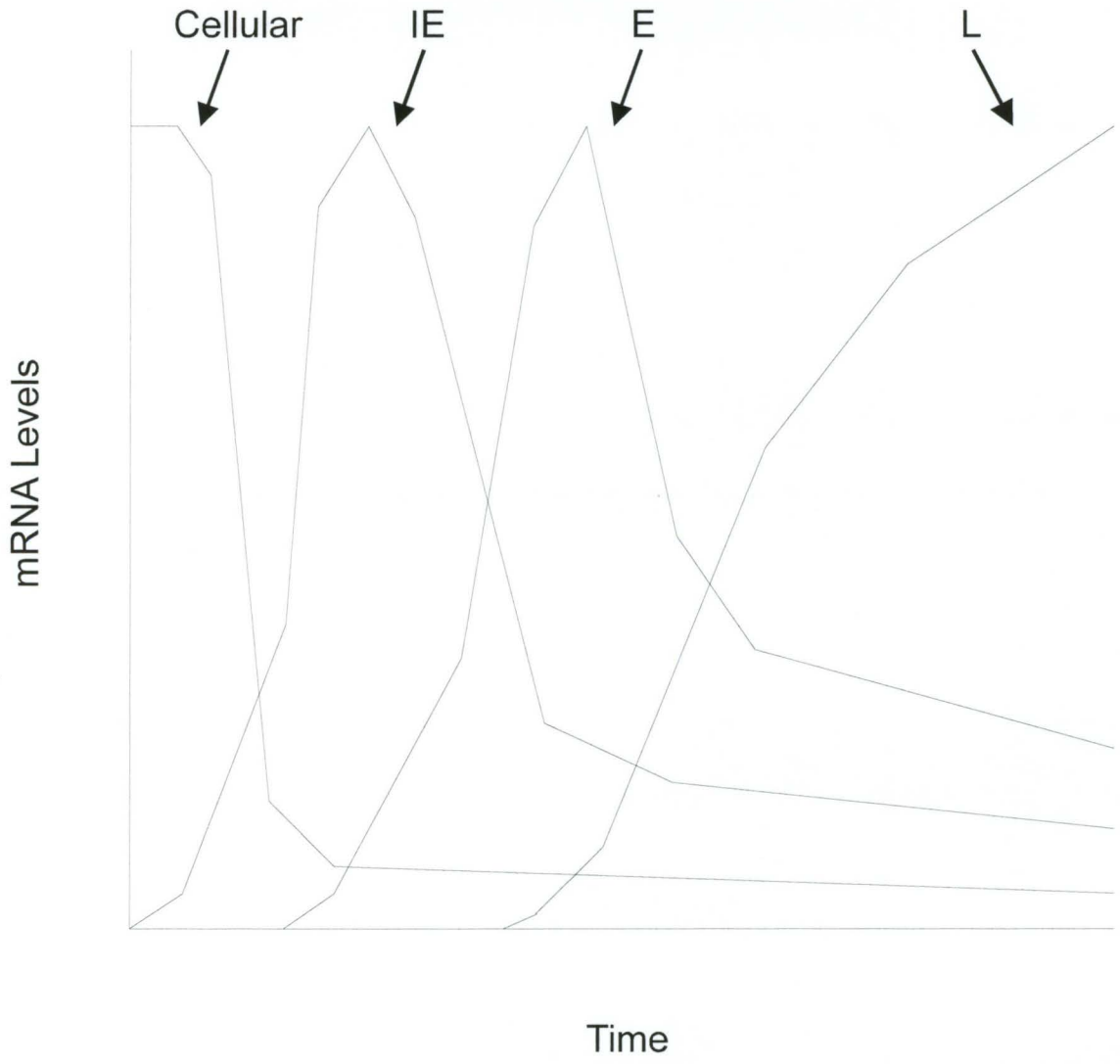


Figure 2. Relative mRNA levels during an HSV-1 infection.
A schematic representation of immediate early (IE), early (E) and late (L) mRNA levels in during an HSV-1 infection. The decline in host mRNA initially present in the cell is represented by the 'Cellular' curve.

(Adapted from Everett, 1987)

initially disables host protein transcription by eliciting two separable stages of host shutoff, an effect that ultimately redirects host transcriptional machinery to produce solely virally encoded mRNA. This initial shutoff has been attributed to the non-specific degradation of mRNA by vhs (Read and Frenkel, 1983; Schek and Bachenheimer, 1985) and besides severely abrogating host protein synthesis, this non-specific degradation ultimately serves to synchronize and accelerate the transition between stages of viral gene expression (Kwong and Frenkel, 1987; Oroskar and Read, 1987, 1989). The secondary shutoff observed in HSV-1 infected cells is dependant on the expression of the IE protein ICP27. Initial studies demonstrated that HSV-1 mutants encoding a defective ICP27 gene exhibited a greatly reduced capacity to shutdown host protein synthesis (Sacks *et al.*, 1985). Furthermore, recent studies suggest that deleterious effects caused by ICP27 occur post-transcriptionally by interfering with cellular splicing events (Hardy and Sandri-Goldin, 1994; Phelan *et al*, 1996).

The method by which capsids containing viral DNA ultimately acquire the tegument and envelope is still unclear. Initial studies have suggested that envelopment may occur when viral capsids bud out of the nucleus and accumulate as mature, enveloped virions between the lamellae of the nuclear membrane (Nii *et al.*, 1968). However, numerous non-enveloped capsids can be seen within the cytoplasm of infected cells suggesting a possible cytoplasmic site for envelopment, or that virions may be de-enveloped once reaching the cytosol (Campadelli-Fiume *et al.*, 1991). Of interest is the observation that a number of herpesviruses produced a second type of particle in addition to infectious virions.

These 'L-particles' consist of the tegument and envelope components of virions yet lack intact nucleocapsids and viral DNA (McLauchlan et al., 1992). How L-particles form during viral replication is still not fully understood, but studies using a temperature sensitive mutant demonstrated that their genesis can be uncoupled from virion formation (Rixon et al., 1992). However, the ability of these particles to deliver vhs and VP16 to cells with apparent wild-type activity suggest that the process of viral attachment, fusion and release are the same for both L-particles and mature capsid containing virions (Rixon et al., 1992, Dargan *et al.*, 1997).

Recent evidence has further suggested that the viral glycoprotein K (gK) also may be involved in nucleocapsid envelopment and the translocation of viruses to the extracellular space (Jayachandra *et al.*, 1997). Since deletion mutations in gK prevent fusion between viral and cellular membranes, it has been postulated that it may function to reorganize the nuclear membrane and as such increase the efficiency of virion envelopment. Additionally, viruses lacking a fully functional copy of this protein were found to be stranded in the perinuclear space or were located in the cytosol as multiple capsid, single envelope virions (Jayachandra *et al.*, 1997).

1.3. Sub-nuclear organization of HSV-1 infected cells

The nucleus of HSV infected cells appears to be organized in a dynamic fashion whereby individual events such as HSV replication and/or capsid production have been ascribed a given sub-compartment of the nucleus.

The early (β) proteins U_L5, U_L8 and U_L52 are key viral products important in directing DNA replication (Bataille and Epstein, 1995). Furthermore these proteins have been shown to localize evenly across the nucleus in a pattern of distinct, punctuate, nuclear foci termed *replicons* (Lukonis and Weller, 1996).

Structural proteins such as ICP5, ICP35 and U_L38 comprise another spatially distinct region of the nucleus near the periphery (*assemblons*), overlapping with regions containing a high density of tegument proteins (Ward *et al.*, 1996). This sub-compartment overlap suggests that there may indeed be a functional/spatial link between different classes of proteins in the assembly of new virions.

In considering viral DNA replication, capsid production, and ultimately virion assembly, it appears that the organization of the nucleus and the spatial co-ordination of viral gene products are of vital importance. In this respect, HSV appears to encode several proteins, all of which are of indispensable for the viability of the virus. These proteins all may co-ordinate different processes within the infected cell, but are similar in the fact that they all seem to facilitate processes from within the nucleus. The functionality of these gene products then is dependent upon two factors: (i) an intact, fully active protein and (ii) its proper localization within the infected cell.

Studies have suggested that the interaction between nuclear proteins and viral glycoproteins may play an essential role in virion particle assembly, envelopment and ultimately egress from the infected cell (Zhang and McKnight,

1993). The tegument protein VP16 is one candidate protein to serve this function as it has been implicated in maintaining the topography of a glycoprotein skeleton in the viral envelope (Zhu and Courtney, 1994). In addition, it has been further postulated that VP16 may also provide a fixed framework around which other viral proteins may assemble (Spear, 1985).

1.4. The viral transactivator VP16

Upon infection, HSV-1 genes are expressed in a temporally regulated manner. Induction of the immediate early (α) genes relies upon the action of VP16 (VMW65, αTIF, ICP25, U_L48), a tegument protein introduced into the cell upon viral fusion. This 490 amino acid phosphoprotein has been shown to contain two functionally distinct regions (figure 3, panel A).

The carboxyl-terminal region (CTD) consists of a highly acidic stretch of approximately 65 amino acids key to its transcriptional enhancement properties (Triezenberg *et al.*, 1988; Cousens *et al.*, 1989). This acidic activation domain (AAD) has been shown to alleviate chromatin mediated inhibition, as well as interact with the basal transcription machinery to stimulate and stabilize the transcription initiation complex (TIC; Stringer *et al.*, 1990; Lin *et al.*, 1991). Furthermore, this domain, independent of the amino terminal region, activates transcription of a reporter if tethered to a heterologous binding domain (Sadowski *et al.*, 1988; Cousens *et al.*, 1989; Berger *et al.*, 1990).

The amino-terminal 424 amino acids of VP16 facilitate functional protein-protein interactions necessary for its activity. Intrinsically, VP16 has only weak DNA binding capabilities to the consensus TAATGARAT (R=purine) sequence present in the enhancer region of immediate early genes (Mackem and Roizman, 1982; McKnight *et al.*, 1987). In order to bind the TAATGARAT element then, VP16 assembles into a multi-component VP16 induced complex (VIC) with at least two other ubiquitous cellular factors: OCT-1 (McKnight *et al.*, 1987; Gerster and Roeder, 1988; Preston *et al.*, 1988) and VCAF-1 (also known as HCF, C1 or CCF; Kristie *et al.*, 1989; Katan *et al.*, 1990; Xiao and Capone, 1990).

It is thought that the POU homeodomain protein OCT-1 is responsible for the recruitment of VP16 and HCF to the target element. Selectivity at the TAATGARAT sequence then is not dependant upon the ability of VP16 to recognize a flanking motif, but rather an allosteric effect of the DNA sequence such that it induces a conformational change in bound OCT-1 enabling it to efficiently interact with VP16 (Walker *et al.*, 1994). To establish a role for HCF in the VIC, studies using VP16 modelled synthetic peptides corresponding to the VP16-HCF binding interface were synthesized. With these peptides, VP16 transactivation was inhibited *in vitro* suggesting that the HCF-VP16 complex is absolutely required for transactivation, and the assembly of the VIC on its response element is in deliberate order (Wu *et al.*, 1994). Conversely, it has been demonstrated that point mutants which disrupt the HCF-VP16 complex still retain their ability to transactivate a chloramphenicol acetyltransferase reporter

construct albeit at reduced levels as compared to wild type. Rather than a distinct assembly pathway then, it was proposed that there is a degree of flexibility in the assembly of the VIC (Shaw *et al.*, 1995).

The true nature of VIC assembly still remains a matter of debate. However, it appears as if VP16 is positioned between HCF and OCT-1, where the function of OCT-1 is to tether VP16 to the DNA element, while HCF stabilizes the interaction (Lai and Herr, 1997). It has been postulated that the acidic activation domain of VP16 is not required for this interaction and as such appears to be free to transactivate downstream targets (Greaves and O'Hare, 1989). However, recent studies suggest that this may not be the case as it appears that transactivation ability of VP16 in the context of the VIC is limited to promoter proximal regions of the target gene (Hagmann *et al.*, 1997). This is interesting considering that when the AAD of VP16 is fused to a DNA binding protein (GAL4), transcription can be activated over both short and long distances (Sadowski *et al.*, 1988; Cousens *et al.*, 1989; Berger *et al.*, 1990). To explain this discrepancy, it has been suggested that the acidic activation domain of VP16 may be masked by inhibitory interactions within the VIC when bound remotely, away from the promoter. However, when it occupies a promoter proximal position, interactions between the pre-initiation complex and the VIC may alter its conformation, converting the previously inactive C-terminal tail of VP16 to an active form (Hagmann *et al.*, 1997). This hypothesized conformational switch of the AAD may be linked to its ability to interact with the basal transcription factor TFIIB as previous studies have demonstrated that this factor changes

conformation and promotes the assembly of the pre-initiation complex when bound by VP16 (Roberts and Green, 1994). It is possible that this interaction also serves to "free" the activation tail of VP16 from other inhibitory interactions within the VIC hence permitting the activation of transcription.

In order to transactivate immediate early genes, it appears as if VP16 activates multiple downstream targets within the pre-initiation complex. Studies have demonstrated that the acidic tail of VP16 interacts with TFIIB (Lin and Green, 1991), TFIID (TATA binding protein; TBP) (Horikoshi *et al.*, 1988), hTAFII31 (Uesugi *et al.*, 1997) and possibly the ADA2 co-activator (Lin and Green, 1991). How these interactions function to activate transcription still remain to be elucidated. However, three possible mechanisms have been proposed: (i) VP16 may serve to promote the formation of open transcription complexes permitting the transcriptional machinery easy access to the target gene (Jiang *et al.*, 1993); (ii) it is possible that it may increase elongation processivity assisting in the initial synthesis of mRNA (Yanakulov *et al.*, 1994); and/or (iii) VP16 may simply facilitate the release of the polymerase upon the initiation of transcription (Payne *et al.*, 1989).

Similar to the virion host shutoff function, the ability of VP16 to transactivate IE genes is not absolutely required for the lytic infectivity of HSV. A viral mutant (*in*1814) containing a four amino-acid insertion at position 379 abolished VIC formation, and as a result, presented a 10^2 - 10^4 reduction in plaquing efficiency per particle depending on the cell line infected. However, this mutant complemented an HSV-2 strain containing a lethal mutation in VP16

(*ts*2203), which was defective in proper virion assembly but capable of immediate early gene transactivation (Ace *et al.*, 1988). Taken together, these studies suggest that aside from its transactivation functions, VP16 has other structural roles within the infected cell, and that functionally distinct regions within VP16 perform these functions.

The absolute requirement for the structural function of VP16 is evidenced by the phenotype of a VP16 null mutant virus 8MA. Although it exhibits a nearly normal level of viral DNA synthesis, capsid production and encapsidation, its ability to produce functional progeny virions is severely attenuated (Weinheimer *et al.*, 1992). Only by propagating 8MA on a specific cell line (16-8) which provides VP16 *in trans* can the virus effectively replicate. By utilizing electron microscopy, comparisons between this 8MA, VP16 null mutant virus, a genetic revertant virus containing a restored copy of VP16 (8MA/R) and wild type HSV-1 (KOS) were performed on complementing (16-8) and non-complementing (A1-3) cell lines. Infections with 8MA/8MA-R and KOS in 16-8 cells and 8MA-R and KOS in A1-3 cells all exhibited a similar phenotype such that a high number of viral particles were seen dispersed throughout the nucleus and cytoplasm. In addition, the predominant form of these capsids had dense cores containing viral DNA, with the majority of the cytoplasmic capsids enveloped and localized at the cellular surface (Weinheimer *et al.*, 1992). In contrast, 8MA infections in A1-3 cells yielded approximately a 50% decrease in the total number of capsids, with the majority of them empty or only partially cored. It appears that VP16 must play a vital role in viral assembly, as a lesion

within the structural portion of this gene leads to defects in newly formed virions (Weinheimer *et al.*, 1992).

These above findings have now been further substantiated by studies using temperature sensitive mutants of VP16. By mutating cysteine residues to glycine at amino acids 78, 102 and 176, HSV could propagate normally at permissive temperatures, but at non-permissive temperatures, viral growth halted at late times during the infection. It appears as if the introduction of these cysteines have little affect on the initial events of the infection such as the genetic cascade or viral DNA replication, but indeed pose deleterious effects during the viral assembly stage (Poon and Roizman, 1995).

1.5. The virion host shutoff protein

Occurring concomitantly with viral gene transactivation in infected cells is the rapid cessation of host cell polypeptide synthesis occurring in parallel with the desegregation of host polyribosomes (Sydiskis and Roizman 1966). Furthermore, HSV infected cells demonstrate an extensive decrease in mRNA levels due to virally induced mRNA degradation (Nishioka and Silverstein, 1977; Schek and Bachenheimer, 1985). This host shutoff and mRNA degradation was shown to be mediated by a structural component of the infecting virion as it occurred in the presence of drugs that precluded the expression of viral genes (Sydiskis and Roizman, 1966; Fenwick and Roizman, 1977; Fenwick and Walker, 1978; Schek and Bachenheimer, 1985). In examining the host shutoff

phenomenon, Read and Frenkel isolated a series of six viable HSV-1 mutants that were deficient in the host shutoff function (Read and Frenkel, 1983). Further characterization of these mutants revealed that vhs activity was not restricted to pre-existing cellular mRNA's as it also accelerated the turnover of virally encoded mRNA as well (Read and Frenkel, 1983; Oroskar and Read, 1987; Kwong and Frenkel, 1987; Strom and Frenkel, 1987). To identify the gene responsible for virion associated host shutoff, Kwong *et al.* mapped the vhs1 mutant to the U_L41 open reading frame of HSV-1 (Kwong *et al.*, 1988), ultimately leading to the construction of vhs null mutants lacking the host shutoff phenotype (Fenwick and Everett, 1990; Smibert and Smiley, 1990).

Using antibodies raised against a synthetic peptide corresponding to amino acids 333-337 of vhs, Smibert *et al.* identified the major product of vhs to be a 58 kDa phosphoprotein (Smibert *et al.*, 1992). This closely resembled the 55 kDa gene product, predicted as a primary translation product (Kwong *et al.*, 1988). However, in infected cells, Read *et al.* identified two vhs species – a major 58 kDa phosphoprotein as well as a less abundant 59.5 kDa gene product. Furthermore, it was demonstrated that only the 58 kDa form of vhs was packaged into newly formed virions suggesting that post-translational modifications may in fact be important for the regulation as well as the packaging of vhs (Read *et al.*, 1993).

Numerous studies have indicated that U_L41 mutants can indeed abolish the host shutoff phenotype of HSV-1. The question remains however, whether vhs requires other proteins, viral or otherwise, to induce its effect. This

question is made more intriguing by the observation that a HSV-1 virion containing a null mutation in what is thought to be a putative Ser-Thr kinase (U_L13) exhibits a vhs null-mutant phenotype, suggesting that vhs may require UL13 to regulate its activity by phosphorylation (Overton *et al.*, 1994). However, recent findings have demonstrated that cells infected with a mutant virus lacking a functional ICP22 gene exhibit the same phenotype as cells infected with a null mutant U_L13 virus. In these cells, vhs mRNA levels were substantially reduced as compared to wild type suggesting that UL13 may indirectly affect vhs expression through post translational modifications of ICP22 (Ng *et al.*, 1997, Ogle *et al.*, 1997).

The role that ICP22 plays in modulating vhs activity still remains unclear. One possible hypothesis suggests that vhs expression in previously infected cells may be abrogated as ICP22 has been implicated as a possible transcriptional activator of late genes. This is supported by the observation that late genes require the co-localization of ICP4, ICP22, RNA polymerase II and an associated cellular factor (EBV small RNA associated protein) to distinct nuclear regions (Leopardi *et al.*, 1997). In the absence of U_L13 however, this characteristic ICP22 localization does not occur leading to a decline in late protein levels (Ng *et al.*, 1997). Newly formed virions with this defect may then package a reduced number of vhs molecules leading to an ineffective host shutoff phenotype in further infected cells.

The effect that vhs has on cellular mRNA expression levels throughout the course of an infection has been well documented, yet the mechanism by

which it acts still remains a matter of debate. To elucidate whether vhs non-specific degradation of mRNA is a direct of indirect event, co-transfections of vhs in concert with a lacZ reporter construct demonstrated a strong suppression of the expression of a β - galactosidase reporter gene. However, this suppression was alleviated by using vhs1, a mutant form of vhs (T214I) compromised in its ability to elicit the host shutoff response in infected cells (Kwong *et al.*, 1988). Based on these findings, it appears as if vhs can indeed act independently, and in addition, does not require the presence of any other virally encoded proteins to retain its activity in transient transfections (Jones *et al.*, 1995).

Three different mechanisms can be envisioned to explain the non-specific mRNA degradation seen in the presence of vhs. (i) It may in fact be an RNAse, and could, on its own, completely destroy mRNA. (ii) It may activate another RNAse present in the host. (iii) It may cleave mRNA at a few critical sites that normally serve to protect mRNA from ribonucleolytic degradation. Consistent with the last hypothesis, Zelus *et al.* demonstrated that vhs is able to cleave a poly A sequence located within a β globin substrate (Zelus *et al.*, 1996). This result is consistent with the observation that infected cells have been shown to accumulate non-polyadenylated mRNA (Nakai *et al.*, 1982).

1.6. The interaction between VP16 and vhs

Given the fact that mRNA is non-specifically degraded in infected cells, a crucial question is how viral mRNA accumulates so as to produce viral proteins

and ultimately new virions. Initial studies demonstrated that virally transduced rabbit β-globin accumulates after transfected rabbit β-globin (Smibert and Smiley, 1990) suggesting that even though vhs mediated mRNA degradation is non-specific, virally transduced genes have a selective advantage within the infected cell. Clearly then, there must be a mechanism by which vhs activity is downregulated to permit the expression of viral proteins after host mRNA has been degraded.

Investigation into possible modes of downregulation led to the hypothesis that other viral proteins may indeed modulate vhs activity by direct interactions. In pursuing this theory, Smibert and co-workers discovered that the viral tegument protein VP16 co-precipitated with vhs from infected cells when using an antibody directed against the vhs polypeptide (Smibert *et al.*, 1994). In addition, it appeared that this interaction blocked VP16 from entering the VP16 induced complex on its TAATGARAT consensus binding sequence suggesting that the region which was responsible for binding vhs overlapped one (or more) of the regions responsible for VIC assembly. Further studies revealed that this was indeed the case as this vhs interaction domain was centered between amino acids 335 to 369 in VP16, a region also implicated in OCT-1 and HCF binding (figure 3, panel A; Smibert *et al.*, 1994).

Subsequent studies were concerned in delineating the precise region of vhs that bound to VP16. These experiments demonstrated that a 21 amino acid stretch of vhs spanning residues 310 to 330 was key to the ability of vhs to

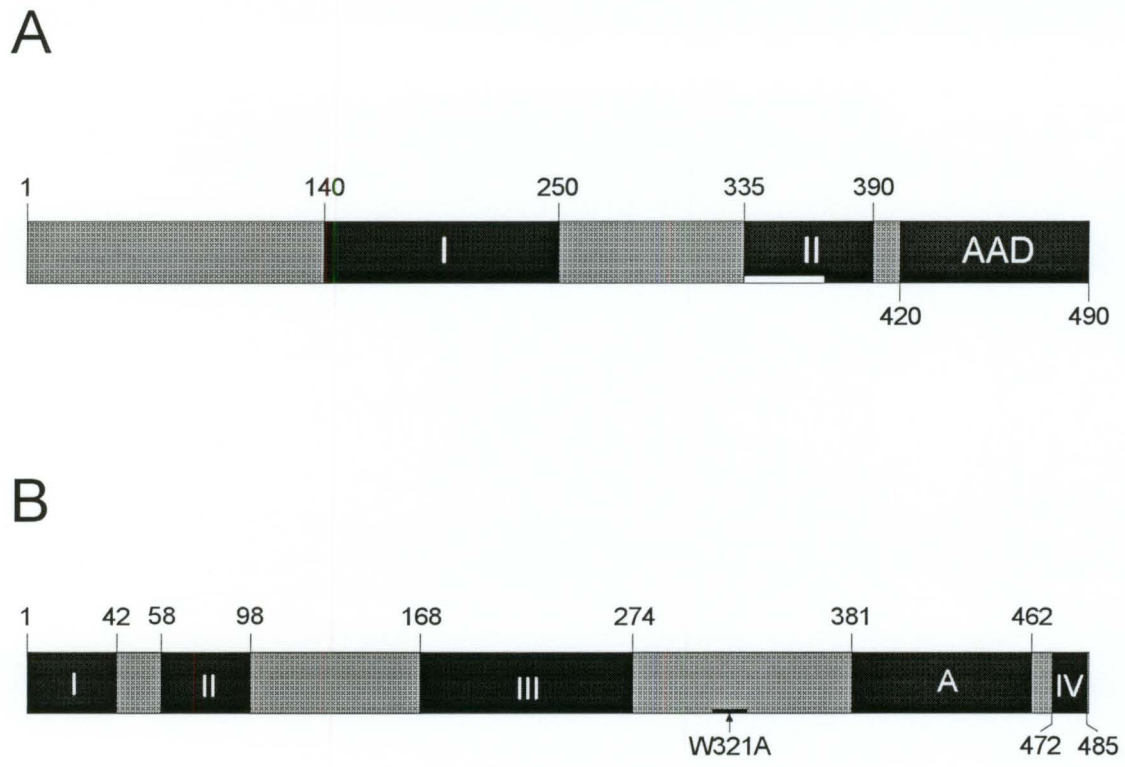


Figure 3. Functional domains of the HSV-1 proteins vhs and VP16.
Panel A. VP16 consists of three main domains. Domain I and II were initially characterized as regions involved in OCT-1 and HCF binding. The third domain consists of a 65 amino acid highly acidic stretch and as such is referred to as the acidic activation domain (AAD). This C-terminal region functions as a transcriptional activating tail, through interactions with basal transcription machinery located downstream from the TAATGARRAT consensus binding sequence (adapted from Shaw *et al.*, 1995). The solid white bar represents the region in VP16 responsible for binding vhs. *Panel B.* Likewise to VP16, vhs consists of multiple domains spanning the entire length of the protein. Characterization of the four domains referred to as box I, II, III and IV were initially made through homology studies and represent regions within vhs that are conserved among the five alphaherpesviruses (Berthomme *et al.*, 1993). More recently, another less conserved, yet mutation sensitive region was identified (box A) located between box III and IV (Jones *et al.*, 1995). The solid bar represents the minimal binding region necessary and sufficient for interacting with VP16 (Schmelter *et al.*, 1996). This region is referred to here as the VP16 binding domain (VBD).

bind VP16, and appeared to constitute a modular binding interface necessary and sufficient to interact with VP16 (Schmelter *et al.*, 1996). To further map the binding determinants of this region, successive point mutations were made ultimately leading to the isolation of a tryptophan 321 to alanine amino acid substitution that completely abolished the interaction. Additional mutations in the surrounding residues severely compromised the interaction yet the fragment still retained the ability to interact with VP16 albeit at a reduced efficiency. (Schmelter *et al.*, 1996).

Following these studies of a possible vhs-VP16 regulatory link that could serve to downregulate vhs activity, Lam and co-workers (1996) used a VP16 null virus (8MA; Weinheimer et al., 1992) to assay the possible accumulation of virally encoded mRNA throughout the infection. They discovered that viral protein synthesis and mRNA levels exhibited a severe decline at late times of infection, ultimately culminating in complete translational arrest. Furthermore, this phenotype was rescued by either inactivating the vhs gene, or by using a VP16 recombinant possessing functional VP16. From these observations, it was hypothesized that the temporal control during HSV infection was a result of newly synthesized VP16 inhibiting or downregulating vhs from the infecting virion via direct protein – protein interactions (Lam *et al.*, 1996).

1.7. Project overview

Vhs enhances the infectivity of HSV by serving to reprogram the synthetic machinery of the host cell to produce solely viral proteins (Oroskar and

Read, 1987; Fenwick and Owen, 1988; Kwong *et al.*, 1988; Oroskar and Read, 1989). However, vhs induced mRNA degradation is non-specific, so HSV has evolved a mechanism by which vhs is downregulated at intermediate times during infection thus permitting viral mRNA to accumulate and ultimately lead to the synthesis of viral proteins (Fenwick and Owen, 1988; Fenwick and Everett, 1990).

Not until the discovery of the interaction between VP16 and vhs did possible theories emerge on how vhs downregulation may occur. One such hypothesis suggested that VP16 may serve to translocate vhs into the nucleus thus compartmentalizing it in a sub-cellular location where its mRNA destabilizing activity is no longer effective in eliciting a host cell shutoff (Lam *et al.*, 1996). In addition, this localization may serve to target vhs to a region of the cell where it may be packaged into new virions to subsequently infect other cells. This is not inconceivable, as previous studies have suggested that vhs mutants lacking the region that interacts with VP16 (missing amino acids 179-344) are not packaged into the tegument of new virions (Read et al., 1993).

Since vhs does not possess a nuclear retention signal, it remains within the cytosol of host cells. If VP16 mediated vhs nuclear localization is possible, the interaction between the two must serve to translocate both proteins in tandem. Hence, the ability of vhs to localize to the nucleus does not solely rely on the VP16-vhs interaction but also upon the region in VP16 that serves to translocate VP16 (and conceivably vhs) into the nucleus.

Using EGFP and immunofluorescence studies, our aim was to not only determine if vhs was capable of relocalizing within the cell to the nucleus but whether any such event was dependant upon its ability to interact with VP16. In addition, we set out to determine the region(s) in VP16 that localize it to the nucleus with the thought that it may possibly serve to translocate vhs as well.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and reagents

Below is a list detailing all the specialized reagents used in this study, and their corresponding manufacturers.

ampicillin	Sigma Chemical Company
amylose resin	New England Biolabs
BioRad protein assay	BioRad Laboratories
2,5-diphenyloxazole (PPO)	Fisher Chemicals
dithiothreitol	Sigma Chemical Company
ECL reagents	Amersham Life Science
fetal bovine serum	Sigma Chemical Company
kanamycinsulfate	Boehringer Mannheim
penicillin(5,000U/ml)/streptomycin(5,000μg/ml) cocktail	Gibco/BRL
deoxyribonucleotides (dATP, dCTP, dGTP, dTTP)	Pharmacia
phenylmethylsulphonylfluoride (PMSF)	Boehringer Mannheim
L-glutamine	Gibco/BRL

bovine serum albumin	Sigma Chemical Company
molecular weight standards: (i) DNA: 1 kbp DNA ladder (ii) protein: prestained low range SDS-PAGE standards	 Life Technologies Bio-Rad Laboratories
Nonidet P-40	U.S. Biochemicals
paraformaldehyde	BDH
salmon sperm DNA	Sigma Chemical Company
trypsin-EDTA	Gibco/BRL
polyoxyethylene-sorbitan monolaurate (Tween-20)	Sigma Chemical Company
XAR film	Kodak Corp.

2.1.2. Radiochemicals

^{14}C-labeled high range protein standards	Gibco/BRL
[α-^{32}P]dATP (3,000 Ci/mmol; 10 μCi/μl)	Amersham Canada Ltd.
L-[^{35}S]-methionine (1151 Ci/mmol; 10μCi/μl)	NEN Life Science

2.1.3. Enzymes

The following enzymes were employed in this study, with all reactions performed as recommended by their corresponding manufacturers.

calf intestinal phosphatase	New England Biolabs
DNA polymerase I (Klenow)	New England Biolabs
lysozyme	Sigma Chemical Company
restriction endonucleases	New England Biolabs

RNAse A	Pharmacia
T7 polymerase	Pharmacia
SP6 polymerase	Promega

2.1.4. Cloning vectors

For the purpose of creating EGFP fusion proteins, pEGFP-c3 was obtained from Clontech Laboratories Inc. Within pEGFP is a red shifted mutant of (*wt*) GFP, optimized for brighter fluorescence and mammalian expression. By cloning the gene of interest (in frame) into the C-terminal MCS, high level expression of the fusion occurs driven by the human CMV immediate early promoter. In addition, an SV40 polyadenylation signal directs the proper processing of the 3' end of the corresponding mRNA transcript.

In order to express EGFP fusions in vitro, pGEM 5zf(-), and pGEM 7zf(+) were obtained from Promega. Cloning of the gene into the MCS of these plasmids can be done in such a way that *in vitro* expression may be carried out by either T7 or SP6 polymerase.

2.1.5. Host bacterial strains

For construction and amplification of all clones, an *E. coli* DH5α host strain of the genotype: *F* $^-$,ϕ80Δ*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *hsd*R17 (r_k^-, m_k^-) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1 was used.

All bacteria was grown in 2YT media (1.6% bacto-tryptone, 1% bacto-yeast and 0.5% NaCl) supplemented with either 30 μg/ml kanamycin or 100 μg/ml ampicillin.

2.1.6. Mammalian cell lines

The SV40 transformed African Green monkey kidney cell (COS-1) obtained from the American Type Culture Collection was the sole mammalian cell line used in this study.

2.1.7. Antibodies

Three different antibodies were used in this study. For Western blots a polyclonal rabbit antibody that recognizes EGFP was obtained from Clontech Laboratories Inc. For immunofluorescence studies, the monoclonal mouse anti-VP16 antibody LP1 was provided by Dr. A. Minson of the University of Cambridge (McLean *et al.*, 1982), while the texas red conjugated donkey anti mouse antibody was acquired from Jackson Immunochemicals Inc.

2.1.8. Oligonucleotides

Oligonucleotides as described in Table 2.1 were used for DNA sequencing. All oligonucleotides were synthesized by the Central Facility of the Institute for Molecular Biology at McMaster University.

Oligonucleotide	Sequence	Purpose
AB10572	$^{5'}$CTG GAG TTC GTG ACC GCC GCC$^{3'}$	EGFP MCS Forward
AB11898	$^{3'}$GTC GGT ATG GTG ATT ACA TCT CC$^{5'}$	EGFP MCS Reverse

Table 2.1 Oligonucleotides utilized in DNA sequencing.
The above oligonucleotides were used to sequence the junction points of each EGFP fusion by the 5' dideoxy sequencing method (Pharmacia).

2.2. Methods

2.2.1. Plasmid construction

2.2.1.1. EGFP-vhs fusions

All plasmids described below are shown in Figure 4.

EGvhs encodes full-length vhs fused to the fluorescent marker protein EGFP. The vhs insert for EGvhs was excised from pUC/CMVvhs, a plasmid containing (*wt*) full-length vhs bounded by various unique restriction sites (Jones *et al.*, 1995). By cutting pUC/CMVvhs with NcoI, the plasmid was linearized with 5' overhangs. These overhangs were subsequently filled in by the Klenow fragment of DNA polymerase I, and cut with KpnI ultimately liberating the full-length vhs insert. This fragment was then cloned in frame into pEGFP-c3, digested with HindIII (filled in using the Klenow fragment) and KpnI.

EGvhs VBD (wt) contains the minimal binding region of vhs capable of interacting with VP16. Conversely, the point mutant *EGvhs VBD mutW321A* encodes the same vhs VP16 binding domain but with a tryptophan 321 to alanine point mutation shown to abolish its ability to bind VP16 (Schmelter *et al.*, 1996). For the construction of EGvhs VBD (*wt*) and EGvhs VBD *mut*W321A, pSPXX (oligo) and pSPXX (oligo) Mut-W321A were used (Schmelter *et al.*, 1996). In each case, the VBD was removed from the pSPXX plasmids and subsequently inserted into EGFP as a SalI/BglII fragment.

2.2.1.2. *In vitro* expression vectors

For the purpose of expressing the EGFP-vhs fusions in vitro, the NheI/SspI fragment of EGvhs, EGvhs VBD (wt) and EGvhs VBD *mut*W321A was inserted into the SpeI/EcoRV sites of pGEM 5zf(-) downstream of the SP6 polymerase promoter. The exception was EGFP itself as it was excised as a NheI/SspI fragment and inserted into XbaI/SmaI sites of pGEM 7zf(+) under the control of the T7 promoter.

2.2.1.3. EGFP-VP16 fusions

All plasmids described here are schematically shown in figure 8.

EGVP250, 299, 335, 345, 355, 369 and *379* all encode EGFP tethered VP16 truncations spanning amino acids 4 to 250, 299, 335, 345, 355, 369 and 379 respectively. VP16 fragments were obtained from the low copy number yeast vectors pCDBVP250, pCDBVP299, pCDBVP335, pCDBVP369, pCDBVP379 (Popova *et al.*, 1995) pCDBVP345 and pCDBVP355 (J. Knez) which contain VP16 segments 250, 299, 335, 345, 355, 369 and 379 fused to the GAL4 DNA binding domain. Each VP16 truncation was liberated as a SalI/SacII fragment and cloned in frame into XhoI/SacII sites in pEGFP-c3 (EGV345, 355 and 379 were constructed by L. Solomon).

EGVP490 encodes full length VP16 fused to EGFP. Full length VP16 was isolated from the *in vitro* expression vector pSPUTK65 by cutting with NcoI, filling in the overhang with the Klenow fragment, and then digesting the resulting

linearized plasmid with KpnI. Ultimately the full-length VP16 insert was cloned into corresponding HindIII (filled in by Klenow) / KpnI sites in pEGFP-c3.

Similar to EGVP490, *EGVP411* encodes a large segment of VP16 spanning amino acid residues 4 to 411. For this construct, the VP16 insert was removed from pSPUTK65 as a SalI fragment, and inserted into the XhoI site of pEGFP-c3.

EGVP74 and *182* contain VP16 truncations spanning residues 4-74 and 4-182 fused to the EGFP gene. Construction of these clones utilized identical internal VP16 and corresponding external pEGFP-c3 MCS restriction sites. Given this, restriction site collapses were performed by digesting EGVP411 with ApaI (74) and KpnI (182). The resulting linearized pEGFP-VP16 fragment was then re-ligated to create the constructs (clones constructed by A. Datta).

All in frame pEGFP-VP16/vhs constructs described above were confirmed both by restriction analysis and 5' dideoxy DNA sequencing.

2.2.2. Plasmid purification

2.2.2.1. Small-scale preparation of plasmid DNA

Small-scale preparation of plasmid DNA was performed by the lysis by boiling method (Holmes and Quigley, 1981). Saturated 1 mL bacterial cultures were pelleted and resuspended in STET (0.1 M NaCl, 10 mM Tris-Cl (pH: 8.0), 1 mM EDTA; pH: 8.0 and 5% Triton X-100). Lysozyme (10 mg/mL in 10 mM Tris-Cl (pH: 8.0) was then added to a final concentration 0.6 mg/mL and the sample was boiled for exactly 45 sec. Cellular debris, was pelleted by centrifugation

(25°C) at 16,000xg for 10 min, and removed by a sterile toothpick. To precipitate the plasmid DNA, 0.2 mL of 7.5M Ammonium acetate (~2.2 M final concentration) and 0.6 mL isopropanol was added to the supernatant, and the sample was frozen in liquid nitrogen for 5 min. Ultimately the nucleic acids were recovered by centrifugation (4°C) at 16,000xg for 30 min, washed once with 70% ethanol and dried under vacuum.

2.2.2.2. Large-scale preparation of plasmid DNA

For large-scale preparation of DNA, the commercially available Qiagen Midi/Maxi Plasmid Prep kit was used. The procedure is based on an optimized alkaline lysis procedure and employs a modified anionic silica-gel resin that binds dsDNA and excludes contaminants such as RNA and cellular debris.

Cellular extracts were first prepared from antibiotic supplemented 100 or 500ml 2YT bacterial cultures grown up overnight at 37°C. These extracts were then applied to the silica-gel resin which retains the plasmid dsDNA. After a series of washes, bound DNA was eluted from the resin column, precipitated and collected by centrifugation. Preparation of the cellular extracts, their application to the resin, column washes and subsequent elution steps were all performed according to the manufacturer's specifications.

2.2.3. DNA quantitation via fluorescence

DNA was quantified using fluorormetry as outlined by Sambrook *et al.*, 1989.

2.2.4. Transformation of DNA into bacterial cells

2.2.4.1. Preparation of electro-competent *E. coli*.

A single colony of freshly streaked DH5α *E. coli* was inoculated into 500 mL of 2YT and grown up to an approximately 0.5 O.D. The culture was then cooled in ice water for 15 min and the bacterial pellet was collected by centrifugation (4°C) at 3000xg for 20 min. Following this, the supernatant was removed and the pellet was resuspended in 50mL of ice cold 10% glycerol in H_2O. This centrifugation / washing step was repeated twice, with the final bacterial pellet resuspended in 1 mL of ice cold 10% glycerol / H_2O per 1 gram of cells.

2.2.4.2. Preparation of chemically competent E. *coli.*

A single colony obtained from a freshly streaked plate of DH5α bacteria was initially inoculated into 1 mL 2YT media and grown up overnight. From this primary culture, 500 μL was further transferred into 100-250mL of 2YT and grown up to an optical density of approximately 0.45. The cells were then cooled on ice water for 10 minutes and collected by centrifugation (4°C) at 3000xg for 20 min). The pellet was gently resuspended in 20 mL ice cold RF1 solution (100 mM RbCl, 50 mM $MnCl_2{\cdot}4H_2O$, 30 mM $K^+CH_3COO^-$, 10 mM $CaCl_2{\cdot}2H_2O$ and 15% Glycerol adjusted to pH: 5.8 with acetic acid) and incubated on ice-water for 1 hr. Following this, the cells were pelleted by centrifugation (4°C) at 3000xg for 20 minutes, and resuspended in 8 mL of RF2

(10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2 2H_2O$ and Glycerol adjusted to pH: 6.8 with NaOH). A volume of 50-100 μL of the resuspended cells was then aliquotted into microfuge tubes and frozen in liquid N_2.

2.2.4.3. Electroporation

Introduction of DNA into bacterial cells by electroporation was performed essentially as outlined by the manufacturers of the Gene Pulser (BioRad). A volume of 15-25 μL of electro-competent cells were placed in a 0.2 cm gap cuvette with 5-15 ng of ligated DNA. The cells were then pulsed at 2.5 kV and 25 μFD while limiting the circuit to a resistance of 200 ohms. The cells were added to 1 mL of 2YT, incubated at 37°C for 30 to 45 minutes and plated on 2YT agar plates containing either 100 μg/mL of ampicillin or 30 μg/mL of kanamycin.

2.2.4.4. Transformation by heat shock

Approximately 20-50 μL of competent DH5α cells were incubated with the target DNA on ice for 30-40 min. DNA was then introduced into the cell via a 90 sec incubation in a 42°C water bath. Following this, the cells were returned to the ice and incubated for an additional 5 min. The cells were then added to 1 mL of 2YT, incubated at 37°C for 1 hr and plated on antibiotic selective 2YT agar plates.

2.2.5. Fusion protein expression in *E. coli*.

2.2.5.1. Overexpression and preparation of MBP fusions

A bacterial strain containing an IPTG inducible plasmid coding for the N-terminal 411 amino acids of VP16 fused in frame to the maltose-binding protein (Shaw *et al.*, 1995) was inoculated into 5 mL of 2YT broth containing ampicillin (100μg/mL) and grown up overnight. The following day 1 mL of this culture was used to inoculate 500 mL of ampicillin supplemented 2YT broth and grown up to a density of 0.45 O.D. units. To induce the expression of MBP-VP16411, 0.5 mM of IPTG was added to the bacteria and the culture was further incubated at 30°C for 3 hours. Following this incubation, the bacteria was pelleted by centrifugation (4°C) at 3000xg for 15 min, resuspended in 10 mLs of column buffer (20 mM Tris-HCl (pH: 7.4), 200 mM NaCl, 1 mM EDTA, 1 mM PMSF and 1 mM DTT), divided into 5mL fractions and left overnight at -20°C. The following day each sample was thawed on ice and probe sonicated (50%) at 4°C, 5 times for 20 sec alternating with 20 sec incubations on ice. Cell debris was removed by centrifugation (7000xg for 30 min), with the remaining unpurified cell extract aliquotted into 1 mL fractions and used for MBP solid phase capture assays.

2.2.6. DNA sequencing

All DNA sequencing was performed using the Pharmacia Biotech T7 sequencing kit. 5 μg of DNA was denatured in 0.22 M NaOH / O.22 mM EDTA

at room temperature for 5 min. Immediately following this, 0.1 M ammonium acetate and 60 μL ice cold absolute ethanol was added and the sample was incubated for 10 min in liquid N_2. Denatured DNA was recovered by centrifugation (4°C) at 16000xg for 30 min, washed once with 70% ethanol, re-centrifuged and dried for 10 min under vacuum. The pellet was stored as a pellet at –20°C or the sequencing reactions were immediately performed. 1-2 pmols of primer (see table 2.1) was incubated with the denatured DNA for 10 min at 60°C and allowed to cool slowly to room temperature. To sequence the sample, 1.375 μM dCTP, dTTP, dGTP and [α-^{32}P] dATP was incubated with the template for 5 min, divided into 4 parts and added to termination mixtures containing 14-17 μM of ddNTP's. After a 5 min, 37°C incubation with the termination mixtures, stop solution (97.5% deionized formamide, 10 mM EDTA (pH: 7.5) with 0.3 % bromophenol blue and xylene cyanol FF) was added. Each sample was then heated to 85°C for 2 min, resolved at 50°C on an 8% acrylamide/7.0 M urea sequencing gel, dried and exposed to film.

2.2.7. Mammalian cell line maintenance

Monolayers of COS-1 cells were maintained in Dulbecco's modified eagle's media supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 2 mM L-glutamine in a 37°C humidified incubator with a 5% CO_2 atmosphere. All media was purchased from the Cancer Research Group at McMaster University.

2.2.8. Transfection of DNA into mammalian cells

DNA was transfected into mammalian cells using a modified version of the calcium phosphate co-precipitation protocol (Graham and van der Eb, 1973). A cocktail of 0.25 M $CaCl_2$, 20 μg of total DNA (including 1-4 μg target DNA with the remainder ssDNA) and dH_2O was combined in a total volume of 250 μL. Subsequently, this mixture was added dropwise to 250 μL of 2x HBSS (280 mM NaCl, 1.5 mM $Na_2HPO_4 \cdot 7H_2O$ and 50 mM HEPES at pH: 7.12) while being gently agitated. Once complete, this 500 μL mixture was incubated for 20 min at room temperature and added to the 0.5% DMSO spiked Dulbecco's supplemented media (+10% FBS) covering the target cells. Transfected cells were then incubated in a humidified 37°C, 5% CO_2 incubator for a further 16 hrs, washed 3x with Dulbecco's modified eagle's supplemented media (-10% FBS) and re-fed with fresh (+10% FBS) media.

2.2.9. Solid phase capture assays

2.2.9.1. Solid phase capture assays using MBP-VP16411 coupled to amylose resin

1mL aliquots of MBP-VP16411 and MBP (diluted 50x) were incubated with 50 μL of amylose resin (New England Biolabs) overnight at 4°C with end-over-end mixing. The following day the samples were washed once with 1 mL column buffer (20 mM Tris-HCl (pH: 7.4), 200 mM NaCl, 1 mM EDTA, 1 mM PMSF and 1 mM DTT), column buffer + 0.2% BSA and column buffer + 0.2% BSA + 0.05% NP-40. The MBP/MBP-VP16411 coupled resin was resuspended

in 500 μL of column buffer and a specified amount *in vitro* translated [^{35}S]-methionine labeled protein was added. After incubating the samples for 1 hr at room temperature with end-over-end mixing, the resin was washed 1x with column buffer, 3x with column buffer + 0.2% BSA and 3x with column buffer, 0.02% BSA + 0.05% NP-40. Any remaining protein bound to the beads was ultimately eluted from the resin with 1x SDS sample buffer (50mM Tris-HCl (pH: 6.8), 0.1% bromophenol blue, 2% SDS, 10% glycerol and 100 mM DTT).

2.2.9.2. Preparation and amplification of the SDS-PAGE

Protein in 1x SDS sample buffer was resolved on SDS polyacrylamide gels performed as previously outlined by Laemmli *et al.* (1970). Basically, separating gels consisting of 10-15% acrylamide were overlaid with a 4% stacking gel. Protein suspended in 1x SDS sample buffer was denatured by boiling for 5 min and added to the lanes in the stacking gel. Gels were run at 200V, and subsequently were incubated in fixing solution (50% methanol / 5% acetic acid) or destaining solution (5% methanol / 5% acetic acid) for 30 min. In the case of [^{35}S]-methionine labeled protein gels, fluorography was performed to intensify bands. After fixing with destaining solution, these radioactive SDS-PAGEs were soaked in DMSO for 1 hr changing the solution intermittently. Gels were then soaked in DMSO containing 22.2% PPO (2,5-diphenyloxazole) for 2 to 3 hrs, re-hydrated in H_2O for 15-20 min, dried and exposed to film at –80°C.

2.2.10. Western blot analysis of proteins

2.2.10.1. Preparation of mammalian cell extracts

Media covering the transfected cells was removed by aspiration and the cells were washed 3x with PBS (136.8 mM NaCl, 2.7 mM KCl, 1.5 mM K_2HPO_4 and 8 mM $Na_2HPO_4{\cdot}7H_2O$). 1 mL of TEN buffer (40 mM Tris-HCl (pH: 7.4), 1 mM EDTA and 0.15 M NaCl) was then added to the plate and incubated at room temperature for 5 min. Following this incubation, the cells were detached from the plate using a cell lifter (Costar), placed in microfuge tubes and pelleted by centrifugation (4°C) at 16000xg for 2 min. The supernatant was removed and the pellet was resuspended in CAT assay buffer (250 mM Tris-HCl (pH: 7.8), 0.5% NP-40 and 1 mM PMSF), incubated on ice for 10 min and periodically vortexed. Following this, the extract was sonicated 3 times for 20 sec alternating with 30 sec incubations on ice. Ultimately cell debris was removed by centrifugation (4°C) at 16000xg for 10 min, while the supernatant was collected and stored at –20°C.

2.2.10.2. Quantification of Protein Concentration

To determine the protein concentration of the prepared cellular extracts in CAT assay buffer, BioRad protein assay reagent was used. Essentially, 200 μL of the BioRad dye reagent (Coomassie brilliant blue G-250) was added to BSA standards and unknown samples diluted in 800 μL of H_20. The samples were incubated at room temperature for 5 minutes at which time

their A_{595} was determined. The absorbance of the BSA standards were used to generate a standard curve from which the concentrations of unknown samples were calculated.

2.2.10.3. Western blot procedure

Western blots were preformed as outlined by Sambrook *et al.*, 1989. Initially, 50 μg of total protein obtained from transfected mammalian cells was loaded and separated on a SDS-PAGE gel. Proteins were then transferred from the gel to a nitrocellulose membrane overnight at 100 mA. The following day, the membrane was blocked for 1 to 2 hr with 3% skim milk powder and washed 3x for 10 min with TBST (20 mM Tris-HCl (pH: 7.5), 150 mM NaCl and 0.05% Tween-20). A suitable dilution of the 1° antibody was then added in TBST + 1% skim milk powder and incubated for 1 hr at room temperature. Again the membrane was washed 3 times with TBST followed by the addition of the 2° antibody diluted in TBST + 1% skim milk powder. After a 1 hr incubation at room temperature the membrane was washed 3x in TBST and 1x in Tris Saline (20 mM Tris-HCl (pH: 7.5), 150 mM NaCl). To detect the bands an ECL western blot detection kit was used as outlined by its manufacturer (Amersham). Briefly, the two ECL reagents were combined and incubated with the nitrocellulose membrane for 1 min. The solution was then poured off the membrane and it was exposed to film for 15 to 60 sec.

2.2.11. Fluorescence microscopy

2.2.11.1. EGFP fluorescence

To observe the cellular localization of proteins tagged with EGFP, COS-1 cells were seeded onto coverslips placed in six well dishes. Generally, the cells were added at an appropriate density so that they would be approximately 30% confluent at the time of transfection. Each of the EGFP constructs were then transfected into the cells as described previously. After 48 hrs, the media was removed and the cells were washed 3 times with PBS. Following this, 4% paraformaldehyde (PFA) in PBS was added to each of the wells and incubated for 30 min at room temperature. Once fixed, the coverslips were washed 3x with H_2O, mounted onto slides with 10% PBS/90% glycerol and sealed with an aqueous slide sealant (ASS; 7.5% Gelatin and 54% Glycerol in H_2O) or transferred to 6 well dishes lined with parafilm for double fluorescent labeling.

2.2.11.2. Immunofluorescence

Fixed cells transfected with VP16 were initially permeabilized with 1% Triton X-100 in PBS for 20 minutes at room temperature. Following this, the cells were washed once with PBS/0.02% Tween-20 and once with PBS / 0.02% Tween-20/1% BSA. 1° antibodies were then diluted in PBS/3% BSA, added directly to the coverslips and incubated for 1 hr at 37°C in a humidified chamber. After washing the cells with PBS/0.02% Tween-20/1% BSA, the 2° antibody (again diluted in PBS/3% BSA) was added to the coverslips and again incubated

for 1 hr at 37°C in a humidified chamber. Subsequently, the cells were washed 1x with PBS/0.02% Tween-20, once in PBS and 2x with H_2O, dried in a 37°C incubator for 20 min and mounted and sealed as described previously.

3. RESULTS

3.1. VHS co-localizes with VP16 to possible perinuclear regions within the cell

3.1.1. EGFP does not interfere with the interaction between vhs and VP16

The deleterious effects seen within infected cells containing a functional virion host shutoff protein have been well characterized. However, how vhs action is downregulated to permit the accumulation of viral mRNA still remains a matter of debate. Recent studies have focused on a possible regulatory interaction between VP16 and vhs as a potential mechanism by which vhs activity is suppressed. Moreover, it has been proposed that through this interaction, VP16 may serve to compartmentalize vhs away from target mRNA (Smibert *et al.*, 1994; Lam *et al.*, 1996). Experiments have demonstrated that a functional interaction between vhs and VP16 may be required to transport vhs to the nucleus so it can be incorporated in the tegument of new virions. In these studies, Western blot analyses on purified KOS (wt), vhs1 and ΔSma HSV viruses suggested that wild type vhs and vhs1 (T214I) were indeed present in viral teguments, but a deletion mutant (ΔSma: Δ179-344) was not (Read *et al.*, 1993). Interestingly, it appears as if the ability of these proteins to be packaged

into new virions coincides with their ability to interact with VP16 suggesting that nuclear localization of vhs then may not only be a means of downregulating vhs activity, but may also serve to facilitate its packaging into new virions.

In order to determine if VP16 could serve to translocate vhs into the nucleus, and whether this translocation was dependent on the ability of vhs to bind VP16, the cellular distribution of full length vhs as well as vhs VBD (*wt*) and vhs VBD *mut*W321A was monitored in the absence and presence of VP16 (Schmelter *et al.*, 1996). Both the vhs VBD constructs consist of the 21 amino acid minimal binding domains of vhs that were deemed necessary and sufficient to interact with VP16. The one difference between the two is a single amino acid substitution (alanine 321 to tryptophan) which was demonstrated to abolish its ability to interact with VP16 (Schmelter *et al.*, 1996).

To monitor the cellular distribution of each of these constructs in the presence and absence of functional VP16, vhs, vhs VBD (*wt*) and vhs VBD *mut*W321A were fused in frame to the enhanced green fluorescent protein (EGFP; figure 4). An initial concern was that the addition of a large (27 kDa) protein to a small fragment such as vhs VBD may alter of even abolish the ability of the fusions to interact with VP16. To investigate this possibility, *in vitro* translated [^{35}S]-Met EGvhs, EGvhs VBD (wt) and EGvhs VBD *mut*W321A were tested for their ability to bind MBP and MBP-VP16$_{411}$ immobilized on amylose resin. As expected, EGvhs bound strongly to the MBP-VP16$_{411}$ conjugated resin, suggesting that the fusion of the bulky EGFP onto the N-terminal of vhs did not affect its ability to interact with VP16 (figure 5, lane 6). In addition, the

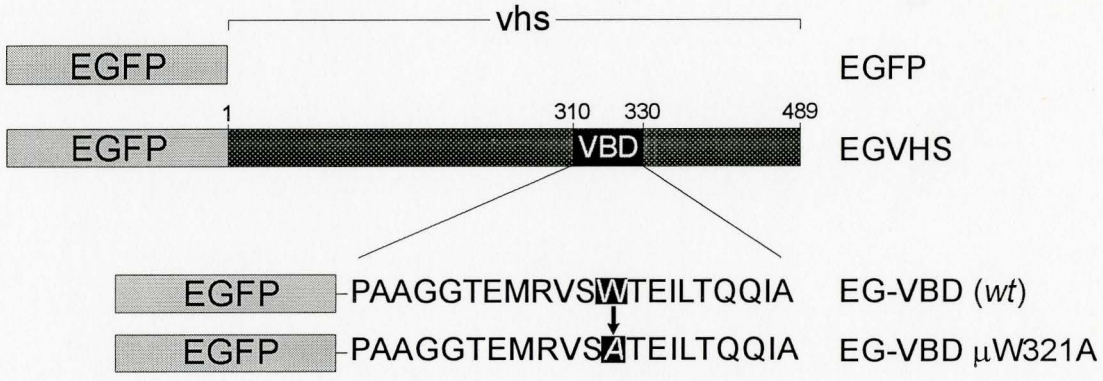


Figure 4. Schematic Representation of EGFP-vhs Fusions.
Illustrated above are the constructs that were used to assay the ability of vhs to relocalize within the cell in the presence of VP16. EGvhs consisted of full-length vhs protein fused to the C-terminal end of the fluorescent marker protein EGFP. By attaching this protein, the position of vhs within the cell could be monitored at all times by using fluorescence microscopy. Two smaller EGvhs constructs were also created by fusing EGFP to a small region of vhs (spanning amino acids 310 to 330). EGvhs VBD (*wt*) consisted of this 21 amino acid stretch of vhs that was previously demonstrated to be able to interact with VP16 as a modular binding domain. EGvhs VBD *mut* W321A was almost identical to EGvhs VBD (*wt*) with the exception of a single point mutation (W321A) that abolished the interaction between vhs and VP16.

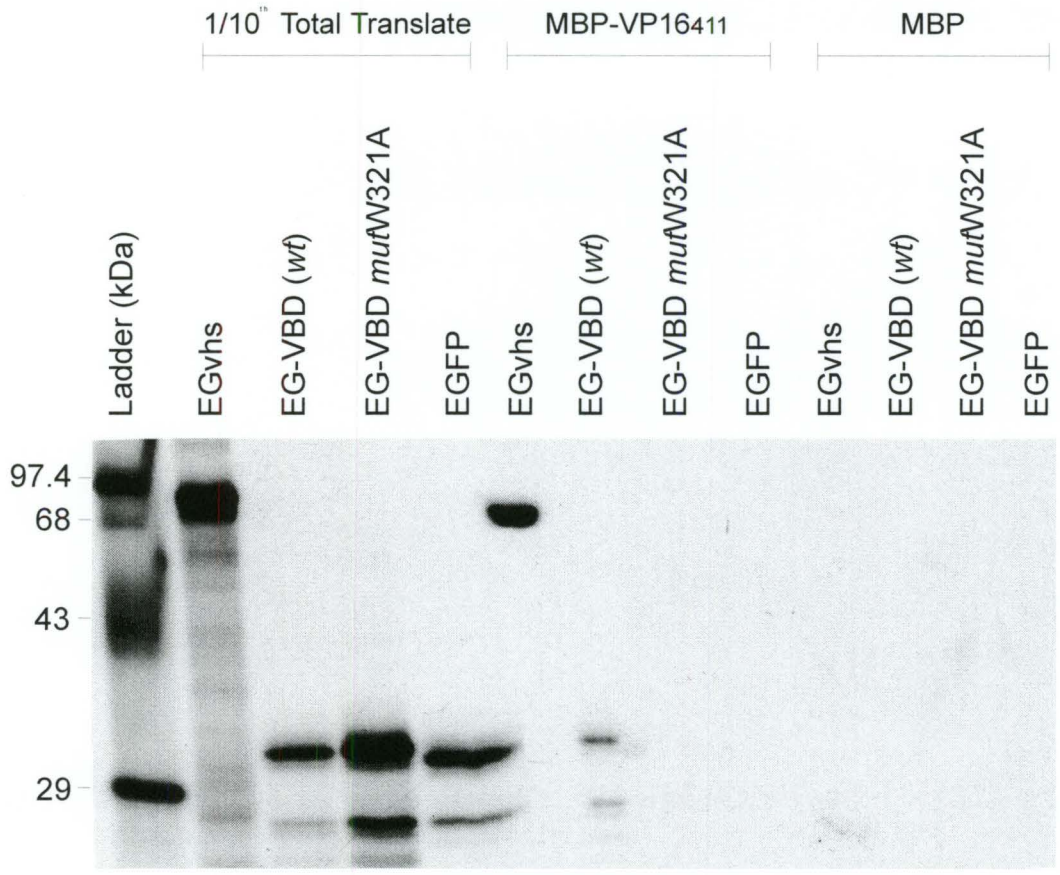


Figure 5. EGvhs and EGvhs VBD (wt) bind specifically to VP16411.
Equivalent aliquots of in vitro translated proteins were incubated with MBP or MBP-VP16411 coupled amylose resin for 1 hr at 25°C in column buffer (20 mM Tris-HCl (pH: 7.4), 200 mM NaCl, 1 mM EDTA, 1 mM PMSF and 1 mM DTT). The resin was extensively washed with combinations of column buffer, column buffer + 0.02% BSA and column buffer +0.02% BSA + 0.05% NP-40. Ultimately, bound proteins were eluted by boiling the resin for 5 min in SDS-PAGE sample buffer and resolved on a 12.5% polyacrylamide SDS gel. The first four lanes represent 1/10th of the total programmed reticulocyte lysate that was incubated with the MBP of MBP-VP16 conjugated resin. In each case, EGFP was used as a negative control to monitor any non-specific binding to the conjugated amylose.

EGvhs VBD (*wt*) also bound to VP16 but with a lower affinity (figure 5, lane 7). This reduced binding is not surprising, as other studies have reported that larger fragments of vhs (ie. 179-344) seem to bind VP16 with a greater affinity. Perhaps the flanking regions surrounding the vhs VBD play a role in stabilizing its interaction with VP16 and/or stabilize vhs structure (Schmelter *et al.*, 1996).

The interaction between these constructs and MBP-VP16411 was deemed to be specific according to three observations: (i) both EGvhs and EGvhs VBD (*wt*) could bind to MBP-VP16411 but did not bind to the MBP conjugated resin (figure 5) nor the resin alone (data not shown), (ii) the point mutant EGvhs VBD *mut*W321A did not bind in any of the conditions and (iii) EGFP alone did not non-specifically interact MBP-VP16, MBP, nor the resin itself. These results suggest that the EGvhs interactions with the MBP-VP16411 conjugated resin are mediated by specific vhs-VP16 binding and not by extraneous regions present in EGFP. It appears then that the fusion of the EGFP protein to the N-terminal portion of the vhs fragments does not alter their ability to interact with VP16.

3.1.2. EGvhs, EGvhs VBD (*wt*), EGvhs VBD *mut*W321A and VP16 express well *in vivo*

In order to assay the relative levels of expression between the vhs constructs and co-transfected VP16, Western analyses were performed on extracts obtained from VP16/vhs co-transfected cells (figure 6). All the EGvhs constructs appeared to express to high levels, however, it appears as if EGvhs

Figure 6. Western blot analysis of EGvhs/VP16 co-transfected cellular extracts.

50μg of total protein obtained from the cellular extracts of mock, EGvhs/VP16, EGvhs VBD (wt)/VP16, EGvhs VBD *mut*W321A/VP16 and EGFP/VP16 co-transfected COS-1 cells were initially resolved on two separate 12.5% polyacrylamide gels. The proteins were then transferred to nitrocellulose membranes overnight and probed with either a (A) rabbit anti-GFP antibody or (B) an antibody raised against the C-terminal 411 amino acids of VP16 (αSal) to identify the bound proteins. Above each lane is the EGvhs fusion that was transfected along with full length VP16.

was not transcribed and translated as well as the other smaller constructs. The reason for this is unclear but it is unlikely that any residual mRNA destabilizing activity would account for this discrepancy as the expression of VP16 in the same group of cells is apparently unaffected (figure 6; panel B, lane 2). It is more likely that full length vhs was degraded as it may be more susceptible to proteolytic degradation considering it is larger than the other constructs, and protrudes farther away from the highly stable β-barrel structure of EGFP (Ormo *et al.*, 1996).

In examining VP16 expression in these cells (figure 6, panel B), its concentration is relatively consistent regardless of what vhs construct was co-transfected along with it. Even taking into account the fact that each of the antibodies have different affinities to their target protein, it seems that vhs is still expressed to higher levels than VP16 within these transiently transfected cells. This result does not parallel the ~4:1 VP16 to vhs stoichiometric expression levels seen in an infection (Zhang and McKnight, 1993), but does indicate that VP16 and vhs are being expressed at adequate levels to monitor any changes in cellular localization.

3.1.3. EGvhs and EGvhs VBD (wt) co-localize with VP16 in co-transfected cells

Transient transfections of viral proteins such as vhs have been shown to be biologically relevant in delineating the role that HSV-1 viral proteins play within the cell (Jones *et al.*, 1995, Zelus *et al.*, 1996). Therefore, co-transfections

were used in this study to determine whether the presence VP16 was capable of altering the subcellular distribution of vhs. These co-transfection experiments were performed utilizing the three previously described EGFP-vhs fusions transfected into COS-1 cells either alone or in tandem with VP16.

Transfection of EGvhs VBD (*wt*), EGvhs VBD *mut*W321A and EGFP led to a uniform distribution of fluorescence throughout the entire cell including the nucleus. This result is not entirely surprising as small proteins with molecular weights below 40 kDa passively diffuse through the 10nm aqueous channel located in the nuclear pore complex (Silver, 1991). In the case of the larger, EGvhs protein, no nuclear fluorescence could be noted suggesting that it is too large (~85 kDa) to diffuse into the nucleus and furthermore that there are no regions within EGFP nor vhs that serve to translocate the protein into the nucleus (figure 7).

It is interesting to note that VP16 itself does not completely localize to the nucleus when transfected (figure 7, VP16 alone), a result consistent with previous studies (Friedman *et al.*, 1988; Elliot *et al.*, 1995). Moreover, it has been demonstrated that VP16 assumes a concentrated distribution around the periphery of the nucleus referred to here as a 'halo-like' cellular distribution. The function of this localization is still unclear, but it could constitute another sub-nuclear compartment important in the final assembly of the virion (Ward *et al.*, 1996; Zhu and Courtney, 1994).

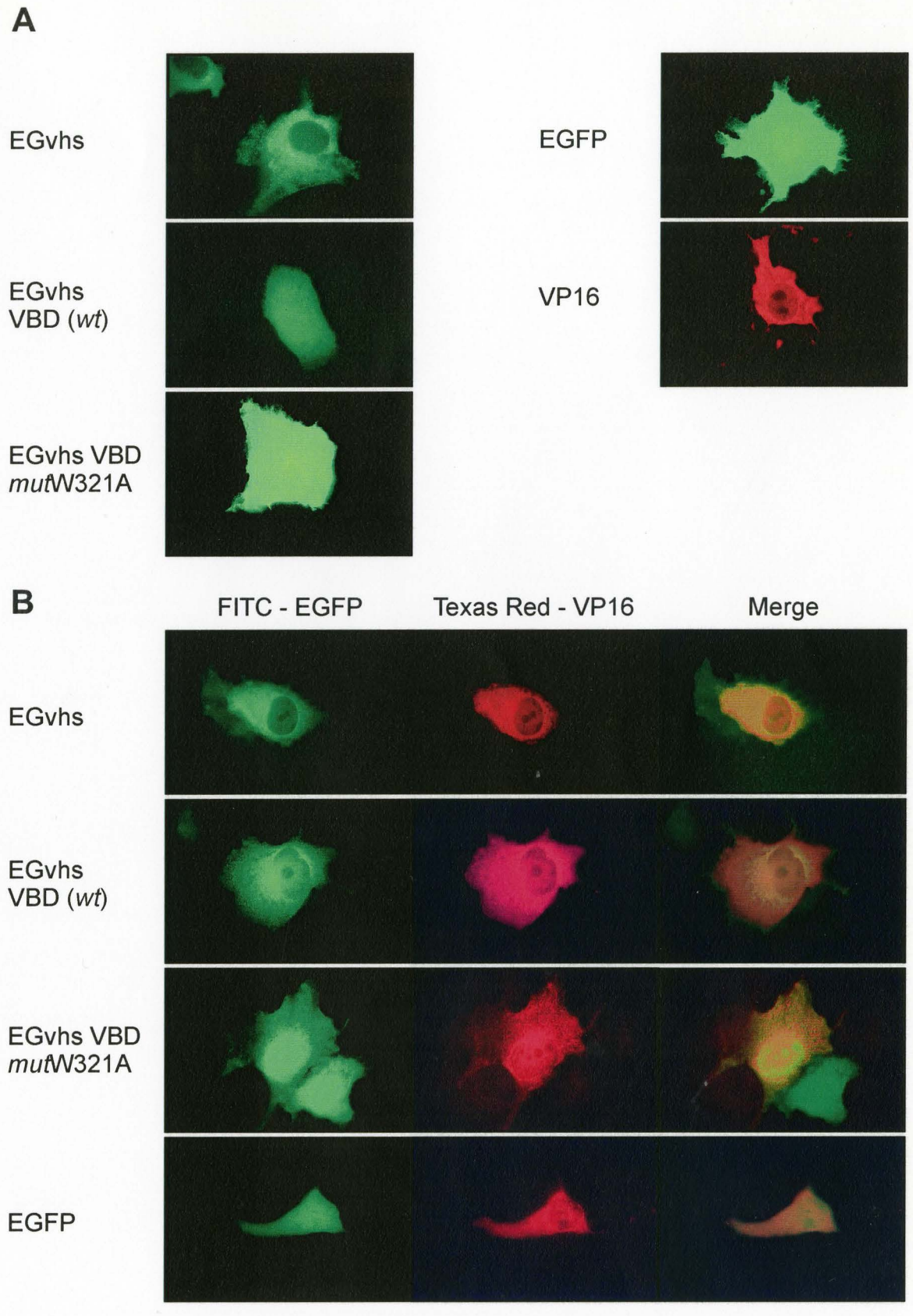
When VP16 was co-transfected with FL EGvhs (*wt*), the construct co-localized to what appeared to be perinuclear and cytoplasmic regions along with

VP16 (figure 7, EGvhs Merge). EGvhs VBD (*wt*) / VP16 co-transfections also yielded a redistribution of the vhs construct, but interestingly it did not match the results obtained in the EGvhs / VP16 co-transfections. In this case, vhs re-distributed to the periphery of the nucleus, but much more efficiently than its EGvhs (*wt*) counterpart (figure 7, EGvhs VBD (*wt*); FITC). Transfection of full length EGvhs as compared to the EGvhs VBD (*wt*) appears then to result in very different cellular distributions in the presence of VP16.

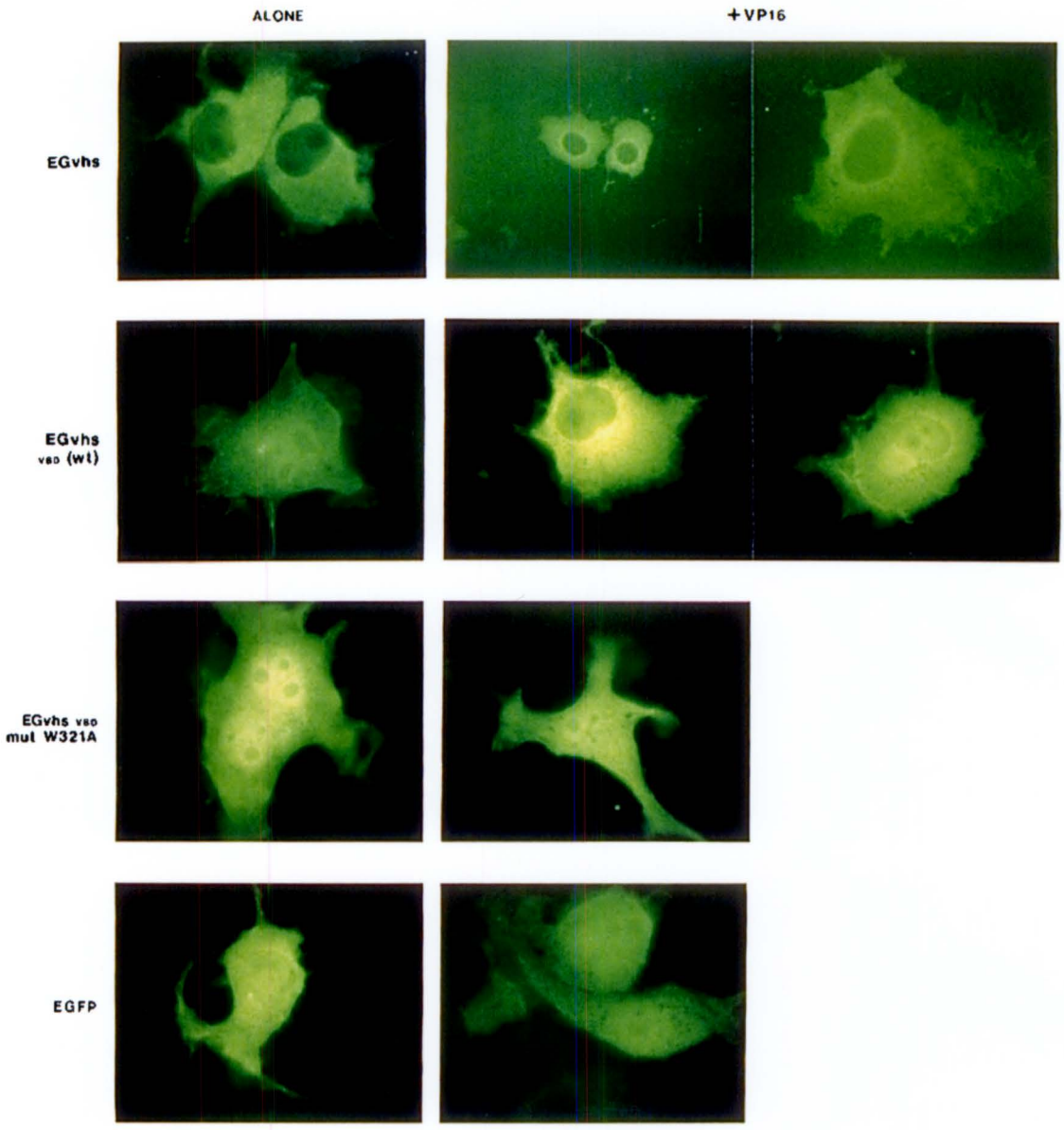
Further control studies were conducted to ensure that there was no cross channel leak over when using the two filters on the epi-fluorescent microscope (data not shown). From these experiments we could conclude that EGFP and the texas red fluorophore spectra were not overlapping and thus did not significantly contribute to the background nor demonstrate false overlapping regions.

The ability to bind VP16 is an essential feature of both the EGvhs and EGvhs VBD (*wt*) constructs in order to re-distribute within the cell. In these experiments, localization of each construct altered in the presence of VP16. However, when EGvhs VBD *mut*W321A and EGFP were transfected into cells with VP16, no notable re-distribution occurred. This coincides with the results of the solid phase capture assay (figure 5) as only those vhs fragments shown to interact with VP16 *in vitro*, (namely EGvhs VBD (*wt*) and EGvhs), relocated to the nucleus and co-localized with VP16 *in vivo*.

Figure 7. EGvhs and EGvhs VBD (*wt*) co-localize with VP16 *in vivo*.
COS-1 cells were transfected via the calcium co-precipitation method (Graham and van der Eb, 1973) with the specified EGvhs construct alone (panel A) or in concert with full length VP16 (panel B). Once transfected, the cells were incubated at 37°C for 36 hours and ultimately fixed and permeabilized for immunofluorescence. In each case, the location of VP16 was determined through probing the cells with LP1 (a mouse anti-VP16 antibody at a 1:60 dilution in PBS) and a donkey anti-mouse antibody conjugated to a texas red fluorophore (diluted 1:70 in PBS). Cells were viewed under a Zeiss epi-fluorescence microscope equipped with both a FITC (ex. 450-490 nm; em. 510-520 nm) and Rhodamine (ex 515-565 nm; em. 580-590 nm) filter set. The fluorescent cells were photographed using Kodak Elite Ektachrome 400 Film. Developed pictures were ultimately scanned onto a Kodak Photo CD and were subsequently processed using Adobe Photoshop 4.0. For the purpose of creating the merged images, corresponding red and green fluorescent pictures were overlaid with the top layer set at 50% opacity. Ultimately, the finished images were imported into CorelDRAW! 8.0 where they were arranged and labeled for subsequent printing on a Canon Fiery colour laser printer. (Panel C) Additionally, photographs of EGvhs transfected and EGvhs/VP16 co-transfected (FITC fluorescing) cells were taken to further corroborate merged results. All of the images were arranged and displayed in panel C.


A
EGvhs
EGvhs VBD (wt)
EGvhs VBD mutW321A
EGFP
VP16
B
FITC - EGFP
Texas Red - VP16
Merge
EGvhs
EGvhs VBD (wt)
EGvhs VBD mutW321A
EGFP

C



3.2. The region spanning amino acids 335 to 355 is important for the nuclear localization of VP16

The role of VP16 as a transactivator may be only of limited importance to the viability of the virus. A virus lacking a copy of VP16 is compromised in its ability to transduce viral genes, but is only slightly less viable when infected at a high MOI (Ace *et al.*, 1989). However, there seems to be an essential structural role for VP16 during infection. How VP16 contributes structurally to virion assembly is still unclear, but there is a strong body of evidence to suggest that it may indeed serve as a glycoprotein scaffold (Zhu and Courtney, 1994) and/or co-ordinate the assembly of the tegument (Ward *et al.*, 1996). What is common among these studies is the hypothesis that VP16 functions from within the nucleus to co-ordinate these events, as evidenced by the accumulation of VP16 at the nuclear periphery of infected cells (Ward *et al.*, 1996).

Since recent studies have suggested that VP16 may downregulate and ultimately package vhs by translocating it to the nucleus (Lam *et al.*, 1996; Read *et al.*, 1993) the elucidation of the region in VP16 responsible for its localization is now of added importance. As such, the NLS of VP16 may not only function to position VP16 so it can activate genes but the possibility exists that it also may serve to indirectly downregulate and package vhs.

3.2.1. EGFP-VP16 fusions are expressed *in vivo*

In order to delineate the region(s) responsible for the nuclear localization of VP16, various truncations of VP16 were fused to the fluorescent

marker protein EGFP, and tested for their ability to localize to the nucleus when transfected into COS-1 cells. Initially nine separate EGFP-VP16 (EGVP) fusions were generated spanning the N-terminal 74, 182, 250, 299, 335, 369, 379, 411 and 490 (full length) amino acids of VP16 (figure 8). To ensure that each of the constructs expressed well and as the proper size *in vivo*, a Western blot was performed on COS-1 cells transfected with each of the clones (figure 9). All of the constructs appeared to express to sufficient levels in vivo (figure 9). Each fusion was deemed to be in frame and encode the proper fragment of VP16 as evidenced by N and C terminal sequencing of the cloning junction points. However, some of the constructs (EGVP250, 299, 335, 369 and 379) were fused to EGFP utilizing different upstream and downstream restriction sites than the remaining clones (EGVP74, 182, 411 and 490). As a result, EGVP250, 299, 335, 369 and 379 possess extra amino acids introduced as a result of the cloning procedure and as such contributed to a higher apparent molecular weight. EGVP345 and 355 also appeared to express well in vivo but migrated to a position corresponding to a protein of slightly higher molecular weight (data not shown). This is not surprising however, as these clones also possessed additional amino acids introduced as a result of the cloning procedure.

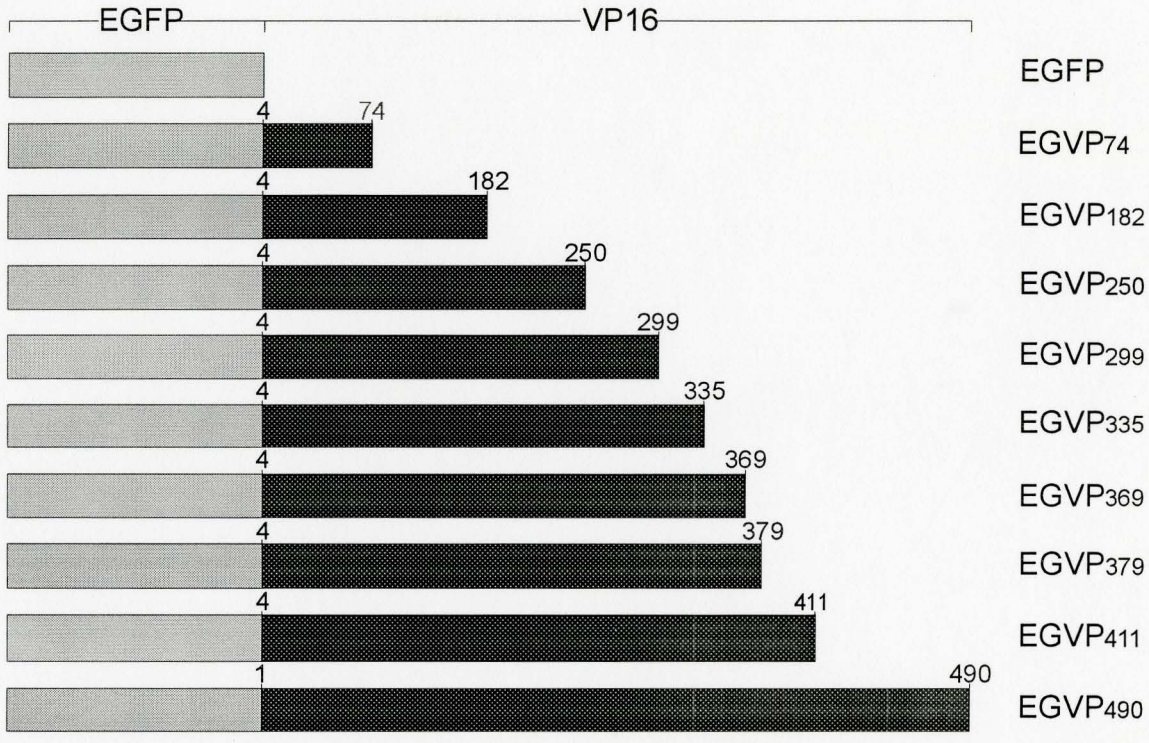


Figure 8. Schematic representation of EGVP constructs.
VP16 truncations used for the determination of the region(s) responsible for nuclear localization are depicted above. Each clone was subsequently transfected into COS-1 cells and visualized using both the intrinsic fluorescence of EGFP and another conjugated antibody targeted to the VP16 portion of the fusion. All constructs (except the full length EGFP - VP16 construct) span from amino acid 4 to the one indicated above the C-terminal end of each clone.

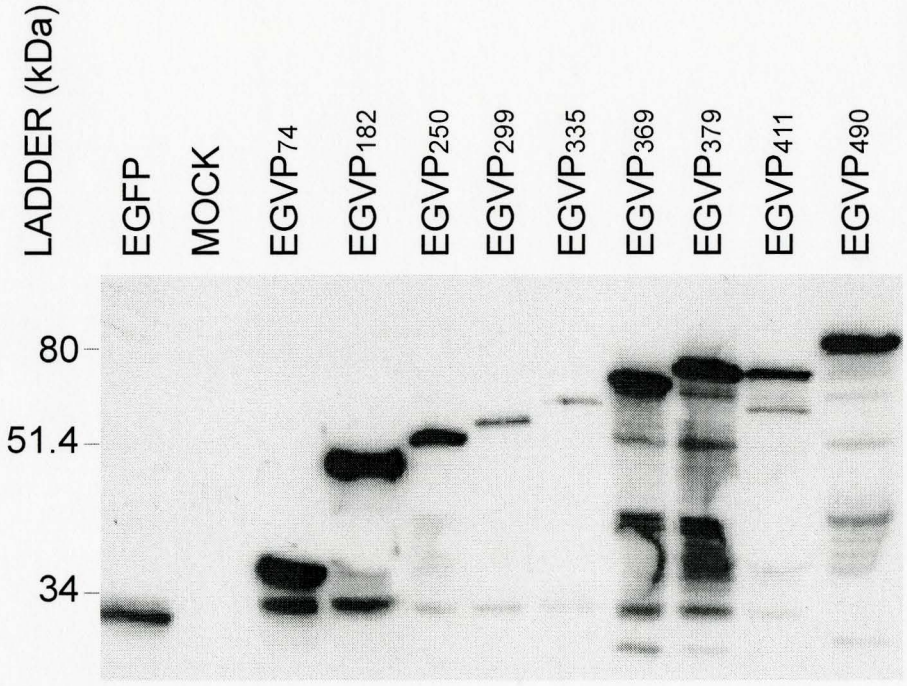


Figure 9. Expression of the EGVP constructs *in vivo.*
Western blots were performed by loading 50μg of total protein isolated from cells transfected with each specified construct onto a 12.5% SDS polyacrylamide gel. Once resolved, the proteins were transferred to a nitrocellulose membrane, extensively washed as described previously, and probed with a rabbit anti-GFP antibody. After further washes, an anti-rabbit antibody coupled to horseradish peroxidase was added and ultimately identified using ECL detection reagents (Amersham).

3.2.2. The N-terminal 355 amino acids are sufficient for the localization of VP16 to the nucleus

In order to observe each of the EGFP-VP16 truncations, they were transfected into COS-1 cells, and prepared for fluorescence. Transfection of EGVP74 and 182 both yielded a similar uniform distribution of fluorescence throughout the entire cell. As each of these constructs are smaller than 40 kDa, both are capable of diffusing into the nucleus through the aqueous channel in nuclear pores, and hence move freely into the nucleus (figure 10). Localization of these clones to the nucleus then is not a case of specific active transport, but rather a result of non-specific passive diffusion.

EGVP250, 299 and 335 all exhibit similar phenotypes as each are almost completely excluded from the nucleus (figure 10, panels A and B). In order for these constructs to localize to the nucleus, the proteins would have to be specifically imported as their molecular weight exceeds the nuclear pore exclusion size of 40 kDa. Of further interest is the speckled appearance of the FITC fluorescence in each of the cells. One possibility is that these clusters of EGFP fluorescence may be aggregates of insoluble proteins. As inclusion bodies of EGFP have been shown to be non-fluorescent (Clontech), these insoluble clusters may originate from the aggregation of the VP16 portion of the fusion. Supporting this hypothesis is the observation that removal of the acidic activation tail in some cases causes VP16 to become insoluble (L. Donaldson, unpublished observations).

The distribution of the EGFP fluorescence is practically identical in cells transfected with EGVP250, 299 and 335. However, in EGVP369, 379 and 411 a distinctive change in EGVP localization occurs. While the EGFP-VP16 constructs encoding a fragment of VP16 spanning the N-terminal 335 amino acids or less assumed a cytoplasmic distribution, all of the constructs encoding 369 amino acids or more partially localized to the nucleus of the transfected cells (figure 10, panel B). In addition, the full length EGVP490 also partially localized to the nucleus, but unlike the smaller constructs, accumulated at the periphery of the nucleus.

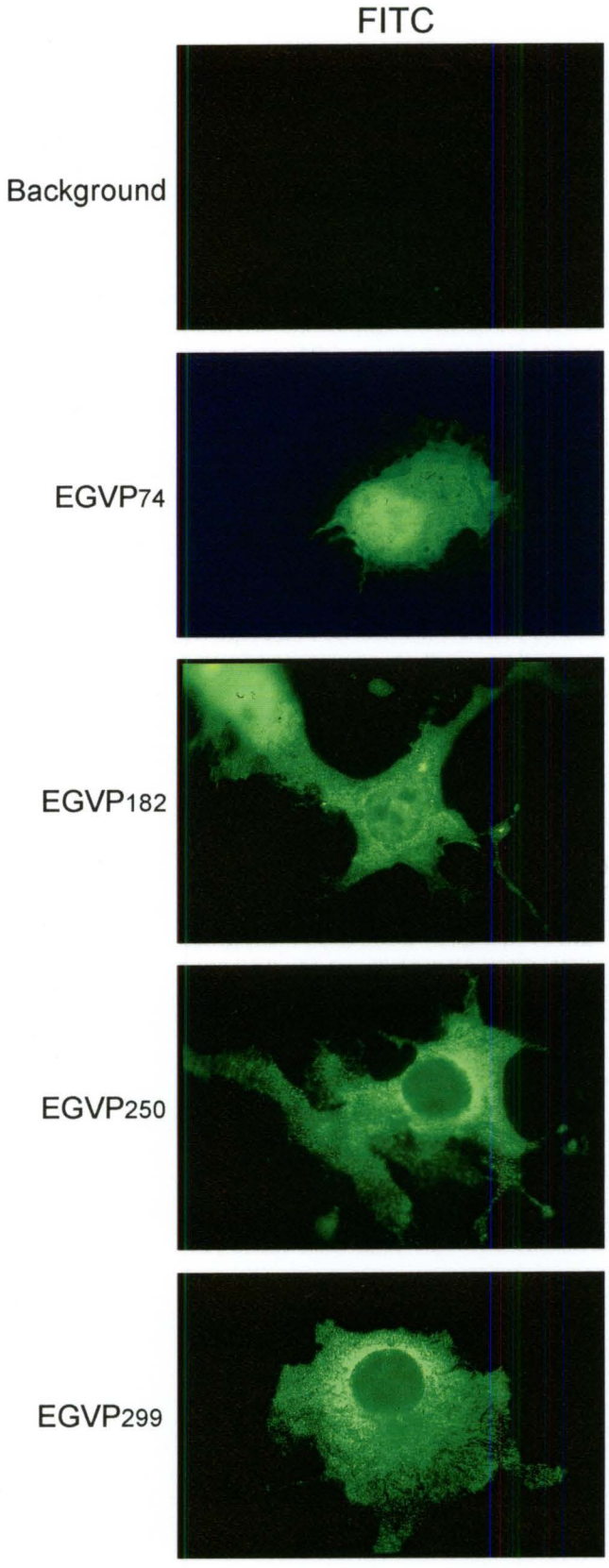
As mentioned previously, possible 'insoluble' proteins appear to be present in cells expressing EGVP369, 379 and 411. In these cells however, the proteins appear to aggregate in distinct foci just on the edge of the nucleus (figure 10, panel B, EGVP369, 379 and 411). Whether these foci constitute a distinct compartment or if they are just an artifact of the system is unclear. Elliott *et al* (1995) previously described similar structures as tegument bodies. However, their results indicated that formation of these tegument bodies absolutely required the 37 C-terminal amino acids of the acidic activation domain.

It appears that the region spanning amino acids 335 to 369 is absolutely necessary for VP16 localization. However recent experiments have narrowed down this region even further. Preliminary results using two new EGFP-VP16 fusions spanning the N-terminal 345 and 355 amino acids of VP16 have indicated that the twenty amino acids between 335 and 355 also seem to

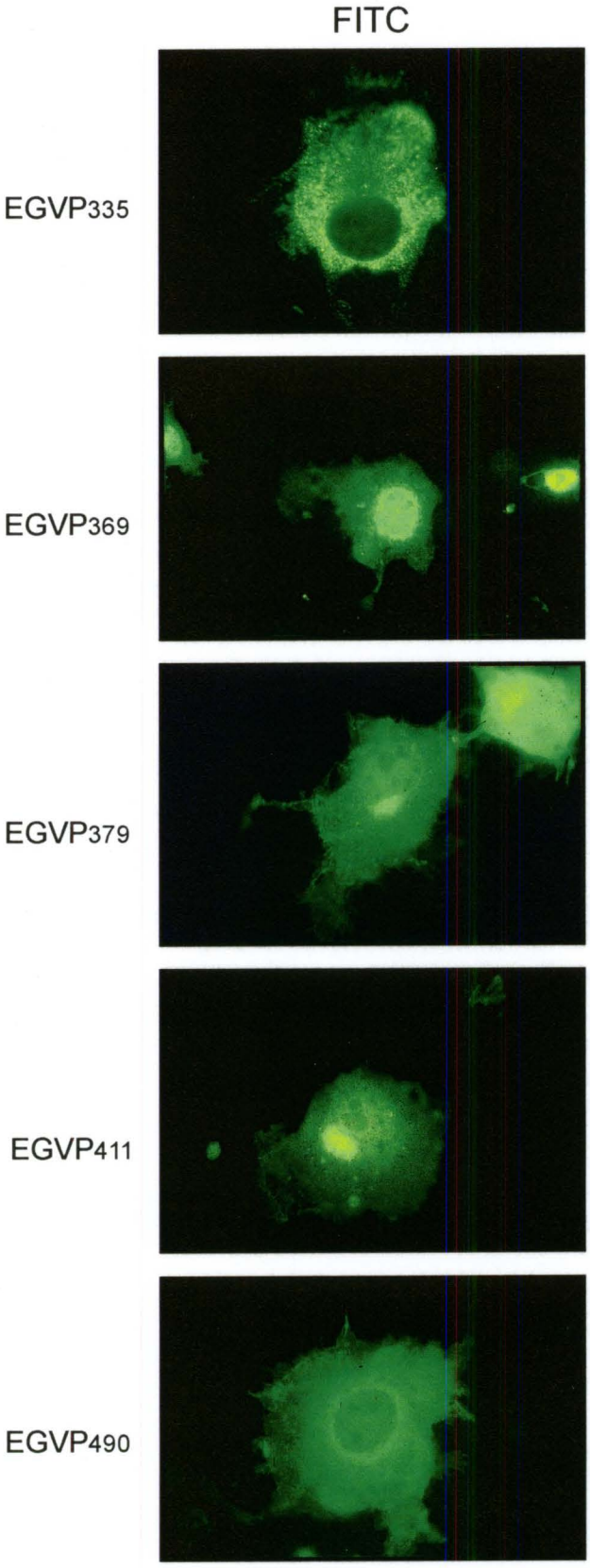
Figure 10. The region between amino acids 335 to 355 is necessary for nuclear localization of VP16.

COS-1 cells seeded onto coverslips were transfected with the specified construct using the calcium co-precipitation method (Graham and van der Eb, 1973). Following this, the cells were incubated at 37°C for 36 hrs, fixed using 3.7% paraformaldehyde in PBS. Transfected cells expressing the fluorescent EGVP74, 182, 250, 299 (panel A) and EGVP335, 369, 379, 411 and 490 (panel B) were ultimately mounted onto slides, photographed and digitally processed as previously described.

A



B



be important for nuclear localization of VP16 (data not shown).

Results initially obtained with the EGVP345 fusion have been conflicting as fluorescence within the cells appears to be nuclear in some instances, and in others completely cytoplasmic. The reason for these contradicting observations still remains unclear, but it is possible that truncating VP16 to amino acid 345 may have in fact created a conformationally constrained protein which may alter its properties as a whole and thus cause it to behave erratically *in vivo*.

4. DISCUSSION

4.1. VP16 possibly serves to shunt vhs to a virion assembly pathway.

VP16 has long been known to play an important role throughout the lifecycle of Herpes Simplex Virus Type 1. Many studies have focused on how VP16 initiates the characteristic genetic cascade of viral genes in infected cells, while others are beginning to elucidate possible structural roles that VP16 may play at later times during virion assembly. One key phenomenon linked to VP16 is its ability to downregulate the messenger ribonucleolytic action of vhs (Lam *et al.*, 1996). Initial immunofluorescence studies investigating whether compartmentalization could account for this vhs downregulation proved to be inconclusive as vhs localization patterns were seen either confined within the body of the nucleus or located in the perinuclear region depending on what antibody was used. The reason for these conflicting localization patterns remains unclear, but it has been hypothesized that different vhs epitopes may have been exposed depending upon the physical environment of each cellular compartment (Jones, Ph.D. thesis, 1995). As these experiments were complicated by the fact that vhs was being monitored in the context of an

infected cell, we set out ascertain if VP16 alone could serve to localize vhs to the nucleus without any other viral proteins. The advantage of this system over the previously described one, was that it utilized a very stable fluorescent maker protein (EGFP) rather than a reacting antibody to locate the position of vhs. By using this marker, any potential problems previously observed with possible masked epitopes were alleviated.

Utilizing the minimal binding domain also limited our study to regions in vhs solely responsible for VP16 binding. As it is highly unlikely that this region is an interaction domain for another host cell protein, any indirect effects that VP16 may have within the cell that may function to localize vhs could be discounted. Consequently, cellular redistribution of vhs in the presence of VP16 could be attributed to a direct effect such as VP16 mediated translocation across the nuclear membrane via protein-protein interactions.

Co-transfection studies presented here suggest that vhs can indeed relocalize within the cell in the presence of VP16. Furthermore this re-distribution seems to be absolutely dependent on the ability of the two proteins to interact with each other as the cellular distribution of a point mutant (EGvhs VBD *mut* W321A), previously shown to be unable to bind VP16 (Schmelter *et al.*, 1996; figure 5), remained unaltered when co-transfected with VP16 (figure 7). Besides possessing transactivation and structural roles during HSV infections then, the results presented here appear to assign VP16 a new role as a shuttle protein, possibly translocating both itself and vhs across the nuclear membrane into the nucleus. The ability of VP16 to translocate another protein in tandem is

not a foreign possibility as hsp70, for example, has been shown to facilitate the localization of other proteins into the nucleus (Shi and Thomas, 1992).

Since the merged FL-vhs / VP16 images do present an overlap of EGFP/Texas Red fluorescence within cytoplasmic/perinuclear regions of the cell (figure 7, Panels B and C), VP16 mediated vhs downregulation via compartmentalization in the nucleus could be a possible mechanism of vhs regulation. However, as physiologically relevant stoichiometric expression levels of vhs and VP16 were not achieved in these co-transfection experiments we can only hypothesize that compartmentalization may be a valid mechanism of vhs downregulation (figure 6). Furthermore, these observations do not disprove the possibility that VP16 may inactivate vhs by simple protein-protein interactions. Downregulation in this case could be envisioned as a direct inhibition of vhs activity via a conformational alteration or by blocking the substrate from entering the active pocket of the protein.

In comparing the distribution of EGvhs VBD (*wt*) and EGvhs an interesting feature emerges. In these experiments, the 20 amino acid fragment encoding the wild type VP16 binding domain of vhs appeared to assume a more complete distribution around and possible within the perinuclear region of transfected cells in the presence of VP16 (figure 7, Panels B and C; table 4.1 column 3). Conversely, full-length EGvhs co-transfected with VP16 assumed a distribution more intermediate between the nucleus and the cytosol. One possibility for this discrepancy is that full-length vhs may in fact be partially retained in the cytosol

Construct	Location of Fluorescence[a]		Relocates in Presence of VP16	Able to Bind VP16[b]	Fluorescent Statistics (%)[c]	
	EGvhs Clone Alone	Construct in Presence of VP16 (Merge)			Cells Expressing vhs & VP16[i]	Similar Cellular Distribution[ii]
EGFP	Uniform	Uniform	No	No	65% ± 2.2	93% ± 0.7
EGvhs VBD μW321A	Uniform	Uniform	No	No	72% ± 3.1	94% ± 0.9
EGvhs VBD (wt)	Uniform	Perinuclear (+++) Nuclear (+)	Yes	Yes	70% ± 2.6	89% ± 1.6
EGvhs (FL)	Cytosolic	Perinuclear (+++) Cytosol (+++)	Yes	Yes	62% ± 2.5	81% ± 1.1

Table 4.1 Relocalization of vhs is dependent upon its ability to bind VP16. Results from fluorescent imaging shown in figure 7 (column a above) and the EGvhs / VP16 solid phase capture assay (figure 5 - column b above) was complied and summarized above. In addition, the percentage of observed cells which exhibited both EGFP and Texas Red fluorescence were also included as well as the relative percentage of cells exhibiting a similar nuclear localization to the imaged cells in figure 7 (column c above). The relative percentages were tabulated by (i) counting the number of fluorescent cells exhibiting both EGFP and Texas red fluorescence and dividing them by the total number of fluorescing cells, and (ii) Counting the cells exhibiting similar cellular distributions of EGFP fluorescence and dividing by the total number of fluorescing cells. In each case the standard deviation was also calculated and appended to each statistic.

by some as of yet unidentified interacting partner. Since EGvhs VBD (*wt*) lacks almost the entire coding region of vhs, regions capable of facilitating protein-protein interactions would clearly be lost. At present, studies utilizing the yeast two-hybrid system in our laboratory are underway to determine if there are indeed host cell specific proteins that may indeed bind vhs. Whether possible candidate proteins emerge that may be capable of interacting with vhs and possibly retain the protein within the cytosol however remains to be seen. Another explanation is that full-length functional vhs may be retained within the cytosol through its interactions with a substrate. Again by the previous arguments, the majority of vhs is removed in the EGvhs VBD (*wt*) construct, so it would be unable to interact with mRNA and thus not be capable of moving to the perinuclear region of the cell.

Another mutant form of vhs unable to interact with VP16 is vhs-ΔSma (Read *et al.*, 1993). This mutant is a collapsed form of vhs containing an internal deletion from residues 149-344 and likewise to EGvhs VBD *mut*W321A, is unable to bind VP16 (Smibert *et al.*, 1994). The viability of viruses containing this mutated form of vhs is compromised as they do not elicit the characteristic host shutoff observed in wt virus, and that ΔSma is not efficiently packaged into newly formed virions (Read *et al.*, 1993). In considering both the results presented here and the fact that ΔSma does not get packaged, the ability of vhs to interact with VP16 may not solely be responsible for downregulation of its activity, but also to shunt the protein into the viral assembly pathway.

The perinuclear region of HSV infected cells has been shown to contain a high concentration of tegument proteins (Ward *et al.*, 1996) and as such has been long suspected to direct the final enveloping of new virions (McLauchlan *et al.*, 1992; Rixon *et al.*, 1992; Jayachandra *et al.*, 1997). As vhs does not appear to localize to the nucleus on its own, it is conceivable that its interactions with VP16 may indeed carry it to this region so it may be efficiently captured by exiting capsids and go on to infect new cells. By disrupting this interaction then, it is possible that newly formed virions may contain fewer copies of vhs, which would lead to a decreased host shutoff in newly infected cells. In addition, the characteristic downregulation of vhs within these cells would now be abolished, as VP16 would be unable to bind and inactivate vhs.

It appears as if the ability of VP16 to localize to the nucleus may play a key role in the packaging and conceivably downregulation of vhs. In light of this, identifying the region(s) responsible for this localization is of great importance since disrupting its function could have widespread consequences on HSV infectivity and viability.

4.2. VP16 partially localizes to the nucleus via a possible bipartite nuclear localization signal located between residues 335 and 355

In order for VP16 to initiate the cascade of viral protein synthesis, it must initially localize to the nucleus in order to transactivate its target genes. What is interesting is the observation that VP16 only partially localizes to the nucleus (Friedman *et al.*, 1988) but yet drastically enhances the expression of

immediate early genes (Batterson and Roizman, 1983; Campbell *et al.*, 1984; Bzik and Preston 1986; Pellett *et al.*, 1985). This partial localization of VP16 is not novel as other proteins such as the human ubiquitin activating enzyme E1 also appears to distribute between both nuclear and cytoplasmic subpopulations (Handley-Gearhart *et al.*, 1994). How in fact VP16 localizes to the nucleus at all is a mystery however, considering the fact that throughout its entire sequence the are no evident nuclear localization signals.

Evidence presented here may now implicate VP16 as a shuttle protein that serves to translocate vhs into the nucleus in order to downregulate its activity or package it into the tegument of new virions. The region responsible for VP16 localization then is of added importance as it may not only serve to facilitate the transactivation of immediate early genes but also may conceivably downregulate vhs and/or facilitate its packaging into new virions.

To date, three different nuclear localization signals have been classified. The classical NLS of the SV40 large-T antigen relies on a KKKRK polybasic region to localize it to the nucleus (Kalderon *et al.*, 1984), while in contrast, the unclassified NLS such as that found in the M9 region of the heterogeneous ribonucleoprotein (hnRNP) consists of 38 amino acids with three basic amino acids interspersed among the entire sequence (Sionmi and Dreyfuss, 1995). The third NLS consists of two basic regions separated by approximately 10 amino acids. These 'bipartite' localization sequences have been found in the Retinoblastoma gene product (KRPAATKKAGQAKKKK) and

Construct	EGFP Nuclear Localization	Texas Red Nuclear Localization	Intensity of Nuclear Localization
$EGVP_{74}$	Yes	Yes	++
$EGVP_{182}$	Yes	Yes	+
$EGVP_{250}$	No	No	N/A
$EGVP_{299}$	No	No	N/A
$EGVP_{335}$	No	No	N/A
$EGVP_{355}$	Yes[(a)]	Yes[(a)]	+[(a)]
$EGVP_{369}$	Yes	Yes	+++
$EGVP_{379}$	Yes	Yes	+
$EGVP_{411}$	Yes	Yes	++
$EGVP_{490}$	Yes	Yes	+

Table 4.2 Amino acids spanning residues 335 to 355 are necessary for the nuclear localization of VP16.

The above table presents summarized data from figure 10 and data not shown (column a). In all cases, nuclear translocation of the EGVP constructs were far from absolute. Therefore, the intensities shown above do not reflect a complete translocation of the VP16 fragments into the nucleus but rather the relative ability of each construct to localize to the nucleus.

Figure 11. Sequence alignment of proteins homologous to VP16.
Homologous transactivators from HSV 1 and 2, BHV (bovine herpes virus), EHV-1 (equine herpes virus) and VZV (varicella-zoster virus) were aligned based on their primary sequences. Shaded (light grey) amino acids denote residues 80% or more conserved between the five proteins. Within the putative nuclear localization domain (residues 330-344 - shaded in yellow) are hydrophobic (φ) and conserved (c) amino acids.

nucleoplasmin (KRPAAIKKAGQAKKKK) (Zakenhaus *et al.*, 1993; Dingwall *et al.*, 1982).

Studies presented here suggest that the region of VP16 responsible for nuclear localization falls between residues 335 and 355 (Figure 9 and table 4.2). At first glance this region does not appear to have any classical NLS but in reviewing the sequence alignment of HSV 1 and 2, BHV, EHV-1 and VZV, (Figure 11) this stretch of amino acids presents two highly conserved basic ends with 9 mainly hydrophobic residues interspersed between them (RASGYFMVLIRAK). This stretch is not entirely unlike the bipartite NLS seen in other proteins, as many also contain a stretch of hydrophobic amino acids in between flanking basic ends.

Studies have shown that nuclear localization signals behave independently and as such can localize cytoplasmic proteins such as pyruvate kinase to the nucleus when the expressed as a NLS fusion (Kalderon *et al.*, 1984). Whether the 15 amino acid sequence presented here is necessary and sufficient for the translocation of VP16 to the nucleus, and furthermore whether it is capable of re-locating an unrelated cytoplasmic protein to the nucleus still remains to be seen. Further studies will hopefully delineate the actual sequence necessary for the characteristic nuclear localization of VP16, and whether or not it functions as an NLS.

Since VP16 has been shown to play an important role in the viral lifecycle and production of new virions, and furthermore appears to mediate its

functions from within the nucleus, a single alteration in the protein inhibiting its nuclear localization could be potentially lethal to the virus. VP16 and vhs as possible targets for antiviral therapies then may indeed present points of weakness in the formidable resilience of HSV infections.

5. BIBLIOGRAPHY

Ace, C.I., Dalrymple, M.A., Ramsay, F.H., Preston, V.G. and C.M. Preston, 1988. Mutational Analysis of the Herpes Simplex Virus Type 1 *Trans* Inducing Factor vmw65. J. Gen. Virol. **69**: 2595-2605.

Ace, C.I., McKee, T.A., Ryan, J.M., Cameron, J.M. and C.M. Preston. 1989. Construction and Characterization of a Herpes Simplex Virus Type 1 Mutant Unable to Transinduce Immediate-Early Gene Expression. J. Virol. **63**: 2260-2269.

Bataille, D. and A. Epstein. 1995. Herpes Simplex Virus Type 1 Replication and Recombination. Biochimie **77**: 787-795.

Batterson, W. and B. Roizman. 1983. Characterization of the Herpes Simplex Virion-associated Factor Responsible for the Induction of α Genes. J. Virol. **46**: 371-377.

Berger, S.L., Cress, W.D., Cress, A., Triezenberg, S.J. and L. Guarente. 1990. Selective Inhibition of Activated but not Basal Transcription by the Acidic Activation Domain of VP16: Evidence for Transcriptional Adaptors. Cell **61**: 1199-1208.

Berthomme, H., Jacquemont, B. and A. Epstein. 1993 The Pseudorabies Virus Host Shutoff Homolog Gene: Nucleotide Sequence and Comparison with Alphaherpesvirus Protein Counterparts. Virol. **193**: 1028-1032.

Bzik, D.J. and C.M. Preston. 1986. Analysis of Sequences which Regulate the Transcription of Herpes Simplex Virus Immediate Early Gene 3: DNA Sequenced Required for Enhancer Like Activity and Response to Trans-activation by a Virion Polypeptide. Nucleic. Acid. Res. **14**: 929-943.

Campadelli-Fiume, G., Farabegoli, F., Di Gaeta, S., and B. Roizman. 1991. Origin of Unenveloped Capsids in the Cytoplasm of Cells Infected with Herpes Simplex Virus 1. J. Virol. **65**:1589-1595.

Campbell, M.E.M, Palfreyman, J.M. and C.M. Preston. 1984. Identification of Herpes Simplex Virus DNA Sequences Which Encode a Trans-acting Polypeptide Responsible for Stimulation of Immediate Early Transcription. J. Mol. Biol. **180**: 1-19.

Chee, M. and B. Barrell. 1990. Herpesviruses: A Study of Parts. TIG **6**: 86-91.

Cousens, D.J., Greaves, R., Gooding, C.R. and P. O'Hare. 1989. The C-Terminal 79 Amino Acids of the Herpes Simplex Virus Regulatory Protein, Vmw65, Efficiently Activates Transcription in Yeast and Mammalian Cells in Chimeric DNA-Binding Proteins. EMBO J. **8**: 2337-2342.

Dargan, D.J. and H. Subak-Sharpe. 1997. The Effect of Herpes Simplex Virus Type 1 L-Particles on Virus Entry, Replication, and the Infectivity of Naked Herpesvirus DNA. Virol. **239**: 378-388.

de Bruyn Kops, A. and D.M. Knipe. 1988. Formation of DNA Replication Structures in Herpes Virus-Infected Cells Requires a Viral DNA Binding Protein. Cell **55**: 857-868.

Dingwall, C., Sharnick, S.V. and R.A. Laskey. 1982. A polypeptide domain that Specifies Migration of Nucleoplasmin into the Nucleus. Cell **30**: 449-458.

Elliott, G., Mouzakitis, G. and P. O'Hare. 1995. VP16 Interacts Via its Activation Domain with VP22, A Tegument Protein of Herpes Simplex Virus, and is Relocated to a Novel Macromolecular Assembly in Co-Expressing Cells. J. Virol. **69**: 7932-7941.

Everett., RD. 1987. The Regulation of Transcription of Viral and Cellular Genes by Herpesvirus Immediate-Early Gene Products. Anticancer Res. **7**: 589-604.

Fenwick, M.L. and B. Roizman. 1977. Regulation of Herpesvirus Macromolecular Synthesis VI. Synthesis and Modification of Viral Polypeptides in Enucleated Cells. J. Virol. **22**: 720-725.

Fenwick, M.L. and M.J. Walker. 1978. Suppression of The Synthesis of Cellular Macromolecules by Herpes Simplex Virus. J. Gen. Virol. **41**: 37-51.

Fenwick, M.L. and R.D. Everett. 1990. Inactivation of the Shutoff Gene (UL41) of Herpes Simplex Virus Types 1 and 2. J. Gen. Virol. **71**: 2961-2967.

Friedman, A.D., Triezenberg, S.J. and S. McKnight. 1988. Expression of Truncated Viral *Trans*-Activator Selectively Impedes Lytic Infection by its Cognate Virus. Nature **335**: 452-454.

Fenwick, M.L. and S.A. Owen. 1988. On the Control of Intermediate Early (α) mRNA Survival in Cells Infected with Herpes Simplex Virus J. Gen. Virol. **69**: 2869-2877.

Gerster, T., and R.G. Roeder. 1988. A Herpesvirus Trans-Activating Protein Interacts with Transcription Factor OTF-1 and Other Cellular Proteins. Proc. Natl. Acad. Sci., *USA*. **85**: 6347-6351.

Graham, F.L. and A.J. van Der Eb. 1973. Transformation of Rat Cells By DNA of Human Adenovirus 5. Virology **54**: 536-539.

Greaves, R.F. and P. O'Hare. 1989. Separation of Requirements for Protein-DNA Complex Assembly from Those for Functional Activity in the Herpes Simplex Virus Regulatory Protein Vmw65. J. Virol. **63**: 1641-1650.

Hagmann, M., Georgiev, O. and W. Schaffner. 1997. The VP16 Paradox: Herpes Simplex Virus VP16 Contains a Long Range Activation Domain but within the Natural Multi-Protein Complex Activates only from Promoter Proximal Positions. J. Virol. **71**: 5252-5962.

Handley-Gearhart, P.M., Stephen, A.G., Trausch-Azar, J.S., Ciechanover, A. and A.L. Schwartz. 1994. Human Ubiquitin-activating Enzyme, E1. J. Biol. Chem. **269**: 33171-33178.

Hardy, W.R. and R.M. Sandri-Goldin. 1994. Herpes Simplex Virus Inhibits Host Cell Splicing, and Regulatory Protein ICP27 is Required for This Effect. J. Virol. **68**: 7790-7799.

Holmes, D.S. and M. Quigley. 1981. A Rapid Boiling Method for the Preparation of Bacterial Plasmids. Anal. Biochem. **114**: 193-197.

Horikoshi, M., Karey, M.F., Kakidani, H. and R.G. Roeder. 1988. Mechanism of Action of a Yeast Activator: Direct Effect of GAL4 Derivatives on Mammalian TFIID Promoter Interactions. Cell **54**: 665-669.

Jayachandra, S, Baghian, A and K.G. Kousoulas. 1997. Herpes Simplex Virus Type 1 Glycoprotein K is not Essential for Infectious Virus Production in Actively Replicating Cells but is Required for Efficient Envelopment and Translocation of Infectious Virions from the Cytoplasm to the Extracellular Space. J. Virol. **71**: 5012-5024.

Jiang, Y., Triezenberg, S.J. and J.D. Gralla. 1993. Defective Transcriptional Activation by Diverse VP16 Mutants Associated with a Common Inability to Form Open Promoter Complexes. J. Biol. Chem. **269**: 5505-5508.

Jones, F.E.. Ph.D. Thesis, 1995

Jones, F.E., Smibert, C.A. and J.R. Smiley. 1995. Mutational Analysis of the Herpes Simplex Virus Virion Host Shutoff Protein: Evidence that vhs Functions in the Absence of Other Viral Proteins. J. Virol. **69**: 4863-4871.

Katan, M., Haigh, A., Verrijzer, C.P., Vandervliet, P.C. and P. O'Hare. 1990. Characterization of a Cellular Factor with Interacts Functionally with OCT-1 in the Assembly of a Multi-Component Transcription Complex. Nuc. Acids. Res. **18**: 6871-6880.

Kalderon, D., Richardson, W.D., Markham, A.F. and A.E. Smith. 1984. Sequence Requirements for Nuclear Location of Simian Virus 40 Large-T Antigen. Nature **311**: 33-38.

Kalderon, D., Roberts, B.L., Richardson, W.D. and A.E. Smith. 1984. A Short Amino Acid Sequence Able to Specify Nuclear Localization. Cell **39**: 499-509.

Kristie, T.M. and B. Roizman. 1984. Separation of Sequences Defining Basal Expression from those Conferring α Gene Recognition within the Regulatory Domains of Herpes Simplex Virus 1 α Genes. Proc. Natl. Acad. Sci., *USA*. **81**: 4065-4069.

Kristie, T.M., Lebowitz, J.H. and P.A. Sharp. 1989. The Octomer-Binding Proteins form Multi-Protein-DNA Complexes with the HSV α-TIF Regulatory Protein. EMBO J. **8**: 4229-4238.

Kwong, A.D. and N. Frenkel. 1987. Herpes Simplex Virus-Infected Cells Contain a Function(s) that Destabilizes Both Host and Viral mRNAs. Proc. Natl. Acad. Sci., *USA*. **84**: 1926-1930.

Kwong, A.D., Kruper, J.A. and N. Frenkel. 1988. Herpes Simplex Virus Virion Host Shutoff Function. J. Virol. **62**: 912-921.

Laemmli, U.K. 1970. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. Nature **227**: 680-685.

Lai, J. and W. Herr. 1997. Interdigitated Residues within a Small Region of VP16 Interact with OCT-1, HCF and DNA. Mol. Cell. Biol. **17**: 3937-3946.

Lam, Q., Smibert, C.A., Koop, K., Lavery, C, Capone, J.P., Weinheimer, S. and J.R. Smiley. 1996. Herpes Simplex Virus VP16 Rescues Viral mRNA from Destruction by the Virion Host Shutoff Function. EMBO J. **15**: 2575-2581.

Leopardi, R., Ward, P.L., Ogle, W.O. and B. Roizman. 1997. Association of Herpes Simplex Virus Regulatory Protein ICP22 With Transcriptional Complexes Containing EAP, ICP4, RNA Polymerase II and Viral DNA Requires Post-Translational Modification by the UL13 Protein Kinase. J. Virol. **71**: 1133-1139.

Lin, Y. and M.R. Greene. 1991. Mechanism of Activation of an Acidic Transcriptional Activator *in vitro*. Cell **64**: 971-981.

Lukonis, C. and S.K. Weller. 1996. Characterization of Nuclear Structures in Cells Infected with Herpes Simplex Virus Type 1 in the Absence of Viral DNA Replication. J. Virol. **70**: 1751-1758.

Mackem, S. and B. Roizman. 1982. Structural Features of The Herpes Simplex Virus α Gene 4, 0 and 27 Promoter-Regulatory Sequence which Confer α Regulation on Chimeric Thymidine Kinase Genes. J. Virol. **44**: 939-949.

McGeoch, D.J., Dalrymple, A., Davison, A.J., Dolan, A., Frame, M.C., McNab, D., Perry, L.J., Scott, J.E. and P. Taylor. 1988. The Complete DNA Sequence of the Long Unique Region in the Genome of Herpes Simplex Virus Type 1. J. Gen. Virol. **69**: 1531-1574.

McKnight, J.L.C., Kristie, T.M. and B. Roizman. 1987. Binding of the Virion Protein Mediating α Gene Induction in Herpes Simplex Virus 1 – Infected Cells to its *cis* Site Requires Cellular Proteins. Proc. Natl. Acad. Sci., *USA*. **84**: 8061-7065.

McLean, C., Buckmaster, A., Hancock, D., Buchan, A., Fuller, A. and A Minson. 1982. Monoclonal Antibodies to Three Non-Glycosylated Antigens of Herpes Simplex Virus 2. J. Gen. Virol. **63**: 297-305.

McLauchlan, J., Addison, C., Craigie, M.C. and F.J. Rixon. 1992. Noninfectious L-Particles supply functions which can facilitate infection by HSV-1. Virol. 190: 682-688.

Nakai, H., Maxwell, I.H. and L.I. Pizer. 1982. Herpesvirus Infection Alters the Steady-State Levels of Cellular Polyadenylated mRNA in Polyoma Virus-Transformed BHK Cells. J. Virol. **42**: 1131-1134.

Ng, T.I., Chang, Y.E. and B. Roizman. 1997. Infected Cell Protein 22 of Herpes Simplex Virus 1 Regulates the Expression of Virion Host Shutoff Gene UL41. Virology **234**: 226-234.

Nii, S., Morgan, C. and H.M. Rose. 1968. Electron Microscopy of Herpes Simplex Virus II. Sequence of Development. J. Virol. **2**:517-536.

Nishioka, Y. and S. Silverstein. 1977. Degradation of Cellular mRNA During Infection by Herpes Simplex Virus. Proc. Natl. Acad. Sci., *USA*. **74**: 2370-2374.

Ogle, W.O., Ng, T.I., Carter, K.L. and B. Roizman. 1997. The UL13 Protein Kinase and the Infected Cell Type are Determinants of Post-Translational Modification of ICP0. Virology **235**: 406-413.

Ormo, M., Cubitt, A.B., Kallio, K., Gross, L.A., Tsien, R.Y. and S.J. Remington. 1996. Crystal Structure of The *Aequoria victoria* Green Fluorescent Protein. Science **273**: 1392-1395.

Oroskar, A.A. and G.S. Read. 1987. A Mutant of Herpes Simplex Virus Type 1 Exhibits Increased Stability of Immediate Early (*alpha*) mRNAs. J. Virol. 61: 604-606.

Oroskar, A.A. and G.S. Read. 1989. Control of mRNA Stability by the Virion Host Shutoff Function of Herpes Simplex Virus. J. Virol. **63**: 1897-1906.

Overton, H., McMillan, D., Hope, L. and P. Wong-Kai-In. 1994. Production of Host Shutoff-Defective Mutants of Herpes Simplex Virus Type 1 By Inactivation of The UL13 Gene. Virology **202**:97-106.

Payne, J.M., Laybourn, P.J. and M.E. Dahmus. 1989. The Transition of RNA Polymerase II From Initiation to Elongation is Associated with Phosphorylation of the Carboxyl-Terminal Domain of Subunit IIa. J. Biol. Chem. **264**: 19621-19629.

Pellett, P.E., McKnight, J.L.C., Jenkins, F.J. and B. Roizman. 1985. Nucleotide Sequence and Predicted Amino Acid Sequence of a Protein Encoded in a Small Herpes Virus DNA Fragment Capable of Trans-inducing α genes. Proc. Natl. Acad. Sci. USA **82**: 5870-5874.

Phelan, A., Dunlop, J. and J.B. Clements. 1996. Herpes Simplex Virus Type 1 Protein IE63 Affects The Nuclear Export of Virus Intron-Containing Transcripts. J. Virol. **70**: 5255-5265.

Poon, A. and B. Roizman. 1995. The Phenotype *in vivo* and in Infected Cells of Herpes Simplex Virus 1 α *Trans* Inducing Factor (VP16) Carrying Temperature Sensitive Mutation Introduced by Substitutions of Cysteines. J. Virol. **69**: 7658-7667.

Popova, B., Bilan P, Xiao, P., Faught. M. and J.P. Capone. 1995. Transcriptional Activation by DNA – Binding Derivatives of HSV-1 VP16 that lack the Carboxyl-Terminal Acidic Activation Domain. Virology **209**: 19-28.

Preston, C.M., Frame, M.C. and M.E.M. Campbell. 1988. A Complex Formed Between Cell Components and an HSV Structural Polypeptide Binds to a Viral Immediate Early Gene Regulatory DNA Sequence. Cell **52**: 425-434.

Read, G.S. and N. Frenkel. 1983. Herpes Simplex Virus Mutant Defective in The Virion Associated Shutoff of Host Polypeptide Synthesis and Exhibiting Abnormal Synthesis of α (Immediate Early) Viral Polypeptides. J. Virol. **46**: 498-512.

Read, G.S., Karr, V.M. and K. Knight. 1993. Isolation of a Herpes Simplex Virus Type 1 Mutant with a Deletion in the Virion Host Shutoff Gene and Identification of Multiple Forms of the vhs (UL41) Polypeptide. J. Virol. **61**: 7149-7160.

Rixon, F.J., Addison, C. and J. McLauchlan. 1992. Assembly of Enveloped Tegument Structures (L Particles) can occur Independently of Virion Maturation in Herpes Simplex Virus Type 1 – Infected Cells. J. Gen. Virol. **73**: 277-284.

Roberts, S.G.E. and M.R. Greene. 1994. Activator-Induced Conformational Change in General Transcription Factor TFIIB. Nature **371**: 717-720.

Roizman, B., and A.E. Sears. 1990. Herpes Simplex Virus and Their Replication. In "Virology". 2nd Ed. (ed. B.N. Fields) pp. 1795-1841. Raven Press, New York.

Roizman, B., and A.E. Sears. 1993. Herpes Simplex Viruses and Their Replication In "The Human Herpesviruses". (ed. B. Roizman) pp. 11-68. Raven Press, New York.

Sacks, W.R., Greene, C.C., Aschman, D.P. and P. Schaffer. 1985. Herpes Simplex Virus Type 1 ICP27 is an Essential Regulatory Protein. J. Virol. **55**: 796-805.

Sadowski, I., Ma, J., Triezenberg, S. and M. Ptashne. 1988. GAL4-VP16 is an Unusually Potent Transcriptional Activator. Nature **335**: 563-564.

Sambrook, J., Fritsdch, E.F. and T. Maniatis. 1989. In "Molecular Cloning" A Laboratory Manual". 2nd Ed. Cold Spring Harbor Press, New York.

Schek, N. and S.L. Bachenheimer. 1985. Degradation of Cellular mRNAs Induced by a Virion-Associated Factor During Herpes Simplex Virus Infection of Vero Cells. J. Virol. **55**: 601-610.

Schmelter, J., Knez, J., Smiley, J.R. and J.P. Capone. 1996. Identification and Characterization of a Small Modular Domain in the Herpes Simplex Virus Host Shutoff Protein Sufficient for Interaction with VP16. J. Virol. **70**:2124-2131.

Shaw, P., Knez, J. and J.P. Capone. 1995, Amino Acid Substitutions in the Herpes Simplex Virus Transactivator VP16 Uncouple Direct Protein-Protein Interaction and DNA Binding from Complex Assembly and Transactivation. J. Biol. Chem. **270**: 29030-29037.

Shi, Y. and J.O. Thomas. 1992. The Transport of Proteins into the Nucleus requires the 70-kilodalton Heat Shock Protein or its Cytosolic Cognate. Mol. Cell. Biol. **12**: 2186-2192.

Silver, P.A. 1991. How Proteins Enter The Nucleus. Cell **64**: 489-497.

Siomi, H. and G. Dreyfuss. 1995. A Nuclear Localization Domain in the hnRNP A1 Protein. J. Cell. Biol. **129**: 551-560.

Smibert, C.A. and J.R. Smiley. 1990. Differential Regulation of Endogenous and Transduced β-Globin Genes During Infection of Erythroid Cells with a Herpes Simplex Virus Type 1 Recombinant. J. Virol. **64**: 3882-3894.

Smibert, C.A., Johnson, D.C. and J.R. Smiley. 1992. Identification and Characterization of the Virion Induced Shutoff Product of Herpes Simplex Virus Gene UL41. J. Gen Virol. **73**: 467-470.

Smibert, C.A., Popova, B., Xiao, P., Capone, J.P. and J.R. Smiley. 1994. Herpes Simplex Virus VP16 Forms a Complex with the Virion Host Shutoff Protein vhs. J. Virol. **68**: 2339-2346.

Spear, P.G. 1985. Glycoproteins Specified by Herpes Simplex Viruses. In "The Herpesviruses" (ed. B. Roizman).Vol 3, pp. 315-356. Plenum Press, New York.

Steiner, I. And P.G. Kennedy. 1993. Molecular Biology of Herpes Simplex Virus Type 1 Latency in the Nervous System. Molecular Neurobiology **7**: 137-159.

Stringer, K.F., Ingles, C.J. and J. Greenblatt. 1990. Direct and Selective Binding of an Acidic Transcriptional Activation Domain to the TATA-Box Factor TFIID. Nature **345**: 783-786.

Strom, T. and N. Frenkel. 1987. Effects of Herpes Simplex Virus on mRNA Stability. J. Virol. **61**: 2198-2207.

Sydiskis, R.J. and B. Roizman. 1966. Polysomes and Protein Synthesis in Cells Infected with a DNA Virus. Science. **153**: 76-78.

Triezenberg, S.J., Kingsbury, R.C. and S.L. McKnight. 1988. Functional Dissection of VP16, The Transactivator of Herpes Simplex Virus Immediate Early Gene Expression. Genes Dev. **2**: 718-729.

Uesugi, M., Nyanguile, O., Lu, H., Levine, A.J. and G.L. Verdine. 1997. Induced α Helix in the VP16 Activation Domain upon Binding to a Human TAF. Science **277**: 1310-1313.

Walker, S., Hayes, S. and P. O'Hare. 1994. Site Specific Conformational Alteration of the OCT-1 POU-Domain DNA Complex as the Basis for Differential Recognition by Vmw65 (VP16). Cell **79**: 841-852.

Ward, P.L. and B. Roizman. 1994. *Herpes Simplex* Genes: the Blueprint of a Successful human Pathogen. TIG **10**: 267-274.

Ward, P.L., Ogle, W.O. and B. Roizman. 1996. Assemblons: Nuclear Structures Defined by Aggregation of Immature Capsids and Some Tegument Proteins of Herpes Simplex Virus 1. J. Virol. **70**: 4623-4631.

Weinheimer, S.P., Boyd, B.A., Durham, S.K., Resnick, J.L. and D.R. O'Boyle. 1992. Deletion of the VP16 Open Reading Frame of Herpes Simplex Virus Type 1. J. Virol. **66**: 258-269.

Wu, T.J., Monokian, G., Mark, D.F. and C.R. Wobbe. 1994. Transcriptional Activation by Herpes Simplex Virus Type 1 VP16 *in vitro* Inhibition by Oligopeptides. Mol. Cell. Bio. **14**: 3484-3493.

Xiao, P. and J.P. Capone. 1990. A Cellular Factor Binds to the Herpes Simplex Virus Type 1 Transactivator Vmw65 and is Required Vmw65-Dependant Protein-DNA Complex Assembly with OCT-1. Mol. Cell. Biol. **10**: 4974-4977.

Yakulov, K., Blau, J., Purton, T., Roberts, S. and D.L. Bentley. 1994. Transcriptional Elongation by RNA Polymerase II is Stimulated by Transactivators. Cell **77**: 749-759.

Zakenhaus, E., Bremner, R., Phillips, R.A. and B.L. Gallif. 1993. A Bipartite Nuclear Localization Signal in the Retinoblastoma Gene Product and Its Importance for Biological Activity. Mol. Cell. Biol. **13**: 4588-4599.

Zelus, B.D., Stewart, R.S. and J. Ross. 1996. The Virion Host Shutoff Protein of Herpes Simplex Virus Type 1: Messenger Ribonucleolytic Activity *in vitro*. J. Virol. **70**: 2411-2419.

Zhang Y. and J.L. McKnight. 1993. Herpes Simplex Virus Type 1 UL46 and UL47 Deletion Mutants Lack VP11 and VP12 or VP13 and VP14, Respectively, and Inhibit Altered Viral Thymidine Kinase Expression. J. Virol. **67**:1482-1492.

Zhang, Y.F. and E. Wagner. 1987. The kinetics of synthesis of individual type one transcripts. Virus Genes. 1: 61-74.

Zhu, Q. and R.J. Courtney. 1994. Chemical Cross-Linking of Virion Envelope and Tegument Proteins of Herpes Simplex Virus Type 1. Virology **204**: 590-599.