NOTE TO USERS

CHARACTERIZATION OF THE ROLE OF HERPES SIMPLEX VIRUS PROTEIN VP16 IN VIRAL GENE EXPRESSION THROUGH INTERACTIONS WITH THE VIRION HOST SHUTOFF PROTEIN (VHS) AND HCF-1

By

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ABSTRACT

Herpes Simplex Virus 1 (HSV1) VP16 transcriptionally activates viral immediate-early (IE) genes, through the formation of a multi-protein structure termed the VP16-induced complex (VIC). VIC is comprised of cellular transcription factors Oct-1 and HCF-1. In addition, VP16 is a pre-formed, structural protein that has a critical role in virion assembly. At least one aspect of its role at late times post-infection includes the downregulation of the virion host shutoff protein (vhs); which functions to indiscriminately degrade mRNA.

The interaction between VP16 and vhs was characterized within this thesis in order to differentiate it from the regions involved in VIC assembly, as well as to investigate its functional importance within HSV1. Refined mutagenesis studies were used to localize a minimal binding domain within VP16; in which the alteration a single residue was sufficient to abrogate binding to vhs. Our results indicate that the interaction between VP16 and vhs could effectively be uncoupled from VIC assembly and the transactivation of IE genes. Moreover, virus complementation assays were used to demonstrate that the interaction with vhs is specifically required to sustain virus growth. We were also interested in characterizing VP16 transactivation. Previous work within our laboratory had determined that the amino-terminal domain of VP16, excluding its potent acidic activation domain (AAD), possessed an activation phenotype in yeast and mammalian cells. To investigate these findings further, mutagenesis experiments were employed to differentiate between the activity in mammalian and yeast cells. HCF-1, a recently identified transcriptional co-

regulatory protein, was identified as a co-activator of the truncated VP16 in mammalian cells. HCF-1 was also capable of synergizing VP16 in the activation of an IE gene construct. These findings identify a novel role for HCF-1 in HSV1 gene expression. Taken together, this work enhances our understanding of the role of VP16 in viral gene expression and pathogenesis.

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ABBREVIATIONS

βgal	β-galactosidase
2YT + amp	2YT media containing 100 μg/mL ampicillin
a.a.	amino acid
Ab	antibody
AD	activation domain
bp	base pair
BSA	bovine serum albumin
cDNA	DNA complementary to mRNA
cdk	cyclin-dependent kinase
Ci	Curie(s)
cpm	counts per minute
Da	Dalton
DB	DNA binding domain
ddH_20	distilled deionized water
DMEM	Dulbecco's modified essential media
DMF	dimethylformamide
dsDNA	double-stranded DNA
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay

GST	Glutathione S-transferase
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
Inr	initiator element
IPTG	isopropylthio-β-D-galactoside
MCS	multiple cloning site
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information (USA)
NE	nuclear extract
NLS	nuclear localization signal
NP40	Nonidet P-40
nt	nucleotide(s)
NTP	nucleoside triphosphate(s)
ONPG	o-nitrophenyl-β-D-glactopyranoside
ORF	open reading frame
Ori	origin of DNA replication
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonylfluoride
Pol II	RNA polymerase II
RNase A	ribonuclease A

rRNA	ribosomal RNA
RT	room temperature
SD	standard deviation
SDS	sodium dodecyl sulphate
ssDNA	single stranded DNA
snRNP	small, nuclear RNA associated protein
SV40	simian virus 40
tRNA	transfer RNA
TAF	TBP-associated factor(s)
TBP	TATA-binding protein
TE	10 mM Tris·HCl, 1 mM EDTA
TF	transcription factor
Tris	tris(hydroymethyl)aminoethane
ts	temperature sensitive
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Chapter 1. Introduction

1.1 Human herpesviruses

Herpesviruses are universally recognized pathogens, with apparent descriptions dating back to the time of the ancient Greeks. Hippocrates (~2400 years ago) first described lesions that appeared to spread along the skin as *herpein* ("to creep"); but it was not until the early 20th century that the nature of the pathogen(s) began to emerge (Roizman and Whitley, 2001). Herpesviruses are classified as part of the *herpesviridae* family, comprising more than 130 identified viruses, found within most animal species examined to date. The group shares common structural and biological characteristics, and have been further divided into three subclasses termed alpha-, beta-, and gammaherpesviruses. Herpes simplex virus 1 (HSV1) is one of nine human herpesviruses that have been identified thus far, along with Herpes simplex virus 2 (HSV2), Varicella zoster virus (VZV), Cytomegalovirus (CMV), Epstein-Barr virus (EBV), and Humanherpesvirus 6a, 6b, 7 and 8 (Kaposi's sarcoma). HSV1 is part of the alphaherpesvirus subfamily, along with HSV2 and VZV (Roizman and Pelletier, 2001).

1.1.1 HSV pathogenesis

Although herpesvirus infections are commonly regarded as being relatively innocuous, the broad social and economic impact of these infections and associated disease(s) are staggering. Epidemiological studies indicate that herpesviruses are essentially ubiquitous throughout the human population. In particular, estimates of seropositivity to HSV1 range from 80 to 90% of adults, with the majority of primary

infections occurring within the first five years of life (Whitley, 2001). Factors that may affect the prevalence of HSV1 (and other herpesviruses) are sex, race, ethnicity and socioeconomic status. HSV1 is acquired through direct physical contact, normally via saliva; typically, infecting the mouth (gingivostomatitis) or lips (labidis) (Whitley *et al.*, 1998). Infection is usually clinically asymptomatic or regarded only as a nuisance (Whitley *et al.*, 1998). However, it can also manifest into more serious infections of the eye (keratoconjunctivitis), skin (whitlow) and brain (encephalitis) (Ustacelebi, 2001). Keratoconjunctivitis is the leading cause of pathogen-induced blindness within the United States, affecting 300,000 people each year (Hwang and Spruance, 1999). It is also the most common cause of sporadic encephalitis, affecting one in 150,000 individuals per year: through a high mortality rate or causing permanent neurological damage (Kennedy and Chaudhuri, 2002). Although immune competent individuals generally deal with an infection without clinical incidence, HSV is an opportunistic pathogen. Neonates and immune-compromised individuals are particularly susceptible to acute infection and disseminated disease, and can be the source of the most serious risk of infection (Whitley *et al.*, 1998). Accordingly, HSV infection is among the most life threatening and devastating of infections in neonates, occurring at a rate of three in ten thousand live births (Malkin and Beumont, 1999); primarily due to HSV2 shedding during labour (Corey, 2002). HSV (predominantly HSV2) has also been found to be approximately 90% co-incident with HIV1, likely promoting more efficient spread of HIV, while also becoming an increasingly dangerous pathogen within AIDS patients (Schacker, 2001). HSV2 is estimated to reside within 20 to 30% of adults in the United States, and reaching

40 to 50% in Africa; thus, aggravating the spread of HIV and AIDS related illnesses (Corey, 2002). Increasingly, HSV infection and its associated life-threatening disease(s) are present in immunocompromised individuals, transplant recipients and those undergoing chemotherapy (Naesens and deClerqc, 2001).

1.1.2 Clinical interventions

In practice, the primary line of defense against HSV1 (and other herpesviruses) is the purine analog acyclovir (also gancyclovir and famicyclovir) (Naesens and deClerq, 2001). It takes advantage of the broad specificity of the viral DNA polymerase for nucleotide analogs, via activation by the viral thymidine kinase, thus blocking viral DNA replication (Fyfe *et al.*, 1978). Acyclovir is also useful as a prophylaxis for immunocompromised patients; however, several clinical strains of acyclovir-resistant viruses have been isolated, thus posing a growing concern among clinicians (Whitley *et al.*, 1998). In fact, drug resistant strains of HSV are present within five to 25% of immunocompromised patients undergoing long-term prophylaxis with acyclovir (Whitley *et al.*, 2001). Thus, the development of novel and improved pharmacological agents against HSV continues to be the focus of a large body of research, with two new pharmaceutical candidates, representing a new class of viral DNA synthesis inhibitors, emerging recently (Kleyman *et al.*, 2002, Crute *et al.*, 2002). In addition, the occurrence of 'biological synergism' between infectious agents may also lead to novel therapeutic interventions into previously unexploited pathogenic connections (eg. Acyclovir and HIV) (Griffiths, 2000).

1.1.3 HSV1 structure

Herpesviruses are comprised of four basic structural components, which are used as a basis for classification of the *herpesviridae* family, and include i) an electron dense core, consisting of a linear, double-stranded DNA, ii) a capsid, iii) a tegument and, iv) an outer membrane layer termed the viral envelope (Figure 1.1.3).

HSV1 contains a linear, double-stranded DNA that is approximately 152kbp in length. Viral DNA is organized into two segments termed U_L and U_S, separated by a region of variable repeats, stated schematically as ab-U_L-b'a'c'-U_S-ca (Figure 1.1.3). The U_L and U_S components of the genome can also invert, to produce four isomers of the genome (Roizman, 1979). The inverted elements are highly conserved and can contain a variable number of elements, thus affecting the size of the DNA. The viral DNA can express over 100 transcripts (or ORFs), thus far corresponding to more than 80 proteins (Ward and Roizman, 1994). The tegument is the amorphous protein layer found between the capsid, the structure that encloses the viral DNA, and the surrounding viral envelope. The proteins within the tegument are required to maintain virus structure, in addition to being important for initiating a productive infection (eg. VP16 and vhs) (Roizman and Knipe, 2001). The proteins present in the outer envelope structure consist of a set of 11 proteins (viral membrane glycoproteins; gB through gM), which are used to mediate viral entry into cells and maintain the structural integrity of the virus.

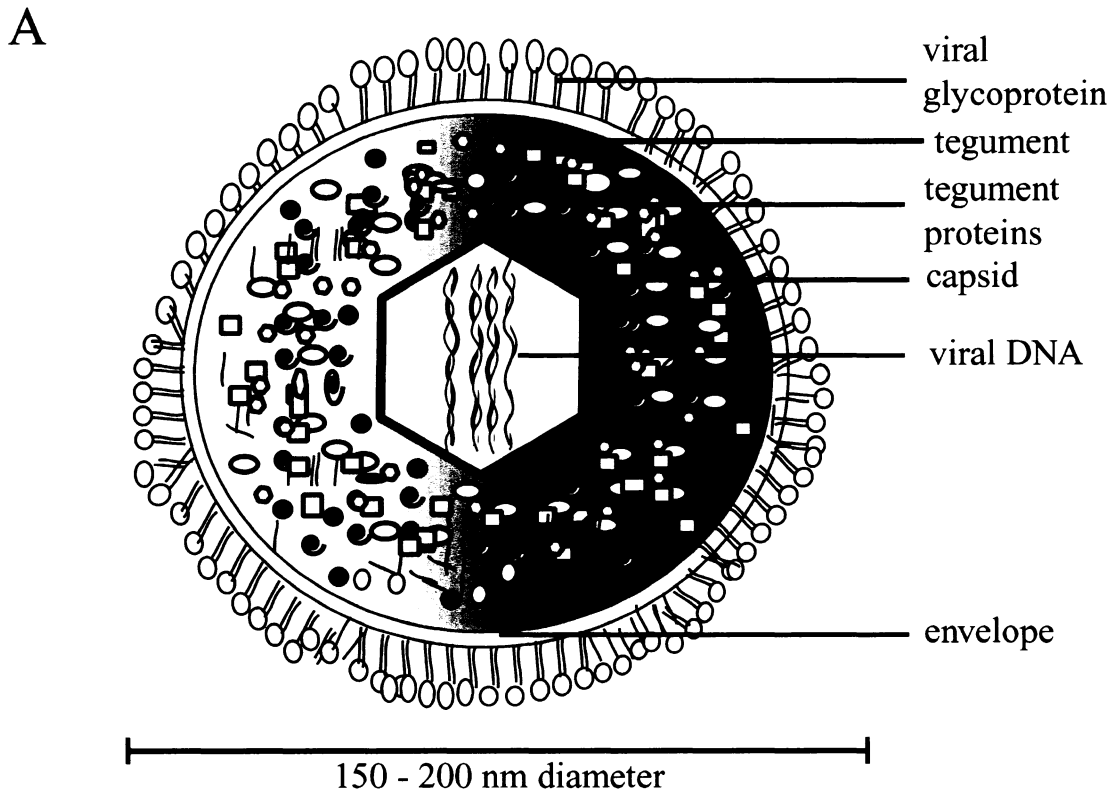

Figure 1.1.3. HSV1 structure and genome organization. A. Schematic diagram of HSV1 structure. The tegument is an amorphous region between the capsid and the outer envelope containing viral structural proteins. The viral envelope accomodates protruding viral glycoproteins. **B.** Linear representation of the HSV1 genome. UL; long repeat, US; short repeat; are flanked by terminal repeat elements ab, and ac, respectively. Adapted from Roizman and Knipe (2001).

1.2 Lytic cycle

The viral lytic cycle, or productive infection, must take place for HSV1 to multiply and propagate. This process follows a complex and highly coordinated series of events, in which the virus takes over the target cell by efficiently utilizing its resources and machinery in order to produce viral particles (Roizman and Knipe, 2001). Once the process is complete, the virus can infect neighboring cells, leaving the host cell to self-destruct. In addition, HSV1 is able to establish a life-long infection (or residency) by maintaining a latent state within the neurons of the trigeminal ganglia that innervate the site of infection. This reservoir of HSV1 then becomes a constant source of re-infection following the reactivation of the latent virus (Whitley, 2001).

1.2.1 HSV1 entry

Initial HSV1 infections primarily occur via the epithelial cells surrounding the mouth and lips, through direct physical contact with another infected individual. Cell recognition and internalization of the virus essentially takes place in three distinct steps. First, glycoprotein C (gC) and/or gB, which protrude from the viral envelope, bind to heparan sulfate containing proteoglycans on the cell surface, resulting in virus attachment to the cell (Laquerre *et al.*, 1998, Shukla *et al.*, 1999). Second, glycoprotein D (gD) interacts with specific cell surface receptors termed HVEM (Herpes Virus Entry Mediator) which belong to the immunoglobulin receptor superfamily (Montgomery *et al.*, 1996, Whitbeck *et al.*, 1997). The binding of gD is critical for virus mediated entry into the cell (Ligas and Johnson, 1988). Third, fusion of the viral and cellular membrane takes

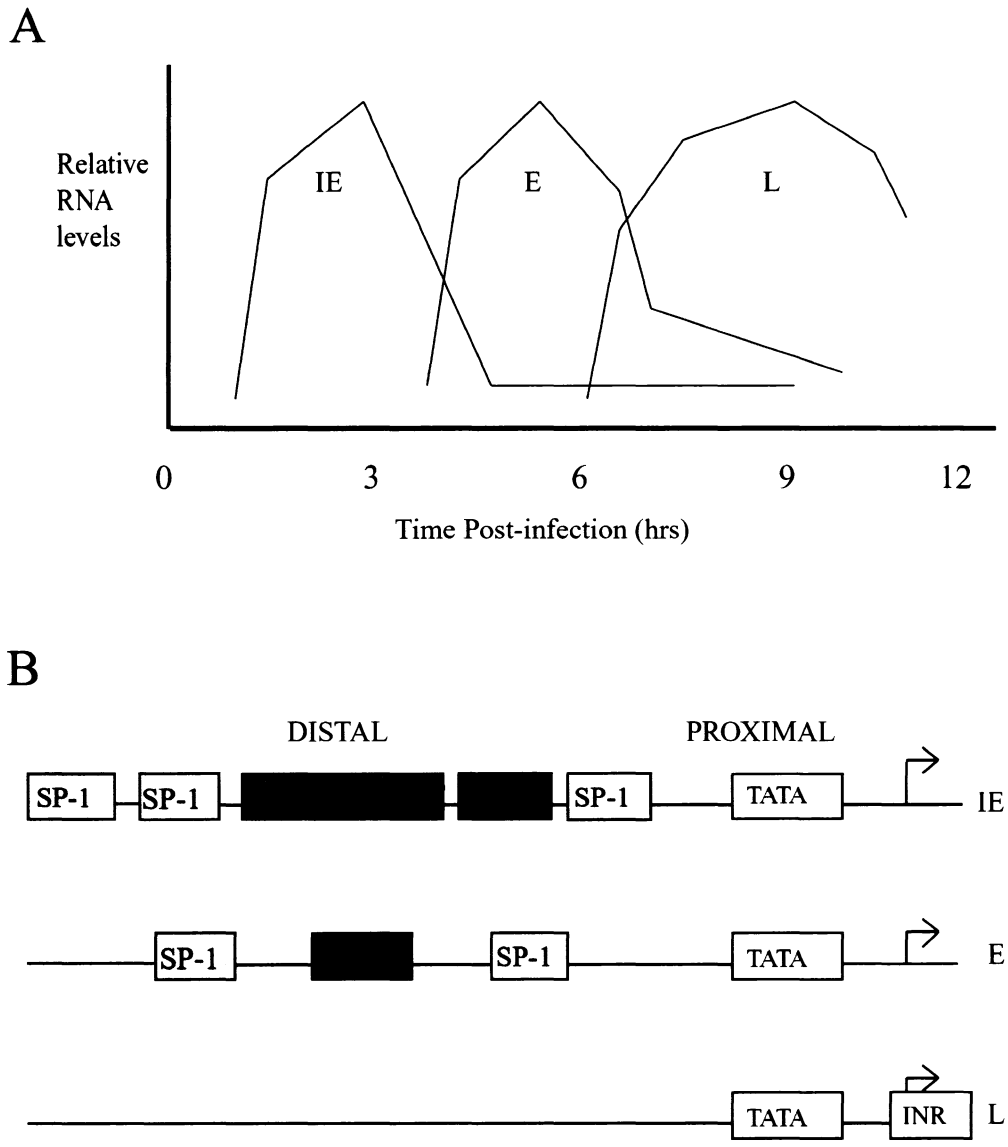

Figure 1.2. HSV1 gene expression. A. HSV1 gene expression can be subdivided into three kinetic classes: IE; immediate-early, E; early, L; late. Transcripts are temporally regulated in a cascade during the course of an infection. Adapted from Weinheimer and McKnight (1987). **B.** Schematic of HSV1 promoter structure. A representation of the proximal and distal regions of HSV1 promoters is shown. The boxed cis-acting elements refer to transcription factor binding sites or the nucleotide sequences corresponding to their respective elements. INR; initiator elements. Adapted from Weir (2001).

place following a conformational change in gD, which then permits an interaction with the gH/gL heterodimer on the surface of the virus. The fusion domain(s) of gH/gL along with the binding of gB, promotes a pH-independent fusion event that allows HSV1 to penetrate the cell (Fuller and Lee, 1992, Roop *et al.*, 1993). As a result, the capsid is brought to the nucleus through a virus-assisted transport mechanism via microtubule structures, ultimately bringing the viral DNA to the nucleus (Sodiek *et al.*, 1997). The proteins that comprise the tegument are then used to prime the cellular environment for viral gene expression and block host defense mechanisms that are geared toward a viral infection (Morrison *et al.*, 1998, Roizman and Knipe, 2001).

1.2.2 Immediate-early genes

The course of an HSV infection can be separated into three stages of gene expression: immediate-early (IE; alpha), early (E; beta) and late (L; gamma) genes, which are expressed in a highly coordinated temporal cascade (Weinheimer and McKnight, 1987) (see Figure 1.2). In general, the role of the alpha genes is to commit the incoming virus to a lytic infection, by regulating the expression of the beta and gamma genes. VP16, which is present in the viral tegument and is brought into the cell upon infection, functions as a potent transcriptional activator of immediate-early gene expression via cis-acting TAATGARAT (R = purine) elements found upstream of IE genes (Post *et al.*, 1981, Batterson and Roizman, 1983) (detailed in section 1.4.1). Expression of the alpha genes commits the virus (and the cell) toward a productive infection. The following genes: ICP0, ICP4, ICP22, ICP27, and ICP47, encode IE proteins. ICP0 is a promiscuous

transactivator of HSV1 genes; although there are no clear cis-acting recognition elements or known DNA binding capability, ICP0 activates expression of reporter genes linked to all classes of HSV promoter elements in transient transfection assays (Cai and Schaffer, 1992). It can also compensate for VP16 in restoring alpha gene expression (Smiley and Duncan, 1997). ICP0 is an important regulatory protein with roles in other cellular processes including the cell cycle, proteolysis, and modulation of the host antiviral response (Mossman and Smiley, 1999, Everett, 2000b). Furthermore, ICP0 localizes to replication compartments within HSV that are adjacent to cellular ND10 domains (discrete structures within the nucleus which may have a role in the regulation of transcription and replication), where ICP0 interacts with SUMO-1; involved in the disruption and degradation of ND10 domains (Everett, 2000a, Everett, 2000b, Everett, 2001). ICP4 (also known as α4, Vmw175) is a large, multi-domain protein involved in the activation of early and late genes, and repression of immediate-early genes (Resnick *et al.*, 1989). It recognizes cis-acting sequences found in all kinetic classes of viral gene expression, and is essential to virus growth and the lytic cycle (Weir, 2001). ICP27 blocks pre-RNA splicing through interaction with host splicing machinery, and promoting the export of viral transcripts out of the nucleus (Sandri-Goldin, 1998, Bryant *et al.*, 2001). In this regard, it is interesting to note that while most of the HSV mRNAs do not contain introns, ICP0, ICP4 and ICP27 do, indicating that these critical genes exploit the existing host environment to its maximal utility (Roizman and Knipe, 2001). ICP22 modifies the host RNA POLII through hyperphosphorylation, which is postulated to direct activity toward viral gene expression (Rice *et al.*, 1995, Jenkins and Spencer,

2001), in addition to being localized to other transcription complexes (Leopardi *et al.*, 1997). ICP47, on the other hand, which is also expressed as an IE protein, protects the invading virus from the host organism immune response through an interaction and sequestration of the MHC1 peptide transporter within the endoplasmic reticulum (York *et al.*, 1994, Tomazin *et al.*, 1996).

1.2.3 Early genes

The early genes encode proteins that are primarily involved in the production of viral DNA, including nucleotide metabolism and replication. Several early gene products are absolutely required for virus growth and replication, while others are not, though the 'non-essential' proteins are likely to provide a significant impact on viral pathogenesis *in vivo* (Whitley, 1998). For instance, thymidine kinase (tk) and ribonucleotide reductase (RR) are involved in modifying cellular pools of nucleotides for use in viral DNA replication, and are not critical to maintain virus growth *in vitro* cell culture systems, but are important for virus reactivation from the latent state (Boehmer and Lehman, 1997).

1.2.3.1 Replication

HSV1 replicates in the nuclei of infected cells. Seven proteins have been found to be necessary and sufficient to conduct origin-dependent viral replication of HSV1, including UL9, UL29 (ICP8), UL30 (DNA polymerase), UL42, UL5, UL8 and UL52 (Skaliter and Lehman, 1994). Replication is initiated with the binding of UL9 (OBP; origin binding protein), which forms a dimer, and contains essential ATP binding and

helicase motifs (Olivo *et al.*, 1988, Bruckner *et al.*, 1991). The dimer binds to the inverted repeats flanking an AT-rich region within the origin, inducing structural changes in the DNA, resulting in single-stranded DNA being disassociated from the complex (Fierer and Challberg, 1992). ICP8 (UL29), a ssDNA-binding protein, is subsequently recruited to UL9 and the ori (Boehmer and Lehman, 1993). Replication can be potentially initiated at three origins of replication, termed 'Ori's. OriL is found within the U_L region, and two copies of OriS are found within the 'c' sequence that flanks the Us segment (also see Figure 1.1.3) (Boehmer and Lehman, 1997). Both OriL and OriS can function as autonomous replicating sequences (Challberg, 1986). The interaction between UL9 and ICP8 functions as the precursor for the entry of the viral replication machinery, including DNA helicase-primase complex, which consists of proteins encoded by UL5, UL8 and UL52. UL5 contains conserved ATP binding and helicase motifs, while UL52 contains a divalent metal binding site and is conserved among other DNA polymerases and primases (Boehmer and Lehman, 1997). The viral DNA polymerase is a heterodimer of the UL30 and UL42 gene products. UL30 contains three motifs (designated I, II, III) that share homology with other DNA polymerases, and also 3'-5' exonuclease activity, while UL42 serves to increase the processivity of UL30 (Gottlieb *et al.*, 1990). Viral DNA replication may be initiated by a 'theta' replication intermediate, which is subsequently converted to a rolling-circle mechanism. This allows for the formation of lengthy concatamers of viral DNA in the 'head-to-tail' formation (Boehmer and Lehman, 1997, Roizman and Knipe, 2001).

1.2.3.2 Replication compartments

Viral DNA replication machinery accumulates at intranuclear foci termed replication compartments, part of distinct nuclear compartments located adjacent to ND10 domains (Everett, 2001). Both the viral DNA and UL29 (ICP8) are sufficient to co-localize to compartments, known as pre-replicative sites, which likely direct the assembly of viral proteins to replication compartments (Lukonis *et al.*, 1997). The role of ND10 in replication is unclear; however, the disruption of ND10 correlates with the loss of growth control while the re-formation of ND10 is indicative of its recovery (Maul, 1998). The goal of an HSV1 infection is to disrupt ND10 domains (Everett, 2000b). ICP0 targets and disrupts ND10 domains, while mutants of ICP0 that lack this function block an infection (Burkham *et al.*, 2001). ND10 structures are postulated to be the source of deposited nuclear proteins, possibly representing a link between transcription and replication functions (Maul, 1998). It is notable that other DNA viruses, including adenovirus and SV40, also form replication compartments adjacent to these domains (Maul, 1998, Everett, 2001).

1.2.4 Late genes

HSV1 late genes are more accurately divided into two subsets, termed leaky-late ($\gamma 1$) and true-late ($\gamma 2$) genes, which are separated on the basis of their corresponding promoter elements and temporal expression patterns. The promoters of leaky-late genes contain upstream Sp1 binding sites, a TATA box, and initiator (Inr) regulatory sites, while the true-late promoters contain only TATA and Inr elements, in addition to a DAS

(downstream activation sequence) element located approximately 20 nucleotides from the transcription start site (Weir, 2001). The DAS binding protein(s) has been identified as the Ku subunit of DNA-PK (DNA protein kinase) (Petroski and Wagner, 1998). The leaky-late genes possess low levels of expression prior to replication, reaching a maximum shortly after replication is initiated. The true late genes are only detected after onset of replication (Lieu and Wagner, 2000). Late gene transcription occurs at discrete sites within viral replication compartments, co-localizing with other viral proteins including ICP4, ICP27 and ICP8 (de Bruyn Kops *et al.*, 1998). The late gene products are primarily structural proteins found within the mature virion.

1.2.5 Assembly and egress

The final stage of virus formation begins with the assembly of the capsid structure, at specialized replication compartment related sites termed 'assemblons' (Ward *et al.*, 1996). The initial stages of capsid organization take place in the cytoplasm, through the construction of a shell containing an internal scaffold (Thomsen *et al.*, 1994, Tatman *et al.*, 1994). Once in the nucleus, the viral DNA inserts into the capsid by displacing the scaffolding proteins, into monomers of unit-length of ~150kB (Varmuza and Smiley, 1985). The concatamers are cleaved to correspond structurally to the capacity of the capsid through recognition of the 'a' sequence at both the L and S termini. Fully assembled nucleocapsids bud through the inner nuclear membrane, and acquire a tegument layer (Vlazny *et al.*, 1982). Viral proteins UL31 and UL34, which are abundant

tegument proteins, are found at the inner nuclear membrane (Reynolds *et al.*, 2001), while other tegument proteins are present in the cytoplasm (Ward *et al.*, 1996).

Two model pathways have been proposed to explain viral envelopment and exit from the cell (Roizman and Knipe, 2001). The original model involves virus envelopment at the inner nuclear membrane (Enquist *et al.*, 1998). In this model, mature capsids bud through the inner nuclear membrane, which already contain viral glycoproteins. The enveloped capsid then moves through the lumen of ER and Golgi vesicles, where the final maturation of the viral glycoproteins can take place, followed by release from the cell. The second model is termed 'envelopment-de-envelopment-re-envelopment' (Skepper *et al.*, 2001). In this scenario, the capsid undergoes a primary envelopment of the inner nuclear membrane, after which a fusion with the outer nuclear membrane results in its de-envelopment, and release into the cytoplasm. The nucleocapsid along with its associated tegument proteins, further accumulates additional tegument proteins, buds into the *trans* Golgi, and is released into the extracellular matrix through secretory vesicles (Cheung *et al.*, 1991). In support of the latter model, studies in which the cellular retention signals of viral glycoproteins were manipulated show differences in virus yield. For instance, construction of a mutant gH protein with an ER retention signal failed to package gH into the virus (Browne *et al.*, 1996), and a gD mutant constructed with an ER localization signal produced significantly less amounts of virus, than with a Golgi retention signal (Whiteley *et al.*, 1999). In addition, viral envelopes are more consistent with a predominantly cytoplasmic membrane composition than one that is nuclear (van Genderen *et al.*, 1994).

Infectious virions can remain cell associated, spread to other cells by virus-induced fusion, or can be released from the cell. Glycoprotein K plays a role in nucleocapsid envelopment and the efficient translocation of virions to the extracellular space, promoting a link between virus egress and virus-induced cell fusion (Hutchinson and Johnson, 1995, Jayachandra *et al.*, 1997). HSV can spread directly from cell to cell, by targeting the viral gE/gI complex to cell junctions (Dingwell and Johnson, 1998), thus utilizing the intracellular spread of virus within non-polarized cells to avoid virion release and recognition by the immune system (Johnson and Huber, 2002). Ultimately, cells productively infected with HSV suffer major biochemical and structural changes resulting from viral replication and the cellular response to infection, which leads to their destruction (Roizman and Knipe, 2001).

1.3 Latency

All herpesviruses share the common ability to establish a latent infection. Latency results in the maintenance of viral DNA within a host in the absence of detectable viral replication, while also retaining the ability to re-infect its host (Jones, 1999). Therefore, the virus is able to persist within its host indefinitely, even in the presence of an existing immune response, until the opportunity to re-establish a productive infection emerges. However, each herpesvirus has a unique biological mechanism and pathology associated with latency and reactivation of the virus from the latent state. HSVI, like other members of the alphaherpesviruses, utilize neurons to establish latency. Researchers are beginning to exploit HSVI neurotropism for gene therapy; in the treatment of brain cancer(s) and

other neurological disorders such as Parkinson's disease (Roizman, 1996). The course of a latent infection can be summarized into three stages: establishment, maintenance, and reactivation.

1.3.1 Establishment

Relatively little is known about the establishment of the latent phase of HSVI. Viruses enter the dorsal root ganglion cells that innervate the site of the productive infection via retrograde axonal transport, and reach the nucleus (Bearer *et al.*, 2000). The viral DNA is then released from the capsid and forms an episome (Rock and Fraser, 1983). Latency is thought to be established through a passive, rather than an active mechanism (ie. IE gene expression is not initiated) (Jones, 1999, Roizman and Knipe, 2001). In addition, the role of VP16 in establishing latency has been investigated, though it did not affect the establishment, nor promote reactivation of the virus from latent neurons (Steiner *et al.*, 1990, Sears *et al.*, 1991). In fact, a number of replication incompetent virus mutants are able to establish latency as efficiently as the wild-type virus (within the animal model systems studied), indicating the latent state can be established in the absence of alpha gene expression (Roizman, 1996).

1.3.2 Virus maintenance during latency

Latency consists of maintenance of viral DNA in a relatively quiescent state within the neuron. The only detectable transcripts from the viral DNA during latency are LATs (latency-associated transcripts) (Stevens *et al.*, 1987). LATs comprise several

transcripts; two minor species of 8.5 and 6.5 kb, and two additional major species of 2 and 1.5 kb (the 2 kb fragment is spliced from the 8.5kb fragment, and 1.5kb is subsequently spliced from the 2kb fragment) (Jones, 1999). LATs have an undetermined role in latency, though their expression is important for virus reactivation (Thompson and Sawtell, 2001). Unsurprisingly, the characterization of the promoter elements that mediate gene expression, termed LAP1 and LAP2, were found to contain a number of neuron specific and other transcription factor binding sites (Wagner and Bloom, 1997, Devireddy and Jones, 2000). Additionally, LAT expression may also be important for establishing latency, since ICP4 is known to represses LAT transcription during the lytic phase (Rivera-Gonzalez *et al.*, 1994). Interestingly, the LAT sequence is complementary to ICPO, ICP4 and γ34.5, which have established roles in the initiation of a productive infection and neurovirulence (Steiner and Kennedy, 1995). LAT(s) may also play a role in preventing apoptosis (Perng *et al.*, 2000, Ahmed *et al.*, 2002).

Previous efforts to detect a translated protein originating from the LATs were apparently unsuccessful (Wagner and Bloom, 1997), although recently, the two kbp LAT fragment was shown to encode a protein (Thomas *et al.*, 1999a). The LAT protein is phosphorylated and localized to the nucleus in punctate, replication-type compartments (Thomas *et al.*, 1999a). Furthermore, the LAT encoded protein appears to enable efficient and selective (compared to HSV2) virus growth of HSV1, in cells that are relatively non-permissive for HSV infection (Thomas *et al.*, 2002). Determination of LAT function remains the primary objective for researchers in understanding the mechanism(s) associated with a latent infection, including reactivation from the latent state.

1.3.3 Reactivation of latent virus

Reactivation from latency occurs in response to "stress". A variety of general stress factors including UV exposure, hypothermia, anxiety, and viral infection have all been shown to trigger HSV1 reactivation (Posavad *et al.*, 1998). The resulting symptoms are usually sub-clinical: 'cold sores' (herpes labidis), localized in proximity to the site of the initial infection (Whitley and Roizman, 2001). The stress response is manifested through signal transduction pathways that can induce virus replication and reactivation from the latent state. In particular, a number of CREB response element (CRE) cis-acting sites contribute to an increase in LAT gene expression upon reactivation (Leib *et al.*, 1991). The virus then travels anterograde along the cells of the trigeminal ganglion to re-establish infection, at a site(s) close to the site of the initial infection (Penfold *et al.*, 1994).

1.4 VP16

1.4.1 Role of VP16 in IE gene expression

HSV1 immediate-early (IE) gene expression is stimulated by a structural component of the virion, termed VP16 (also known as Vmw65, α-TIF, ICP25) (Batterson and Roizman, 1983). It targets a consensus cis-acting promoter element, that was recognized and identified within the upstream promoter of all IE genes, having the consensus sequence 5'GyATGnTAATGArATTCyTTGnGGG-3' (where y; pyrimidine, r; purine, n; any) (Post *et al.*, 1981). VP16 binds DNA relatively inefficiently; it recognizes its target promoter element through the generation of a multi-component complex with

(at least) two cellular factors, the octamer binding protein Oct-1 and HCF-1 (reviewed in Herr, 1998; described in greater detail below). VP16 can activate IE gene transcription in the absence of other viral proteins as demonstrated by its activity in transient transfection assays (Campbell *et al.*, 1984). In addition, there are distinct cis-acting DNA binding elements for mammalian transcription factors GABP (LaMarco *et al.*, 1991, Brown and McKnight, 1992) and SP1 (Jones and Tjian, 1985) within IE promoters, which in conjunction with VP16, function to promote immediate-early gene transcription (Triezenberg *et al.*, 1988b).

1.4.2 VP16-induced complex (VIC)

Paradoxically, although it possesses a highly potent transcriptional activation domain, VP16 lacks the ability to intrinsically localize to the nucleus and to efficiently bind DNA. Therefore, it is through the formation of a multi-component protein complex (referred to as the VP16-induced complex; VIC), that VP16 binds the viral cis-acting TAATGARAT (R = purine) DNA element found upstream of viral IE genes (Post *et al.*, 1981, O'Hare *et al.*, 1988, Spector *et al.*, 1990) (see figure 1.4.2). This complex contains two pre-existing cellular transcription factors, Oct-1 (Kemp and Latchman, 1988, O'Hare *et al.*, 1988, Gerster and Roeder, 1988, Kristie and Sharp, 1990, Verrizijer *et al.*, 1992) and HCF-1 (Katan *et al.*, 1990, Xiao and Capone, 1990, Kristie and Sharp, 1993, Wilson *et al.*, 1993), that are required for complex assembly, and as a consequence for VP16-mediated transcriptional activation (Preston *et al.*, 1988, Goding and O'Hare, 1989, Werstuck and Capone, 1989b, Haigh *et al.*, 1990, Greaves and O'Hare, 1990, Preston *et*

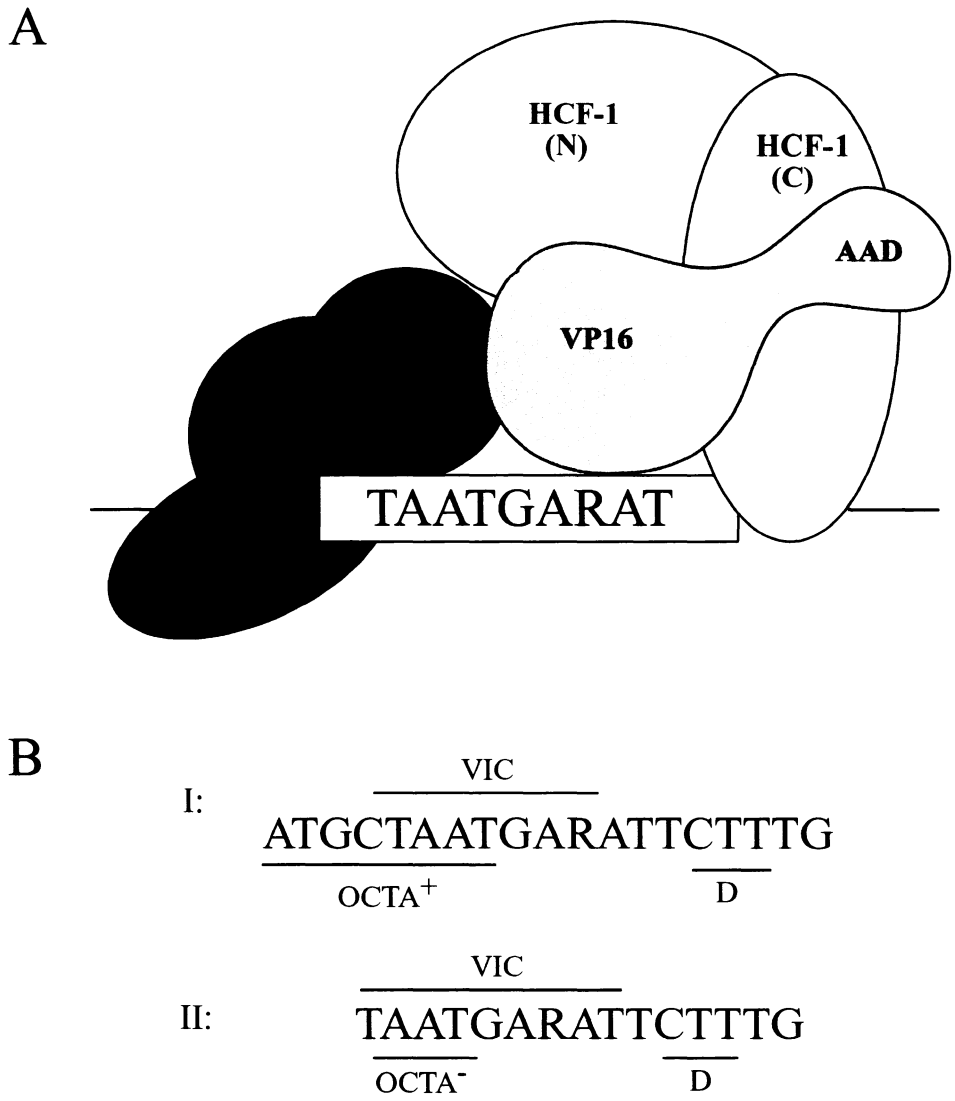

Figure 1.4.2. VP16-induced complex (VIC) A. Schematic representation of the VP16-induced complex (VIC), a multi-protein complex containing cellular factors Oct-1 and HCF-1. Oct-1 bi-partite DNA binding domain; consisting of sub-domains POUh and POUs, and HCF-1 is made up of non-covenlently linked polypeptides HCF(N) and HCF(C). The carboxyl-terminal region of VP16 encodes the acidic activation domain (AAD), which is not required for complex formation. TAATGARAT (R=purine) is the minimal cis-acting DNA element for VIC assembly. **B.** The TAATGARAT element is further categorized by the presence (octa+) or absence (octa-) of a consensus octamer cis-acting element adjoining its sequence. The D element stabilizes the binding of VIC. Adapted from Babb *et al.* (2001).

al., 1997). Though VP16 only has a very weak intrinsic DNA binding capability (Marsden *et al.*, 1987), it does nevertheless make sequence specific contacts with the DNA through the cis-acting GARAT sequence, since mutations generated within GARAT are non-permissive for complex formation (Werstuck and Capone, 1989a, Kristie and Sharp, 1990). Moreover, VP16 appears to adopt a seat-like structure upon binding DNA, positioning numerous basic residues within the protein to make specific contacts with the DNA (Babb *et al.*, 2001). The 'D element', downstream of the TAATGARAT sequence (Figure 1.4.2), is involved in complex formation by VP16, while also functioning as a selectivity determinant for VP16 orthologs (Babb *et al.*, 2001).

In order to form VIC, Oct-1 recognizes the TAAT half-sequence of the TAATGARAT element. The TAATGARAT element can be further differentiated into an $octa^+$ element, which possesses an octamer binding site, and $octa^-$, which does not (apRhys *et al.*, 1989) (refer to Figure 1.4.2). The VP16 complex can distinguish between these since it binds to the $octa^+$ sequence more efficiently, which then correlates with more robust activation (apRhys *et al.*, 1989). However, within VIC, Oct-1 can bind to the TAAT sequence in the presence or absence of an adjacent upstream octamer-binding sequence (AGCT) (Verrijzer *et al.*, 1992). VP16 subsequently interacts with Oct-1 through its POU homeodomain (POUh), which undergoes a conformational change to facilitate its recognition (Stern and Herr, 1991, Walker and O'Hare, 1994, Cleary and Herr, 1995). Through its prior association with HCF-1, VP16 then recognizes DNA-bound Oct-1. Although VP16 can interact with DNA-bound Oct-1 in the absence of

HCF-1, the stability of VIC is greatly enhanced by its presence (Stern and Herr, 1991). Moreover, the role of HCF-1 in complex assembly not only affects the stability of VIC, but also functions to localize of VP16 to the nucleus (LaBoissiere *et al.*, 1999), and was found more recently to function as a co-activator of viral gene expression (Luciano and Wilson, 2002).

1.4.2.1 Mechanism of transcriptional activation

The carboxyl-terminal 80 residues of VP16 are necessary for transcriptional activation (Triezenberg *et al.*, 1988a, Greaves and O'Hare, 1989, Werstuck and Capone, 1989b). This region is enriched for acidic residues, and as such, is part of the larger group of 'acidic' transcriptional activation domains (AAD) (Ptashne, 1988, Blau *et al.*, 1996). The AAD of VP16 is also an independent, modular domain, that is capable of robust transcriptional activation when appended to a heterologous DNA binding domain (Sadowski *et al.*, 1988, Ptashne, 1988). Functional studies of the AAD demonstrate that two sub-regions (termed H1 and H2) are involved in mediating transcriptional activation (Cress and Triezenberg, 1990, Sullivan *et al.*, 1998), while the structural details of the VP16 acidic activation domain remain unclear (Donaldson and Capone, 1992, O'Hare and Williams, 1992). Surprisingly, despite possessing predominantly acidic residues, the alteration of a single hydrophobic residue (F442) to proline, largely abrogates transcriptional activation by the AAD (Regier *et al.*, 1993). Moreover, other hydrophobic residues are also involved in mediating the activity of the AAD (Regier *et al.*, 1993). Binding studies have indicated that the activation domain binds specifically to a number

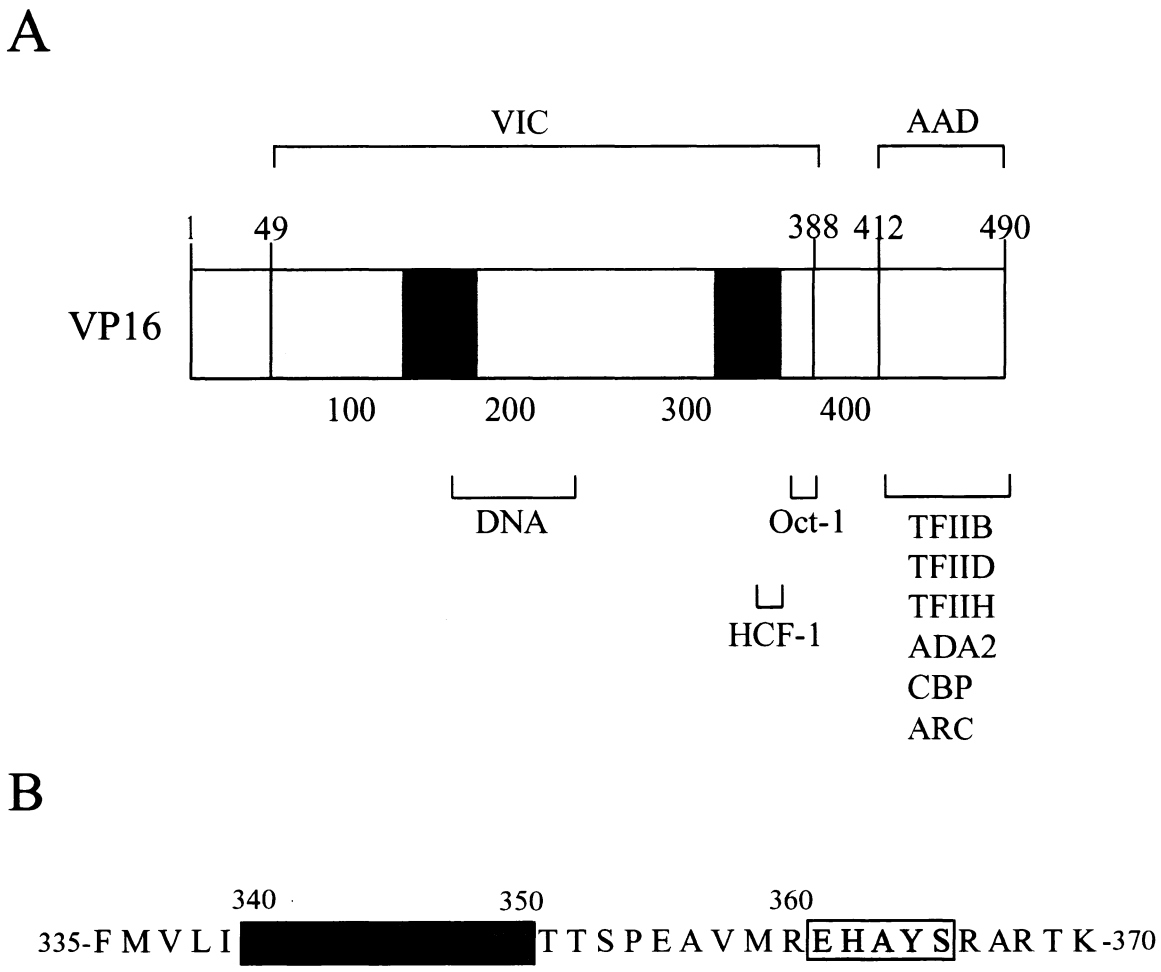

Figure 1.4.2.1. VP16 structural and functional domains. A. Schematic diagram of VP16 structural and functional domains. I and II; regions spanning residues 141-178 and 335-369, respectively, were previously determined to be important for VIC assembly, the yeast transactivation phenotype and interaction with vhs (Werstuck and Capone, 1989a, Smibert *et al.*, 1994, Popova *et al.*, 1995). The localization of critical binding regions for each of the interacting partners are indicated. The references regarding the binding partners of the AAD are provided elsewhere within the text. **B.** Expanded region encompassing residues 335 to 370. Those boxed in green were analyzed by site-specific mutagenesis within this thesis. The HCF-1 binding motif is outlined in the orange box (Freiman and Herr, 1997). VIC; VP16-induced complex, AAD; acidic activation domain.

of basal transcription factors, including TFIIB, TFIID and TFIIH (Lin *et al.*, 1991, Roberts *et al.*, 1993, Xiao *et al.*, 1996), as well as a number of co-regulatory proteins (Berger *et al.*, 1990, Yankulov *et al.*, 1994, Chang and Gralla, 1994, Ghosh *et al.*, 1996, Kraus *et al.*, 1999, Naar *et al.*, 1999), suggesting that its activity is likely regulated through various protein interactions (refer to figure 1.4.2.1).

Deletion mutants of VP16 lacking the AAD are fully capable of forming VIC, indicating that complex formation and promoter recognition can be separated from its activation domain (Freidman *et al.*, 1988, Triezenberg *et al.*, 1988a, Greaves and O'Hare, 1989, Werstuck and Capone, 1989a, Haigh *et al.*, 1990). This is not surprising since transcription factors possess modular functional domains (Ptashne and Gann, 1997), and experiments with a GAL4-AAD fusion protein demonstrate that the VP16 AAD is capable of robust activation in the absence of the amino-terminal domain (Sadowski *et al.*, 1988, Douville *et al.*, 1995). However, VP16 activation appears to be muted to some extent within VIC, leading to speculation that its activity is more tightly regulated within this context (Hagmann *et al.*, 1997, LaBoissierre *et al.*, 1997). Furthermore, there is functional interplay between VP16 and other IE promoter bound factors, including GABP and Sp1, which significantly contributes to IE gene expression (Triezenberg *et al.*, 1988b, Douville *et al.*, 1995). A schematic representation of the VP16 domain structure is outlined in Figure 1.4.2.1. The protein and DNA interactions within VIC represent an exquisite level of co-operativity and regulation of complex formation, providing for multiple levels of differential control and higher-order structures, that may optimize the strength and flexibility of VP16 activation of gene expression (Wu *et al.*, 1994, Shaw *et*

al., 1995, Simmen *et al.*, 1997, Herr, 1998). The mechanism of VP16 induced transactivation of viral immediate-early genes serves as a model for eukaryotic transcriptional regulation (Goding and O'Hare, 1989, Wysocka and Herr, 2003).

1.4.3 Oct-1

Oct-1 is a 766 amino acid protein; a member of the POU domain of transcription factors, which is involved in the transcriptional regulation of a diverse set of genes modulating cellular growth and proliferation (Pruijn *et al.*, 1987, Tanaka *et al.*, 1988, Roberts *et al.*, 1991, Sturm *et al.*, 1993, and refer to Figure 1.4.3). The classification of Oct-1 into the POU domain family is based on a recognized sequence identity among the proteins **P**it-1, **O**ct-1/2 and **U**NC86, which possess a highly conserved bipartite DNA binding domain encompassing 160 amino acids (Herr *et al.*, 1988). The POU domain consists of two structurally independent sub-domains that function co-operatively to bind DNA (Herr and Cleary, 1995). One of the sub-domains, POUs (POU specific domain) is located toward the amino terminal of the protein spanning 68 amino acids, and contains a DNA binding domain that is analogous to bacteriophage lamba and 434 repressor (Assa-Munt *et al.*, 1993, Dekker *et al.*, 1993). The other sub-domain, POUh (POU homeodomain), spans 57 amino acids, and has the structural characteristics of proteins within the homeodomain superfamily of DNA binding proteins (DeLano, 2002). An unstructured and flexible hyper-variable linker region (ranging from 15 to 56 residues) is present between the two domains, and is also involved in determining the specificity of DNA binding (Herr and Cleary, 1995). Both sub-domains are structurally independent,

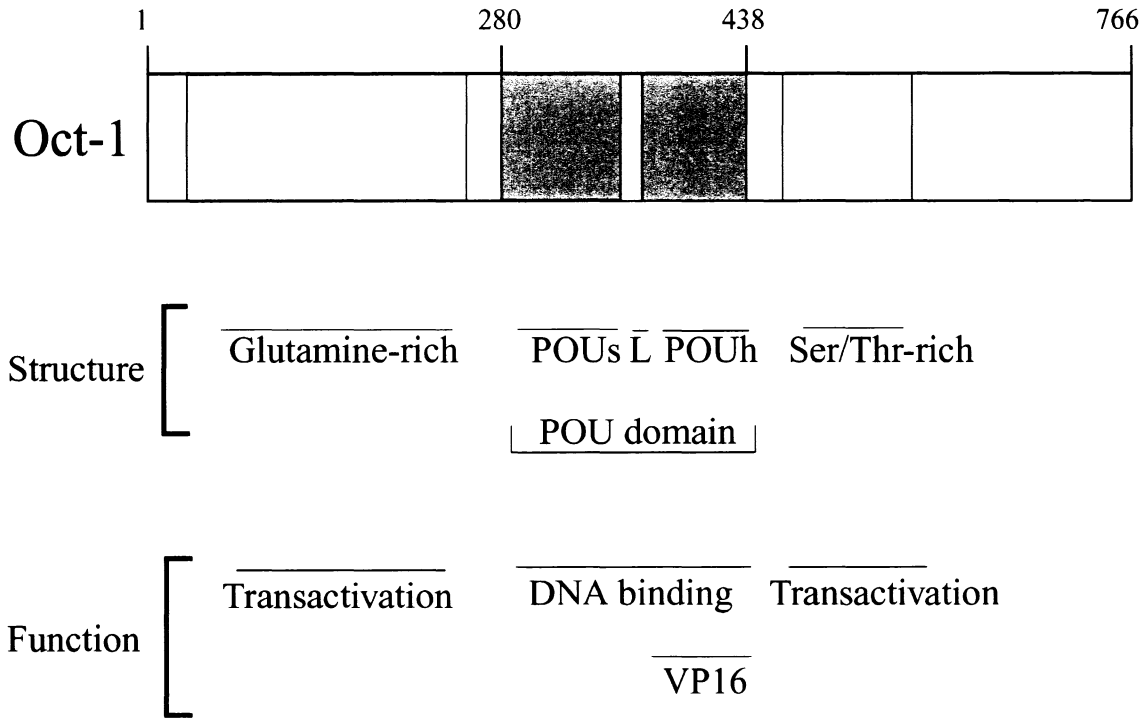

Figure 1.4.3. Oct-1 structural and functional domains. Full-length Oct-1 contains a bi-partite DNA binding domain: consisting of POUs; POU specific domain, POUh; POU homeo domain, L; linker, VIC; VP16-induced complex. The regions indicating various structures and functions are shown and further detailed in section 1.4.3 (and references therein).

and each can make sequence specific contacts with DNA, thus allowing for flexibility, co-operativity and functional diversity in DNA binding (Herr and Cleary, 1995). The natural high affinity binding site for Oct-1 corresponds to the sequence: 5'-ATGCAAAT-3' found in the histone H2B promoter (Klemm *et al.*, 1994), with the POUs domain recognizing the 5'-ATGC, and POUh independently binding the 3'AAAT element (Cleary and Herr, 1995). In addition, a common feature among POU domains is the ability to recognize a diverse set of DNA sequences selectively in association with regulatory cofactors, which include Sp1 (Janson and Pettersson, 1990), AP1 (Kim *et al.*, 1993), PR/GR (Bruggemeier *et al.*, 1991), E1A (Chellappan and Nevins, 1990), OCAB (Babb *et al.*, 1997), and TBP (Zwilling *et al.*, 1994). VP16, E1A, and OCAB do not bind DNA efficiently, and therefore must utilize the DNA binding capability of the POU domain to bind their respective target elements.

Oct-2, which shares significant homology with the POU domain of Oct-1, is selectively expressed in B-cells and neurons (Dent and Latchman, 1991), and is capable of transactivation from TATA-linked Pol II containing promoters (Tanaka and Herr, 1990, Tanaka *et al.*, 1992). VP16 recognizes Oct-1 but not Oct-2, although the two POUh sub-domains differ at only seven residues on the exposed surface of the DNA bound homeodomain (Lai *et al.*, 1992). A glutamic acid at position 22 of helix 1 in Oct-1 (alanine in Oct-2) is the primary binding determinant for recognition by VP16. Since Oct-2 is selectively expressed in neurons, it is postulated to have a role in the establishment of the latent phase of HSV1, through competition for the octamer containing TAATGARAT elements (Lillycrop *et al.*, 1993).

1.4.4 HCF-1

HCF (Host Cell Factor; also known as VCAF-1, CFF, C1, and hereafter HCF-1) was initially identified, and then cloned, as a cellular component of the VP16-induced multi-protein DNA binding complex (Wilson *et al.*, 1993b). It encodes a large precursor protein of 2035 residues that undergoes an autocatalytic proteolytic cleavage reaction, governed by a series of six repeats (termed PRO repeats) within the middle of the protein (Wilson *et al.*, 1995, Vogel and Kristie, 2000). The resulting polypeptides interact non-covalently through self-association domains (Wilson *et al.*, 1995, Vogel and Kristie, 2000). The self-association domain comprises two segments within each of the amino- and carboxyl- terminal fragments, with the carboxyl dimerization region demonstrating homology with the type III fibronectin repeats (Wilson *et al.*, 2000). There are several structural and functional domains within HCF-1 (refer to Figure 1.4.4), identified by an analysis of its primary sequence: a Kelch domain, basic region, Pro repeats, acidic region, FN3 repeats, and a NLS (Wysocka *et al.*, 2001b). The Kelch domain contains a structural motif having a β-propeller-like structure, as in G proteins, which is grouped within the Drosophila Kelch protein superfamily (Adams *et al.*, 2000). This domain encompasses the amino-terminal 380 residues of HCF-1, and is both necessary and sufficient for the assembly of the VP16-induced complex (Hughes *et al.*, 1999); however, the carboxyl-terminal region of HCF-1 is also involved in promoting stable formation of VIC (LaBoissiere *et al.*, 1997). The carboxyl-terminal polypeptide also possesses an acidic region, which was recently shown to contain a transcriptional activation domain (Luciano

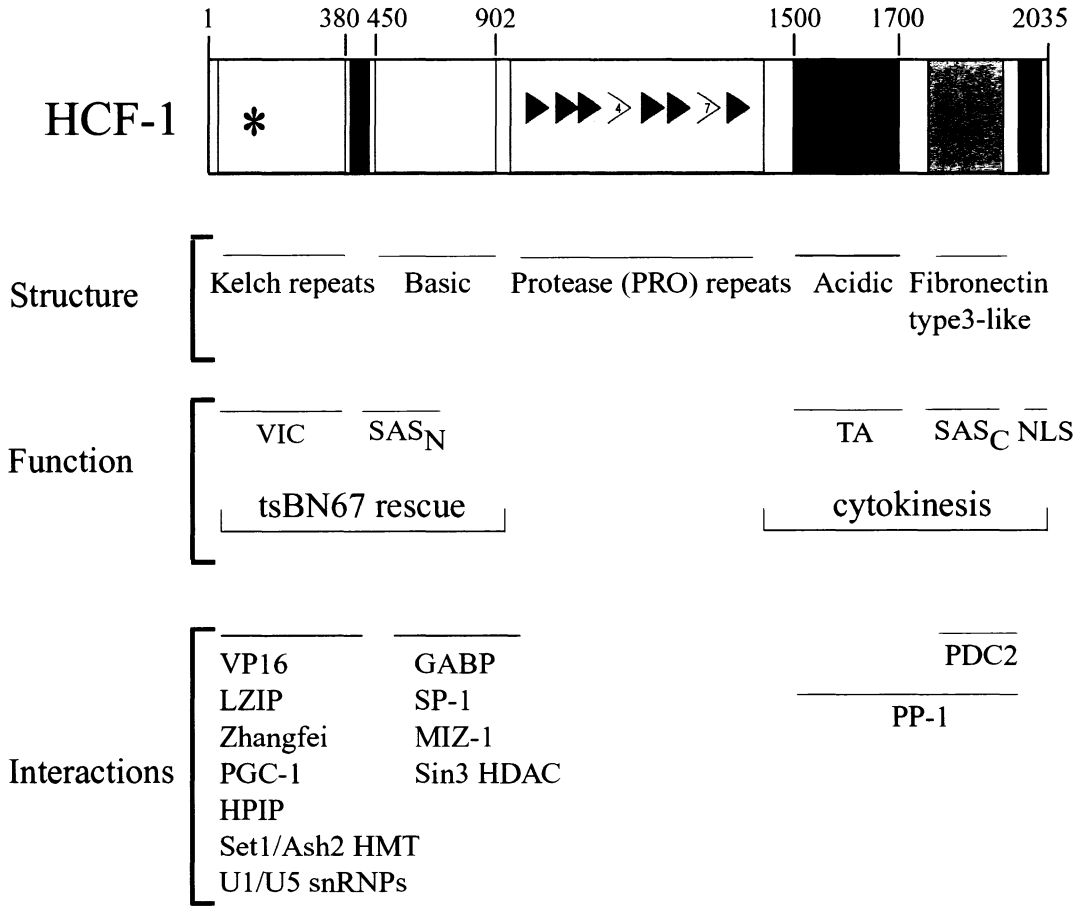

Figure 1.4.4. HCF-1 structural and functional domains. Full-length HCF-1 encodes a 2035 amino acid protein that undergoes auto-proteolysis; forming two polypeptides that interact non-covalently. The regions indicating both the structure and function are further described in section 1.4.4 (references therein). The * denotes the location of the P134S mutant in tsBN67 cells. The triangles represent homology sequences comprising 26 a.a. segments termed PRO (protease) repeats; shaded triangles correspond to segments that are functionally important for proteolysis. TA; transcriptional activation, SAS; self-association sequences, HMT; histone methyl-transferase, HDAC; histone deacetyl-transferase. Adapted from Wysocka et al. (2003).

and Wilson, 2002), and a NLS at its carboxyl-terminal end (LaBoissiere *et al.*, 1999). HCF-1 also interacts with a nuclear export factor (Mahajan *et al.*, 2002).

Homologs of HCF-1, designated HCF-2 and ceHCF (Johnson *et al.*, 1999, Liu *et al.*, 1999b), share similarity with the amino-terminal Kelch domain and carboxyl region of HCF-1, but lack a significant amount of the sequence corresponding to the basic and acidic domain, as well as the region involved in proteolytic processing (Johnson *et al.*, 1999, Liu *et al.*, 1999). Alternatively, dHCF, a recently identified ortholog of HCF-1 from Drosophila, shares more extensive similarity with HCF-1 than with the other homologs (Mahajan *et al.*, 2003). For instance, HCF-1 is exclusively a nuclear protein, while HCF-2 is found in both the nucleus and cytoplasm (Johnson *et al.*, 1999). Interestingly, while HCF-2 and ceHCF retain the Kelch domain and can efficiently promote VP16 complex, they appear to lack a determinant that is necessary to enhance transcriptional activation of the VP16-induced complex relative to a similar fragment of HCF-1 (Lee and Herr, 2002).

1.4.4.1 HCF-1 and the cell cycle

HCF-1 has an important role in cellular proliferation and cytokinesis (Julien and Herr, 2003). An initial indication as to the native function of HCF-1 was obtained through a cell line (tsBN67); possessing a temperature-sensitive induced growth arrest phenotype attributed to HCF-1, which resulted in a block at G_0/G_1 of the cell cycle. This defect resulted from a single point mutation in HCF-1 altering residue 134 from proline to serine (termed P134S) (Goto *et al.*, 1997). The P134S mutant was also unable to

support complex formation with VP16 (Goto *et al.*, 1997). However, subsequent mutagenesis studies have shown that binding to VP16 does not correlate exclusively with the cell cycle defect attributed to HCF-1, and a region beyond the Kelch domain, including the basic region, is also required for complementation of the cell cycle defect (Wilson *et al.*, 1997, Mahajan and Wilson, 2000). The minimal fragment required to rescue tsBN67 cells corresponds to the amino-terminal 902 residues of HCF, in which the kelch and basic domain must remain linked within a single polypeptide (Wilson *et al.*, 1997). Subsequent analysis of the tsBN67 cell defect indicated that a loss of chromatin binding and re-localization of HCF-1 to the cytoplasm precedes the temperature-sensitive defect in those cells (Wysocka *et al.*, 2001b). Furthermore, HCF-1 also appears to play a role in cellular cytokinesis (Reilly and Herr, 2002, Julien and Herr, 2003), a function recently localized to the carboxyl-terminal fragment of the protein. Intriguingly, it appears that the carboxyl-terminal fragment dissociates from the amino-terminal polypeptide during progression through the cell cycle, thus mediating both cytokinesis and G1 progression, respectively (Julien and Herr, 2003).

Though the significance of VP16 binding to HCF-1 with respect to the cell cycle is not clear, it is hypothesized to function as a 'biological sensor' within cells in order to monitor the suitability of establishing a productive infection (eg. in neurons) (Goto *et al.*, 1997, Kristie *et al.*, 1999, Wysocka *et al.*, 2001b, Wysocka and Herr, 2003). Notably, the signals that induce HSV reactivation from the latent state can also induce intracellular trafficking of HCF-1 from the cytoplasm to the nucleus (Kristie *et al.*, 1999).

1.4.4.2 HCF-1 as a co-regulator of gene expression

In addition to, or in conjunction with its role in the cell cycle, HCF-1 is emerging as a transcriptional co-regulatory protein. A number of cellular transcription factors have recently been found to interact with HCF-1, mostly through a common sequence motif termed the HCF-binding motif (HBM) ([D/E]HXY; X is any residue). This motif is found in VP16 and cellular transcription factors including Luman (Lu *et al.*, 1998), Zhangfei (Lu *et al.*, 2000) and PGC-1 (Lin *et al.*, 2002), which interact with HCF-1 and in some cases were shown to be sensitive to the P134S mutant within the Kelch domain (Freiman and Herr, 1997, Lu *et al.*, 2000). Other transcription factors, such as GABP (Vogel and Kristie, 2000), SP1 (Gunther *et al.*, 2000) and MIZ-1 (Piluso *et al.*, 2002) interact with HCF-1 through its basic region. HCF-1 also interacts with snRNPs, which is also sensitive to the P134S mutation (Ajuh *et al.*, 2002), and protein phosphatase 1 through its carboxyl-terminal fragment (Ajuh *et al.*, 2000), indicating a potential role for HCF-1 in post-transcriptional gene regulation. Interestingly, HCF-1 also has both co-activator and co-repressor functions, which appear to be context-dependent (Vogel and Kristie, 2000, Luciano and Wilson, 2000, Luciano and Wilson, 2002, Scarr and Sharp, 2002, Piluso *et al.*, 2002, Wysocka *et al.*, 2003). Future work will undoubtedly uncover an increasingly complex and significant role for HCF-1 not only in viral, but also in cellular gene expression.

1.4.5 VP16 structural role

Despite its well-established role in transcriptional activation of HSV IE genes, an essential facet of VP16 biology involves its role in virus assembly. Ace and colleagues (Ace *et al.*, 1988, Ace *et al.*, 1989) had initiated an investigation into the structural role of VP16 within HSV by generating two important findings: First, a temperature-sensitive (ts) mutant in HSV2, localized to VP16 (ts2203), was critical to virus propagation during virus assembly (Ace *et al.*, 1988). Second, a series of in-frame linker mutants (disrupting segments of the VP16 coding sequence) were found to be important for virus propagation, but not for VP16-complex assembly or transcriptional activation (Ace *et al.*, 1989). The structural defect corresponding to VP16 was further underscored by the construction of a VP16-null virus (8MA), which was shown to possess deficiencies in DNA encapsulation and the production of enveloped viruses, but not the transactivation of IE genes (Weinheimer *et al.*, 1992). An analysis of the VP16 structure (see Figure 1.4.5) has also revealed that the residues important for virion formation appear on opposite sides of the residues implicated for interactions involving VIC, suggesting potentially distinct roles in virus assembly and transactivation (Liu *et al.*, 1999a). Other experiments have also underscored this segregation; the AAD of VP16 is dispensable for virus growth *in vitro*, suggesting that VP16-complex assembly and transcriptional activation can be uncoupled from the structural defect associated with VP16 (Ace *et al.*, 1989, Smiley and Duncan, 1997). Moreover, Poon and Roizman (1995), through construction of temperature-sensitive mutants within VP16, found only a minimal disruption to HSV replication and assembly associated with the inability to transactivate

Figure 1.4.5. The solved VP16 structure. The core VP16 structure generated within PyMOL (pymol.org) using co-ordinates obtained from the Brookhaven Protein Databank (16VP.pdb) (Liu et. al., 1999a). Segments of the ribbon diagram are colour coded in order to contrast the various elements of the structure. The residues corresponding to amino acids 330-350 are shown in orange, the segment between residues 350 and 400 are not present since they are disordered within the original structure. Residues 335,345 and 350 are indicated.

IE genes. However, these results are complicated by the fact that transactivation defective VP16-virus mutants do not completely abolish IE gene transcription, and that transactivation of IE genes by VP16 becomes increasingly unimportant at higher MOI (Ace *et al.*, 1989, Poon and Roizman, 1995, Smiley and Duncan, 1997). In addition, EHV-1 (equine herpesvirus) gene 12, an ortholog of VP16, can complement a defect in the activation of HSV IE genes, though it is not assembled into the virus (Thomas *et al.*, 1999b). It should be noted, however, that a transcription-defective VP16 viral mutant was attenuated using animal models, demonstrating that VP16 transactivation of viral IE genes is required under more appropriate physiological conditions (ie. low MOI) (Tal-Singer *et al.*, 1999). Nevertheless, VP16 possesses an undetermined structural role in virus assembly that appears to be functionally distinct from the transactivation of viral IE genes.

The mechanism by which VP16 is involved in virus assembly is still not clear, although it is synthesized as a late gene and is packaged as a relatively abundant protein within the viral tegument (Spear and Roizman, 1972, Heine and Roizman, 1973). Specific interactions between VP16 and other components of the tegument including vhs (Smibert *et al.*, 1994), VP22 (Elliot and O'Hare, 1995), and Us11 (Diefenbach *et al.*, 2002), may be structurally or functionally important. Additionally, cross-linking studies have also implicated viral glycoproteins gB, gD and gH in complex formation with VP16 (Zhu and Courtney, 1994). Similarly, the UL48 gene in pseudorabies virus (PrV), a VP16 ortholog, is required to co-ordinate the capsid and outer envelope structure of the virus (Fuchs *et al.*, 2002). Thus, VP16 may serve as a link to the components of the capsid and

the viral envelope, in addition to providing a scaffold to organize and (or) package proteins within the viral tegument. Recently, Mossman *et al.* (2001b) outline a role for VP16 in viral egress, downstream of the initial envelopment of the capsid. Since VP16 is found in both the cytoplasm and the nucleus, and has a perinuclear distribution at late times of an infection (Freidman *et al.*, 1988, Elliot *et al.*, 1995, LaBoissiere *et al.*, 1999), a role for VP16 during viral egress following the initial envelopment at the inner nuclear membrane is consistent with its role in virus assembly (Mossman *et al.*, 2001b).

1.5 Virion host shutoff protein (Vhs)

Vhs is a 490 amino acid phosphoprotein encoded by the UL41 gene of HSV1 with an apparent molecular weight of 58kD; which is altered by differential phosphorylation (Smibert *et al.*, 1992, Read *et al.*, 1993). Orthologs of vhs are also present in other alpha-, but not beta- and gammaherpesviruses (Strelow and Leib, 1995). As part of the tegument, vhs immediately prepares the host cell for conversion toward viral gene expression upon infection. It functions by selectively destroying host (and viral) mRNA through the disaggregation of polyribosome structures and the destruction of mRNA (Fenwick and Clark, 1982, Read and Frenkel, 1983, Schek and Bachenheimer, 1985, Strom and Frenkel, 1987, Kwong and Frenkel, 1987, Oroskar and Read, 1987, Kwong *et al.*, 1988, Fenwick and Owen, 1988, Oroskar and Read, 1989). Vhs is functional in the absence of other viral proteins (Jones *et al.*, 1995, Pak *et al.*, 1995).

The mechanism of vhs activity is beginning to emerge with the development of *in vitro* assays (Smiley *et al.*, 2001). Since vhs is specific for mRNAs, the 5' methyl cap and

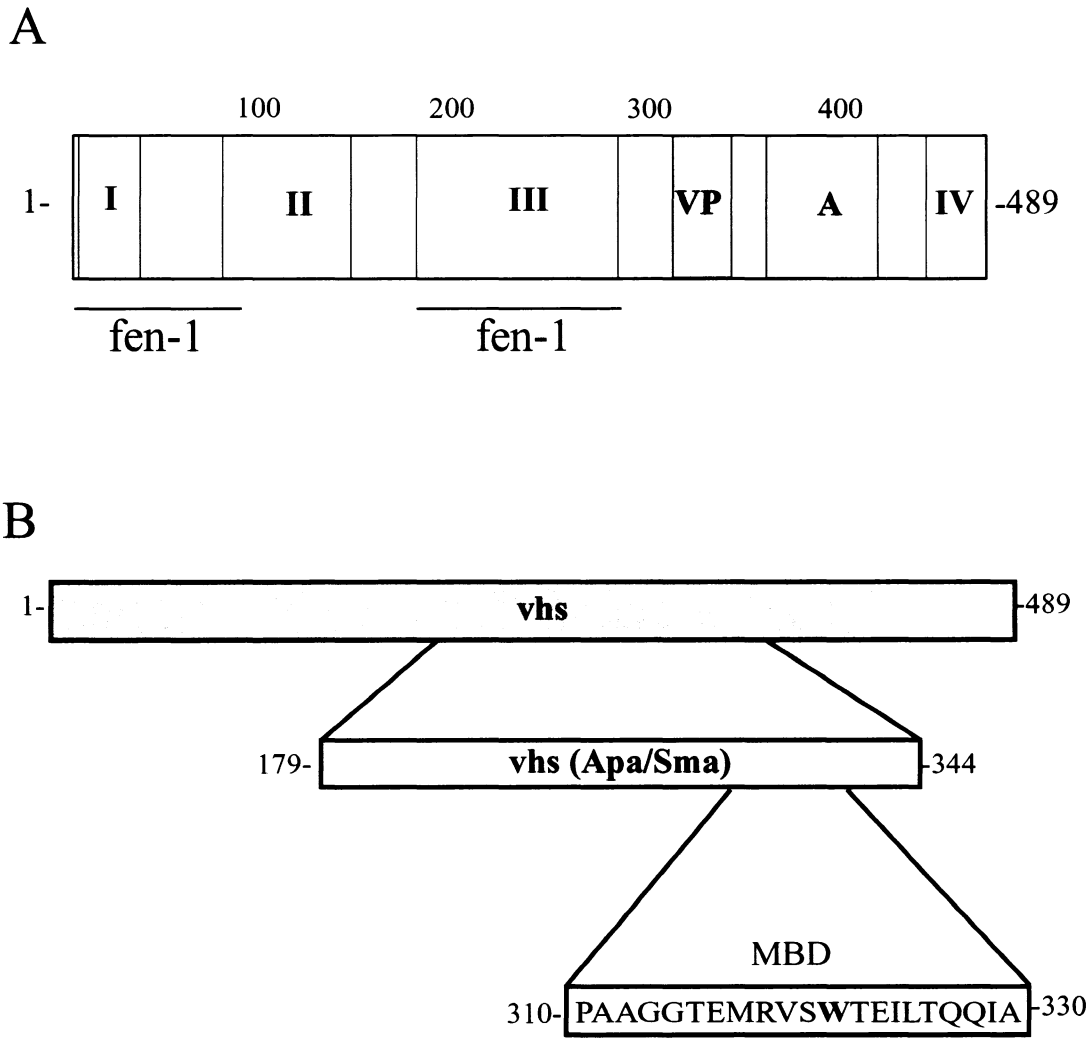

Figure 1.5. Vhs functional domains. A. Schematic diagram of vhs functional domains.Segments I-IV and A represent regions important for vhs activity (Jones *et al.*, 1995). Regions of sequence similarity to fen-1 nuclease are indicated and based on the analysis of Everly *et al.*, 2002. VP; indicates the minimal VP16-interaction domain. **B.** Successive delineation of the regions within vhs that bind to VP16. The polypeptide corresponding to the Apa/Sma fragment of vhs interacts with VP16 (Smibert *et al.*, 1994) and is used throughout this thesis to assess binding to VP16. MBD; VP16 minimal binding domain (MBD) that is sufficient to interact with VP16 when appended to the GAL4 DNA binding domain (Schmelter *et al.*, 1996). Tryptophan (W) 321 is critical for maintaining the interaction with VP16 (shown in bold type).

polyadenylation signals were initially targeted as a source of this specificity. Deadenylated mRNAs accumulate within HSV1 infected cells, leading Zelus *et al.* (1999) to postulate that vhs preferentially degrades polyadenylated mRNA. Moreover, Read and colleagues were subsequently able to demonstrate that the 5' end is targeted initially, followed by the 3' polyadenylation sequence (Karr and Read, 1999, Read *et al.*, 2001). However, a comprehensive set of *in vitro* experiments by Elgadi and others established that 5'- endoribonucleolytic cleavage by vhs can take place in the absence of ribosomes, the 5'-cap and 3'-polyadenylation sequence (Elgadi *et al.*, 1999). The authors also noted that vhs specifically requires both Mg^{2+} and ATP for optimal activity, with mRNA cleavage occurring at discrete sites between 30-40 nucleotides downstream of the 5'cap structure. There was also an apparent preference for GA and/or AG dinucleotides. Vhs also targets and degrades mRNA containing a poliovirus IRES (internal ribosome entry site) (Elgadi and Smiley, 1999, Lu *et al.*, 2001b). The segments of the vhs sequence important for its activity are shown in Figure 1.5. Based on the experiments conducted to date, it is not yet clear if vhs functions as a ribonuclease. Vhs does have homology with the fen-1 family of DNA nucleases, implying that it may function as an mRNase (Everly *et al.*, 2002), though it also appears that an additional factor from mammalian cell extracts is required for full activity (Feng *et al.*, 2001, Lu *et al.*, 2001a). Residues functionally important for vhs activity also correlate with those required for binding to eIF4H (Feng *et al.*, 2001) and a conserved nuclease activity (Everly *et al.*, 2002).

Vhs is not an essential viral gene, although vhs null mutants possess an altered pattern of gene expression *in vitro* (Read *et al.*, 1993). In addition to enhancing the viral

lytic cycle at low MOI, vhs mutant viruses are impaired for both establishment and reactivation from latency, and possess increased immunogenicity (Geiss *et al.*, 2000). The latter may be due to an absence of a non-specific evasion of host defenses (Lalic-Koppers *et al.*, 2001, Lu *et al.*, 2001a), which could be particularly important in the development of vaccines targeted against HSV (Geiss *et al.*, 2000, Keadle *et al.*, 2002).

1.5.1 Interaction with VP16

Vhs is synthesized as a late gene product, and it must be regulated in order to preserve the expression of late viral genes. Since VP16 has previously been shown to interact with vhs, and is more abundant within the virus, it was initially postulated that VP16 would regulate vhs activity (Smibert *et al.*, 1994). Alternatively, an excess of vhs was shown to prevent VIC formation when pre-incubated with VP16, suggesting that vhs may also have a role in VP16 transcriptional activation (Smibert *et al.*, 1994). In an effort to elucidate the functional significance of this interaction, Smiley and colleagues (Lam *et al.*, 1996) demonstrated that VP16 serves to downregulate vhs function at intermediate and late times of an infection. Significantly, the results indicated a novel post-transcriptional role for VP16 in blocking vhs function. Briefly, a summary of their results were as follows: First, a VP16-null virus (8MA) is permissive for destruction of viral mRNA at late times on an infection, while a double mutant possessing an inactive vhs (8MAΔSma) is not. Second, a VP16 revertant of 8MA restored viral gene expression, while superinfection of a VP16 expressing cell line blocked vhs mediated translation arrest (Lam *et al.*, 1996). The interaction with vhs at late times post-infection is also

noteworthy since the structural role for VP16 at late times of an infection may be to block vhs mediated destruction of viral mRNAs. However, the construction of virus with an inactivated vhs gene in the absence of VP16 (8MAΔSma) exposes at least one other function for VP16 in virus assembly that is independent of its interaction with vhs (Lam *et al.*, 1996, Mossman *et al.*, 2001b).

1.6 Project Outline

At the onset of this work, two novel and intriguing aspects of VP16 function had just emerged from our laboratory. First was the interaction between VP16 and vhs (Smibert *et al.*, 1994). The biological significance of this interaction, though initially unclear, was hypothesized to involve VP16 regulation of vhs activity, through an undetermined structural role in virus assembly or regulation of HSV1 gene expression. Second was the discovery of an autonomous activation domain within VP16 (Popova *et al.*, 1995). The amino-terminal region of VP16 (lacking the AAD) was fused with the GAL4 DNA binding domain, and shown to be a robust activator of gene expression in yeast, though only marginally active in mammalian cells. The basis for the differing levels of activity in yeast and mammalian cells was unclear. Furthermore, VP16 orthologs also lacked the potent AAD present in HSV VP16, potentially adding to the significance to those findings. Therefore, an investigation of VP16 mediated regulation of gene expression within HSV, through an interaction with vhs, and an identification of an alternative activation domain within VP16, served as the framework for this thesis.

Previous mutagenesis experiments had isolated two regions within the primary sequence of VP16 containing determinants for binding to vhs and for the yeast transactivation phenotype (Werstuck and Capone, 1989a, Werstuck and Capone, 1989b, Smibert *et al.*, 1994, Popova *et al.*, 1995). We focused on the segment within VP16 between residues 335 and 369, since structural studies and mutagenesis experiments had indicated that the internal deletions at the amino terminus of VP16 were likely to be unstable (Hayes and O'Hare, 1993, Poon and Roizman, 1995). A detailed understanding of the biological significance of the interaction with vhs, and characterization of a putative activation domain within the core VP16 protein was therefore sought through a set of additional mutagenesis studies. As a result, the yeast two-hybrid system (Fields and Song, 1989, Fields and Sternglanz, 1994) and *in vitro* assays were used to map interaction domains within VP16, and serve as a screen to identify mutants of each function. We rationalized that the localization of smaller, distinctly defined regions of activity within VP16, could serve to effectively dissect various aspects of protein structure and function.

Chapter three encompasses the results of the investigation into interaction between VP16 and vhs. Expanding upon previously established carboxyl-terminal deletion mutants, construction of additional deletions resulted in localization of a region between residues 340 and 350 that retained a critical determinant for vhs binding. The alteration of a single residue within this region was sufficient to abolish the interaction. Subsequent structure and function analyses were also able to demonstrate that the interaction with vhs could also be uncoupled from its role as a transcriptional activator.

Moreover, complementation assays of mutant viruses confirmed that the vhs-binding mutant of VP16 is incompatible with virus growth in the presence of a functional vhs, though the mutant can complement virus growth in the absence of vhs.

The investigation of an alternative activation domain in VP16 is presented in chapter four. A region between residues 345 and 350 of VP16 was found to contain a critical determinant for the activation phenotype in yeast; which uncoupled it from the modest activation observed in mammalian cells. Consequently, we found that HCF-1 functions directly as a co-activator of a GAL4-DNA binding domain fusion of VP16. HCF-1, recently described as a transcriptional co-regulator (Luciano and Wilson, 2002), is the only known cellular interaction partner of VP16 within mammalian cells. HCF-1 was shown here to synergize with VP16 in the activation of an IE gene promoter-linked reporter gene, even promoting expression of an IE gene even in the absence of VP16. These results indicate a novel role for HCF-1 in promoting viral IE gene expression, which is illustrated in a model termed the 'VP16-enhanceosome'.

VP16 is a multi-functional protein that has a critical role in establishing a productive viral infection, through the transcriptional activation of IE genes and a structural role in virus assembly. Within this thesis, HSV1 VP16 was investigated with respect to its interactions with the virion host shutoff protein (vhs) and the mammalian cell co-regulator, HCF-1; resulting in a further elucidation and characterization of the role of VP16 in viral gene expression. This work also establishes a framework to further address critical aspects of VP16 function within HSV.

Chapter 2. Materials and Methods

2.1 MATERIALS

2.1.1 Specialized chemicals and reagents

The following is a list of specialized chemicals or reagents that were used in this thesis:

5-bromo-4-chloro-3-indoyl-β-D-galactoside (Xgal)	Biosynth
ampicillin	Sigma
BioRad protein assay reagent	BioRad
bovine serum albumin	Pharmacia Biotech
isopropyl-β-D-thiogalactoside (IPTG)	Biosynth
luciferin	Biosynth
Coomassie brilliant blue (R250)	BDH Chemicals Ltd.
crystal violet	BDH Chemicals Ltd.
geneticin (G418)	Gibco BRL
glutathione-Sepharose 4B	Amersham
L-glutamine; 200mM	Gibco BRL
hexamethylene bisacetamide (HMBA)	Sigma
Hoechst 33258	Fisher Scientific
Human gamma globulin	Cutter Biologicals
Molecular Weight standards	
1Kb DNA ladder	Gibco BRL
low/high range SDS-PAGE standards	BioRad
nucleoside triphosphates	Pharmacia
O-nitrophenyl-β-D-galactoside (ONPG)	Sigma
penicillin (5000U/mL)/streptomycin (5000μg/mL)	Gibco BRL
phenylmethylsulphonylfluoride (PMSF)	Boehringer Mannheim
poly (dIdC·dIdC)	Pharmacia
protease cocktail tablets	Boehringer Mannheim
proteinG-Sepharose	Roche
reporter lysis buffer (cell culture)	Promega
salmon sperm DNA; sonnicated	Sigma
serum, calf	Gibco BRL
fetal bovine	Sigma
sodium deoxycholate (DOC)	Sigma
trypsin-EDTA	Gibco BRL

2.1.2 Radiochemicals

[α-^{32}P]-dATP (3000Ci/mmol; 10μCi/μL)	Amersham
L-[^{35}S]-Methionine (1151Ci/mmol; 10μCi/μL)	Amersham
^{14}C-protein standards (high range; 14-200kDa)	Gibco BRL

2.1.3 Enzymes

All restriction endonucleases and modifying enzymes were purchased from New England Biolabs and used according to the manufacturer's instructions. The following enzymes, used within the Methods, also used according to the manufacturer's instructions, were obtained from the following sources:

Lysozyme	Sigma
T7 or SP6 RNA polymerase	Promega
T7 DNA polymerase	Pharmacia
PFU DNA polymerase	Stratagene
RNase A	Pharmacia

2.1.4 Antibodies

VP16(SAL)
A rabbit-derived polyclonal raised against the 'SAL' fragment of VP16 (resides 5-411) coupled to protein A, made by P. Bilan. It was diluted 10^3-fold in TBST buffer for Western blot analysis (section 2.13.2).

GAL4DB
A mouse monoclonal antibody directed against the GAL4 DNA binding domain (residues 91-147), purchased from SantaCruz Biotech (RKC51). It was used at a dilution of 0.25 μg/mL for Western blot analysis and 2.5 μg/reaction for co-immunoprecipitations (section 2.2.10.4).

VP16(AAD)
A rabbit polyclonal antibody directed against the VP16 acidic activation domain (residues 413-453) purchased from Clontech (#3844-1), and used at a dilution of 0.1 μg/mL for Western blot analysis.

Secondary antibodies
Horse radish peroxidase (HRP)-linked anti-mouse, or anti-rabbit IgG were purchased from Amersham and were generally used at a dilution of ~1 x10^5 fold.

2.1.5 Oligonucleotides

The following tables display oligonucleotides that were utilized throughout this thesis.

Table 2.1.5.1: Oligonucleotides for site-specific mutagenesis[a]

VP_{R341A}[b]	5'-GTACTTTATGGTGTTGATTGCGGCGAAGTTGGACTCG-3'
VP_{A342G}	5'-GGTGTTGATTCGGGGGAAGTTGGACTCG-3'
VP_{K343A}	5'-GTGTTGATTCGGGCGGCGTTGGACTCGTATTC-3'
VP_{L344A}	5'-GTTGATTCGGGCGAAGCGGGACTCGTATTCCAG-3'
VP_{D345A}	5'-GGGCGAAGTTGGCCTCGTATTCCAGC-3'
VP_{S346A}	5'-GGCGAAGTTGGACGCGTATTCCAGCTTCAC-3'
VP_{Y347A}	5'-GCGAAGTTGGACTCGGCTTCCAGCTTCACGACC-3'
VP_{S348A}	5'-GAAGTTGGACTCGTATGCCAGCTTCACGAC-3'
VP_{S349A}	5'-GTTGGACTCGTATTCCGCCTTCACGACCTCGCC-3'
VP_{F350A}	5'-CTCGTATTCCAGCGCCACGACCTCGGCCTC-3'
$VP_{T351/52A}$	5'-CGTATTCCAGCTTCGCGGCCTCGCCCTCCGAGC-3'
VP_{S353A}	5'-CAGCTTCACGACCGCGCCCTCCGAG-3'
VP_{P354A}	5'-GCTTCACGACCTCGGCCTCCGAGCTC-3'
VP_{E355A}	5'-GCTTCACGACCTCGCCCGCCGAGCTC-3'
VP_{L344P}	5'-GTTGATTCGGGCGAAGCCGGACTCGTATTCCAGCTTC-3'
VP_{L344N}	5'-GTTGATTCGGGCGAAGAACGACTCGTATTCCAGCTTC-3'
VP_{L344K}	5'-GTTGATTCGGGCGAAGAAGGACTCGTATTCCAGCTTC-3'
VP_{L344I}	5'-GTGTTGATTCGGGCGAAGATCGACTCGTATTCCAGCTTC-3'
VP_{L344M}	5'-GTTGATTCGGGCGAAGATGGACTCGTATTCCAGCTTC-3'
VP_{L344V}	5'-GTTGATTCGGGCGAAGGTGGACTCGTATTCCAGCTTC-3'
VP_{L344F}	5'-GTGTTGATTCGGGCGAAGTTCGACTCGTATTCCAGCTTC-3'
$VP_{\Delta sal}$[c]	5'-CGCAGACTGTCTACGGCCCCCCC-3'
VP_{Y364A}	5'-CTCAACCTGGATACCAATGGCCTGGGAGACC-3'

$VP_{\Delta 340\text{-}345}$[d]	5'-GGTACTTTATGGTGTTGATTTCGTATTCCAGCTTCACGACC-3'
$VP_{\Delta 345\text{-}350}$[d]	5'-GATTCGGGCGAAGTTGACGACCTCGCCCTCCG-3'
VHS_{W321A}	5'-CGAGACGCGCGTCTCGGCGACCGAAAATTCTAACC-3'

a. Each oligonucleotide was re-suspended in ddH_20 to a final concentration of 1 mg/mL. Complementary oligonucleotides are not shown.
b. Oligonucleotides are labeled using standard single amino acid codes, with the wild-type amino acid preceding the corresponding numerical position, followed by the altered amino acid. VP; VP16, vhs; virion host shutoff protein
c. Silent mutation constructed at residue 411 to abolish the *SalI* restriction endonuclease site.
d. Site-directed mutagenesis creating an internal deletion (Δ) between the displayed residues

Table 2.1.5.2: PCR primers and oligonucleotides for cloning

VP_{F250}[a]	5'-GAACCGTGTTGGCCAACTTCTGCTC-3'
VP_{R340}[b]	5'-CCCCGAGCTCAATCAACACCATAAAGTACCCAGAGG-3'
VP_{R345}[b]	5'-CCCCGAGCTCGTCCAACTTCGCCCGATCAACAC-3'
VP_{R350}[b]	5'-CCCCGAGCTCGAAGCTGGAATACGAGTCCAAC-3'
VP_{R355}[b]	5'-CCCCGAGCTCGGAGGGCGAGGTCGTGAAGCT-3'
$VP_{335\text{-}355}$[c]	5'-TTTATGGTGTTGATTCGGGCGAAGTTGGACTCGTA TTCCAGCTTCACGACCTCGCCCTCCGAGCT-3'
$ICP4_{TAATGARAT}$[d]	5'-GATCGTGCATGCTAATGAGATTCTTTGGGG-3' (top) 5'-GATCCCCCAAAGAATCTCATTAGCATGCAC-3' (bottom)
$ICP0_{TAATGARAT}$[e]	5' – GATCCCGTCGATCGTAATGATATTCTTTGGG -3' (top) 5'- GATCCCCAAAGAATATCATTAGCATGCACGG -3' (bottom)

a. Forward primer used to generate carboxyl-terminal deletions, underlined nucleotides correspond to a *MscI* restriction endonuclease site around amino acid 200 of VP16
b. Reverse primer denoting the carboxyl-terminal endpoint (as indicated numerically) for the PCR derived deletions within VP16, the underlined nucleotides correspond to the *SacI* restriction endonuclease site
c. Sequence encompassing residues 335 to 355 of VP16. The complementary oligonucleotide is not shown.
d. Oligonucleotides containing an octa$^+$ TAATGAGAT element from the promoter proximal site of the HSV ICP4 gene (Aphrys *et al.*, 1989). The octa$^+$ recognition site is underlined and the TAATGARAT cis-acting element is shown in bold. The oligonucleotides were engineered to yield 'GATC' overhangs for cloning purposes.
e. Oligonucleotides containing the octa$^+$ TAAGARAT from the promoter proximal site of the HSV ICP0 gene (Gerster and Roeder, 1988). The octa$^+$ recognition site is underlined and the TAATGARAT element is shown in bold. 'GATC' overhangs were engineered in order to allow for a radioactively labeled probe for EMSA analysis.

Table 2.1.5.3: Sequencing primers

DBD_{FOR}[a]	5'- TCATCGGAAGAGAGTAG -3'
DBD_{MCS}[b]	5'-GCCGGCGGTGGCGCCACC-3'
VP_{FSEQ}[c]	5'-CCGCTCGTCTTGCGCGTG -3'
VP_{RSEQ}[d]	5'-CACGCGCAAGACGAGCGG-3'
VHS[e]	5'-CCTCTGCCGCCGGCCG-3'
$GL2_{MCS}$[f]	5'- GTATCTTATGGTACTGTAACTG -3'

a. Anneals to residues 140 to 142 of the GAL4 DNA binding domain; used for sequencing amino-terminal fusions of the GAL4DB.

b. Primer used to sequence from the 3' end of a GAL4 DNA binding domain fusion within the pPC97 vector
c. Corresponding to the region around amino acid 220, used for sequencing downstream regions in VP16
d. Corresponding to the region around amino acid 220, used to sequence regions upstream of VP16
e. Corresponds to residues 300 to 303 of vhs for sequencing of downstream residues
f. Used for sequencing upstream cis-acting DNA elements cloned into the MCS of pGL2

2.1.5.1 Preparation of oligonucleotides

Oligonucleotide concentrations were determined by using the formula: **C = OD/ε**; where OD refers to optical density units (provided by the manufacturer), ε (epsilon) is an extinction coefficient, which takes into account the contribution of the component nucleotides to the OD (Ausubel *et al.*, 1990), and C is the concentration (mol/L). All oligonucleotides were obtained from the Central Facility at MOBIX (McMaster University). Those used for site-directed mutagenesis were additionally purified by OPC (trityl column purification). Each sample was re-suspended in ddH_20 at the indicated concentration.

2.1.2 Plasmids

2.1.2.1 Plasmids from other sources

pPC97: A low-copy yeast expression vector used for two-hybrid analysis, was generously provided by Dr. P. Chevray (Johns Hopkins University) (Chevray and Nathans, 1992). The GAL4 DNA binding domain (residues 1-147 of GAL4), contains an adjacent MCS that was used to generate fusion constructs. Constitutive expression of the fusion is driven by the ADH1 promoter, and the plasmid was maintained in yeast auxotrophic for leucine. pPC97, which is a derivative of pPC62, was engineered to contain the MCS of pPC86.

pPC86: A low-copy yeast expression vector used for two-hybrid analysis (Chevray and Nathans, 1992), corresponding to the GAL4 activation domain (residues 768-881), also with a downstream MCS. The GAL4 AD possesses a NLS derived from the SV40 T antigen. The plasmid is selected for in yeast auxotrophic for tryptophan, and constitutive expression of the fusion is driven by the ADH1 promoter.

pCDBVP(335,369,404), pCADVP(335,369,404): Carboxyl-terminal deletions of VP16 that are terminated at the indicated residues, were constructed as GAL4 DNA binding domain or activation domain fusions within pPC97 or pPC86, respectively, by B. Popova (Smibert *et al.*, 1994, Popova *et al.*, 1995).

pCDBvhs(Apa/Sma): The Apa/Sma fragment of vhs, encompassing residues 179 to 344, was constructed as a GAL4 DNA binding domain fusion within pPC97, by B. Popova (Smibert *et al.*, 1994).

pCDBHCF380: The amino-terminal 380 residues of HCF-1 were constructed as a fusion to the GAL4 DNA binding domain of pPC97, by D. Piluso. A PCR derived fragment from a full-length clone of HCF-1 (**pCGNHCF**) (Wilson *et al.*, 1993b), was digested with *EcoRI/KpnI* and cloned into the corresponding sites within the target plasmid.

pGEM5Zf(-): *In vitro* transcription/translation plasmid containing both the SP6 and T7 RNA polymerase promoters with an extensive MCS, was purchased from Promega.

pSPUTK: *In vitro* transcription/translation vector containing a SP6 RNA polymerase promoter, optimized for translation by the addition of the β-globin UTR (untranslated leader region), and constructed with a series of unique restriction endonuclease sites with the MCS (Falcone and Andrews, 1991). This plasmid was kindly provided by Dr. D. Andrews (McMaster University).

pSPUTK65: The DNA sequence corresponding to full-length VP16 (490 residues) was excised as a *NcoI/BamHI* fragment from **pMC1** (Werstuck and Capone, 1989b) and cloned into **pSPUTK**, by Dr. J. Capone. VP16 expression is under the control of the SP6 promoter.

pSPUTKvhs: An *in vitro* transcription/translation vector containing full-length vhs, under the control of the SP6 promoter in **pSPUTK** (Elgadi *et al.*, 1999). This plasmid was kindly provided by Dr. J. Smiley (University of Alberta).

pSPAS**:** The Apa/Sma fragment of vhs (spanning residues 179 to 344) was cloned into **pSPUTK**, and is under the control of the SP6 promoter (Smibert *et al.*, 1994). This plasmid was constructed by C. Smibert and was kindly provided by Dr. J. Smiley.

pGEX2T**:** A plasmid utilizing a glutathione-S-transferase (GST) moiety for the purpose of creating protein fusion constructs for overexpression and purification studies in bacteria, was purchased from Amersham. It harbours a pTAC promoter; based on the *lac operon* system, which permits inducible protein expression with IPTG.

pGEX2T$_{(VP16,\ vhs)}$: An intermediate cloning vector was constructed by placing the *BglII* fragment encompassing the MCS of **pSPUTK**, into the *BamHI* site in **pGEX2T,** in order to create **pGEX2T-SP**$_{MCS}$. Full-length VP16 or vhs were excised from their **pSPUTK** derivatives with *NcoI/SacI*, and cloned into the corresponding sites of **pGEX2T-SP**$_{MCS}$ by Dr. J. Capone and P. Shaw, respectively.

pGEX2T-C1: Carboxyl-terminal 79 residues of VP16 were fused to GST by L. Donaldson (Donaldson and Capone, 1992).

pGEM7Zf(-)-HCF$_{902}$**:** The amino-terminal 902 residues of HCF-1, was generated using a PCR derived segment of **pCGNHCF**, and cloned into the *EcoRI/BamHI* sites of **pGEM7Zf(-)** (Promega) by D. Piluso. Its expression is under the control of the T7 promoter.

pMAL-2c: An expression vector utilizing a cytosolic version of the maltose-binding protein to create fusions for overexpression within bacteria, was purchased from NEB. The expression of the fusion is regulated by the pTAC promoter, which is induced with IPTG.

MBP-VP$_{SAL\ (Rmutants)}$: A series of site-specific amino acid substitutions were constructed to investigate the importance of a series of arginine and other residues (termed R mutants) for VP16 function. The mutants were generated within segments of VP16 referred to as the Sal fragment (corresponding to residues 5-411 of VP16) and subsequently fused downstream of MBP in **pMAL-2c**, by P. Shaw (Shaw *et al.*, 1995).

pEVRF0: A mammalian expression vector containing an immediate-early CMV promoter and SV40 polyadenylation signals. This plasmid was kindly provided by Dr. W. Schaffner (Institut für Molekularbiologie II der Universität Zürich) (Matthias *et al.*, 1989).

pEVRF65: Full-length VP16 under the control of the CMV early promoter and the SV40 polyadenylation site, used for mammalian expression and transactivation studies (Werstuck and Capone, 1989b). Full-length VP16 was placed into **pEVRF0**, by N. Shen.

pGL2$_{SV40}$: Reporter plasmid for gene expression studies in mammalian cells, utilizing the *luciferase* gene isolated from firefly *photonis pyrialis* (de Wet *et al.*, 1987) was purchased from Promega. Cis-acting DNA elements are cloned upstream of the transcription start site within the MCS, in conjunction with the SV40 early promoter and polyadenylation signal.

pGL3$_{BASIC}$: is a *luciferase* reporter plasmid that lacks a basal promoter element but contains the SV40 late polyadenylation signal and an upstream MCS (Promega).

p5xGAL4*luc*: Five tandem 17mer oligonucleotides corresponding to upstream activation sequence elements for GAL4 (Sadowski *et al.*, 1988), were cloned upstream in **pGL2$_{SV40}$**. This plasmid was kindly provided by Dr. J. Hassell (McMaster University).

pSGVP$_{(335,369,404)}$: A series of VP16 carboxyl-terminal deletions were fused to a GAL4 DNA binding domain protein within the mammalian expression vector **pSG424** (Sadowski *et al.*, 1988). These plasmids were constructed by B. Popova (Popova *et al.*, 1995).

pCGNHCF: Cloned full-length HCF-1 (spanning residues 2-2035) in the mammalian expression vector **pCGN** harbouring a CMV early promoter (Wilson *et al.*, 1993), was generously provided by Dr. W. Herr (Cold Spring Harbor Laboratory).

pCGNHCF$_{1011,1011(P134S)}$: Plasmids encompassing the amino-terminal 1011 residues of HCF-1, for both the wild-type and P134S mutant, were provided within the pCGN vector (Wilson *et al.*, 1997). These plasmids were kindly provided by Dr. A. Wilson (New York University School of Medicine)

pα4luc: The promoter sequence upstream of the HSV1 ICP4 (α4) gene was excised using *BamHI* and *BglII* from the CAT reporter construct **pα4CAT** (Werstuck and Capone, 1989b) and cloned into the *BglII* site within the MCS of **pGL3BASIC**, by D. Piluso.

2.1.2.2 Plasmids constructed within this thesis

pCDBVP$_{404(Rmut)}$,pCADVP$_{404(Rmut)}$: Double-stranded DNA fragments corresponding to the amino-terminal 404 residues of VP16 containing the mutants generated by P. Shaw (Shaw *et al.*,1995) were isolated from M13 phage extracts as outlined in Sambrook *et al.* (1989). The DNA was first cut with *NcoI*, and blunted with the Klenow fragment of DNA polymerase I, and subsequently digested with *SacII* and cloned into the *SmaI/SacII* sites of either pPC97 or pPC86.

pCDBVP$_{(340,345,350,355)}$: A PCR based cloning strategy was used to construct various carboxyl-terminal deletions within VP16. A forward primer annealing to the region around residue 200 of VP16, and each corresponding reverse primer, were used to produce a truncation at each indicated deletion mutant. The primers used are listed in Table 2.1.5.2. The products of the PCR reaction were cut with *MscI* and *SacI* and cloned into the corresponding segment in **pCDBVP$_{404}$**. The fragments derived from the PCR reaction was subjected to DNA sequence analysis.

pCADVP$_{(340,345,350,355)}$: To generate the corresponding GAL4 activation domain constructs with the PCR derived deletion mutants of VP16, the *NcoI/SacI* fragments from pCDBVP$_{(340,345,350,355)}$ were used to replace the same fragment in **pCADVP$_{424}$**, and were subjected to DNA sequence analysis.

pCDBVP$_{404(R341\ to\ E355,\ \Delta340\text{-}345,\ \Delta345\text{-}350)}$, pCADVP$_{404(R341\ to\ E355,\ \Delta340\text{-}345,\ \Delta345\text{-}350,\ L344subs)}$: Site-specific mutants were generated within VP16 (see below; pGEM5Zf-VP$_{404}$ mutant constructs) and placed within two-hybrid vectors, pPC97 and pPC86. The mutants described here refer to site-specific mutants within the VP16 sequence, including a number of substitutions of amino acid 344, and internal deletions that were created. The fragments corresponding to each of the mutants were excised from **pGEM5Zf-VP$_{404}$** with *NcoI/SacI*, and used to replace the same sites in **pCDB$_{335}$** or **pCADVP$_{335}$**, in order to generate the appropriate GAL4 DNA binding domain or GAL4 activation domain fusions, respectively.

pCDBVP$_{335\text{-}355}$, pCADVP$_{335\text{-}355}$: The oligonucleotides listed in Table 2.1.5.2, corresponding to the coding sequence for residues 335 to 355 were generated in order to determine if the resulting polypeptide fragment was sufficient to act as an autonomous

activation domain when appended to the GAL4 DNA binding domain, or to specifically interact with vhs fused to the GAL4 activation domain. The oligonucleotides were first annealed and then ligated into the SmaI site of pPC97 or pPC86, respectively. The products were subjected to DNA sequence analysis.

pCDBVHS$_{Apa/Sma\ (W321A)}$: Using the oligonucleotides listed in Table 2.1.5.1, residue 321 of vhs (within the context of the Apa/Sma fragment) was altered from tryptophan to alanine within the two-hybrid vector **pCDBVHS$_{Apa/Sma}$**. This plasmid was subjected to DNA sequencing.

pCDBVP$_{350(\Delta 1\text{-}5)}$, pCADVP$_{350(\Delta 1\text{-}5)}$: A deletion of the amino-terminal 5 residues within VP16 were made in order to assess its role with respect to the activation phenotype of VP16 in yeast and vhs binding. Two *SalI* sites adjacent to each other, one just upstream of the coding sequence of VP16 in **pCDBVP$_{404}$** or **pCADVP$_{404}$** and the other at amino acid 5 of VP16, were collapsed, blunted with Klenow and subsequently re-ligated to generate an in-frame deletion of the first five residues of VP16. DNA sequencing analysis was used to confirm the product.

pGEM5Zf(-)-VP$_{404\ (site\text{-}directed\ mutants)}$: Site-specific mutants within VP16 were constructed using **pGEM5Zf-VP404** as a template plasmid. The amino-terminal 404 residues have previously been shown to be sufficient to interact with vhs and coordinate VIC assembly. **pGEM5Zf(-)** is a transcription/translation vector that contains a set of unique restriction endonuclease sites that were potentially useful for subsequent sub-cloning strategies. The QuickChange (Stratagene) site-directed mutagenesis protocol was used to generate various single amino-acid substitutions, as well as internal deletions at various residues throughout VP16. The mutants are listed with their corresponding oligonucleotides in Table 2.1.5.1. The products were subjected to DNA sequence analysis.

pGEM5Zf(-)-VP$_{(335,\ 340,\ 345,\ 350,\ 355,\ 404)}$**:** The various carboxyl-terminal deletion constructs of VP16 were placed into a transcription/translation vector, in which the expression is controlled by the T7 promoter. These clones were generated by excising the *NcoI/SacI* fragment from each of the respective pCDBVP deletion constructs (previously described) and replacing the *NcoI/SacI* fragment in **pGEM5Zf(-)**.

pGEX2T-VHS$_{Apa/Sma,\ Apa/Sma\ (W321A)}$: GST fusions of the Apa/Sma fragment of vhs (residues 179-344) were generated with both the wild-type (**pSPAS**) and mutant (**pSPAS$_{(W321A)}$**) proteins. The coding sequence for each of the inserts were excised from the indicated plasmids with *NcoI/SacI*, and used to replace the *NcoI/SacI* fragment of **pGEX2T-VP16**.

pGEX2T-VP(335, 340, 345, 350, 355, 404): GST fusions of the VP16 deletion constructs were generated by excising the *NcoI/SacI* fragment from each of the respective **pCDBVP** plasmids and replacing the corresponding sites in **pGEX2T-VHS(Apa/Sma)**.

pGEX2T- VP404(R341 to E355, Y364, Δ340-345, Δ345-350, L344subs): A GST fusion of the amino-terminal 404 residues of VP16, was used to generate various site-specific and deletion mutants. Essentially, the list of the mutants can be obtained from the oligonucleotides present in Table 2.1.5.1. The *NcoI/SacI* fragments excised from each of the respective **pGEM5Zf-VP404** contructs were used to replace the corresponding segment in **pGEX2T-VHS(Apa/Sma)**.

pEVRF65(site-specific mutants): In order to make constructs within **pEVRF65** that contain the various site-specific mutants previously generated in **pGEM5Zf-VP404**, it was necessary to create a silent mutation at amino acid 411 of VP16 within **pEVRF65**, in order to abolish the *SalI* restriction site, so that *SalI/SacII* fragments from each of the respective **pGEM5Zf-VP404** plasmids could be placed in the same sites in **pEVRF65**. The subsequent replacement of the wild-type sequence was verified by DNA sequencing.

p(TG)3*luc*: A luciferase reporter gene plasmid in eukaryotic cells containing three tandem (forward; 5' to 3') TAATGARAT elements was constructed by annealing oligonucleotides labeled ICP4TAATGARAT in Table 2.1.5.2 and cloning into the *BglII* site of **pGL2SV40**. The oligonucleotide sequence was derived from the ICP4 TAATGARAT element (apRhys *et al.*, 1989), which also contains an octamer binding site engineered to leave 'GATC' overhangs in order to facilitate cloning.

pCGNHCF(P134S): To construct the full-length HCF-1 containing the P134S point mutant, the *SpeI/XhoI* fragment was excised from the plasmid containing full-length HCF-1 (**pCGNHCF**) and replaced with the equivalent fragment from **pCGNHCF1-1011(P134S)**. The presence of the mutation was confirmed by DNA sequence analysis.

pSGVP(340,345,350,355): Mammalian two-hybrid expression vectors consisting of GAL4 DNA binding domain fusions of the carboxyl-terminal VP16 proteins were generated by first digesting each of the appropriate pCDBVP plasmids with *NcoI*, blunting with Klenow fragment and subsequently digesting with *SacI*. The resulting fragments were inserted into the *SmaI/SacI* sites of **pSG424**. The presence of an intact fusion junction was confirmed by DNA sequence analysis.

pSGVP369 (Y364A): A tyrosine to alanine at residue 364 in VP16 was generated in order to abolish an interaction with HCF-1 (LaBoissiere *et al.*, 1999). Site-specific mutagenesis of **pSGVP369** was carried out with the oligonucleotides listed in Table 2.1.5.1 (Y364A).

pRC/CMV-VP404: **pSGVP404** was transformed into a *dam* methylase deficient host bacteria (GM48; see below) and digested with *EcoRI/BclII*, blunted with Klenow fragment and sub-cloned into the *HindIII* site of the mammalian expression vector **pRcCMV** (Invitrogen). Protein expression is under the control of the immediate-early CMV promoter and SV40 polyadenylation signal. The VP16 coding sequence also lies downstream of a T7 promoter for *in vitro* transcription/translation reactions.

2.1.3 Organisms and strains

2.1.3.1 Bacteria

Escherichia coli (*E. coli*) strains **DH5α**, **DH10β** were used for subcloning and plasmid maintenance and purification. Though **DH5α** had been used initially, we were able to generate consistently higher transformation efficiencies with **DH10β**, so it was subsequently used in later steps. *E. coli* **GM48** is a *dam* methylase deficient strain that was used for the purposes of digesting with the restriction endonuclease *BclII*. The bacterial strains and associated genotypes are listed in Sambrook *et al.* (1989). *E. coli* **BL21(DE3)** (Novagen) was used for expressing GST fusion proteins, since it contains a disrupted protease and are therefore optimized for efficient expression of the bacterial fusions.

2.1.3.2 Yeast

Saccharomyces cerevisiae **PCY2** (MATα Δ*gal4* Δ*gal80* URA3::GAL1-*lacZ* *lys2*-801^{amber} *his3*-Δ200 *trp1*-Δ63 *leu2* *ade2*-101^{ochre}) mutant strain engineered for use in the analysis of the two-hybrid system (Chevray and Nathans, 1992). It contains a *lacZ* downstream of a UASg (GAL4 upstream activation sequence) and GAL1 promoter, and lack both GAL4 and GAL80 proteins. This strain was a kind gift of Dr. P. Chevray.

2.1.3.3 Mammalian cells

COS-1 cells are **CV-1** derived African green monkey (*Cercopithecus aethiops*) kidney cells that have been transformed with SV40 (Gluzman, 1981) and were obtained from the American Type Culture Collection (ATCC CCL-1650). These cells were propagated in DMEM supplemented with 10% fetal bovine serum (FBS).

Vero cells are African green monkey kidney cells (ATCC CCL-81), propagated in DMEM supplemented with 10% calf-serum (CS).

16-8 cells are a **Vero** derived cell line that is used to complement 8MA and 8MAΔSma mutant viruses (see below). VP16 is constitutively expressed in these cells, under the control of the MMTV LTR (Moloney murine sarcoma virus) promoter and HSV-1 TK polyadenylation signals (Weinheimer *et al.*, 1992). Stable expression of VP16 is maintained by neomycin selection using 450 μg/mL G418. The cells were propagated in DMEM supplemented with 10% CS.

tsBN67 is a temperature-sensitive cell line derived from **BHK21** (baby hamster kidney cells) and possesses a cell cycle defect resulting in a block in G_0/G_1 progression (obtained from Riken Cell Data Bank). The defect is attributed to a missense mutation in HCF-1 altering a proline to a serine at residue 134 (Goto *et al.*, 1997). The cells were propagated in DMEM supplemented with 10% CS, at the permissive temperature of 32 °C. The non-permissive temperature for these cells is 39.5 °C.

HeLa cells are a transformed human cervical cancer cell line (ATCC CCL-2), propagated in DMEM supplemented with 10% FBS.

2.1.3.4 Viruses

8MA is an **HSV-1**κos derived mutant virus lacking the entire VP16 coding sequence, being replaced with β-galactosidase (Weinheimer *et al.*, 1992). HSV-1κos is a wild-type laboratory strain of HSV-1. Due to an observed structural defect in virus assembly, the virus must be propagated on a complementing cell line (16-8 cells; which constitutively expresses VP16 in *trans*). **8MA$_{\Delta Sma}$** contains an additional deletion constructed within 8MA to include an inactivation of the vhs gene (Lam *et al.*, 1996). The inactivated vhs was generated through a collapse of the *SmaI* sites within the coding sequence (Read *et al.*, 1993) and is referred to as ΔSma. Due to the absence of VP16, 8MA$_{\Delta Sma}$ must also be propagated on a complementing cell line.

2.2 METHODS

2.2.1 Plasmid Manipulation

2.2.1.1 Cloning techniques

The plasmid constructs described in the Materials section were generated by standard recombinant DNA techniques as described by Sambrook *et al.* (1989) and according to manufacturers' instructions.

2.2.1.2 Polymerase Chain Reaction (PCR)

The PCR reactions were conducted as outlined in Ausubel *et al.* (1990). Reactions were carried out with up to 100 ng plasmid DNA, 1 μM primers, 1 mM dNTPs, and 2 units Vent DNA polymerase. The reactions conducted herein did not require additional $MgSO_4$. A "hot start" melting cycle was initiated (95 ^oC, 5 min), with subsequent melting cycles programmed for 30 sec intervals. The annealing temperature was calculated using the equation: $\mathbf{T_a=[4(G+C)+2(T+A)]^oC-10^oC}$, generally around 55 ^oC, and was programmed for 1 min. The elongation cycle proceeds at 72 ^oC for a variable time, depending on the size of the target fragment (~1 Kb/min). The PCR reaction was set for a total of 30 cycles. The products of the reaction were re-suspended with agarose-loading buffer (30% glycerol, 0.05% bromophenol blue, 0.05% xylene cynol FF) and run on an agarose gel. The DNA band was then excised (see electroelution; section 2.2.5), and subcloned into the appropriate vector.

2.2.1.3 Site-directed mutagenesis

Site-directed mutagenesis was conducted using the QuickChange PCR mutagenesis kit (Stratagene). Each mutant was generated from two complementary oligonucleotides designed to possess nucleotide change(s) corresponding to the desired mutation (listed in Table 2.1.5.1). Primers can vary in length from 25-45 nucleotides, and were designed to correspond to a melting temperature of around 80 °C, based on the formula: $\mathbf{T_m=81.5+(0.45*\ \%GC)-(675/N)-\%X}$; where '%GC' refers to the relative percentage of either G or C present in each oligonucleotide; N refers to its length; and X refers to the number of nucleotides changed with respect to the wild-type sequence and expressed as a percentage. Briefly, reactions contained 1-100 ng (usually 10 ng) of plasmid DNA, 200 µM dNTPs, 125 ng of each primer, 1x PFU enzyme buffer, 2.5 U PFU DNA polymerase, and ddH_2O to a total of 50 µL. The number of cycles and the temperatures used for reactions were variable, and as such, guidelines provided by the manufacturer were followed. Following the reaction, 1 µl (40 U) of *DpnI* restriction endonuclease was added directly to each mixture and placed at 37 °C for 2hr. Up to 10 µl of the reaction mixture was then used to transform competent bacteria, and colonies were picked for subsequent DNA sequence analysis.

2.2.2 Maintenance and growth of bacterial cells

Bacteria were grown in culture or on solid plates using 2YT at 37 °C, as outlined in Sambrook *et al.* (1989). Plasmids were maintained within bacteria using 100 µg/mL ampicillin.

2.2.2.1 Preparation of competent bacterial cells

Bacteria were made chemically competent to take up plasmid DNA through a RbCl based protocol (Hanahan, 1983). Briefly, a single bacterial colony was used to inoculate a 5 mL starter culture in 2YT media. The following day, 1 mL of that culture was used to inoculate 100 mL 2YT, until an OD_{600} of 0.4 was reached. The culture was centrifuged at low speed (3,000 x g, 4 ^{o}C, 5 min) to harvest the cells. Pellets were re-suspended with 10 mL cold RF1 buffer (100 mM RbCl, 50 mM $MnCl_2{\cdot}4H_2O$, 10 mM $CaCl_2{\cdot}2H_2O$, 30 mM KAc pH 7.5, 15% glycerol, pH brought to 5.8 with HAc), and placed on ice for 1 hr. The cell suspension was centrifuged at low speed (3,000 x g, 4 ^{o}C, 10 min), supernatants discarded and pellets re-suspended with 2 mL of cold RF2 buffer (10 mM MOPS pH 6.8, 10 mM RbCl, 75 mM $CaCl_2{\cdot}2H_20$, 15% glycerol, pH brought to 6.8 with NaOH). Quickly, 200 µL was then aliquoted into pre-chilled tubes, flash frozen with liquid N_2 and stored at –70 ^{o}C. Typically, 100 µL of cells produced transformation efficiencies of ~10^7 colonies/µg DNA.

2.2.2.2 Bacterial transformation

Competent bacteria were transformed as described by Hanahan (1983). Briefly, 100 µL of competent cells were incubated with up to 10 µl of extract (containing the plasmid DNA) and left on ice for 30 min. The bacteria were subjected to a heat shock (37 ^{o}C, 45 sec), immediately placed back on ice for an additional 2 min, followed by the addition of 900 µL of 2YT and incubated at 37 ^{o}C for 1 hr with continuous shaking. A

100 µL aliquot of the reaction was absorbed onto 2YT plates (containing the appropriate antibiotic selection) and incubated overnight at 37 °C.

2.2.3 Small scale plasmid preparation

Small scale plasmid preparations ('minipreps') were prepared using the lysis boiling method outlined by Sambrook *et al.* (1989). Briefly, 1.5 mL of an overnight bacterial culture (containing the plasmid of interest, under antibiotic selection) was centrifuged at high speed (12,000 x g, 4 °C, 2 min), and pellets were re-suspended in 350 µl Lysis buffer (10 mM Tris·HCl pH 8.0, 1 mM EDTA pH 8, 100 mM NaCl, 5%$_{(v/v)}$ Triton X-100) and 30 µl of 10 mg/mL lysozyme (freshly prepared in 10 mM Tris·HCl pH 8.0). The cell suspension was incubated in a boiling water bath for 45 sec, centrifuged (12,000 x g, 4 °C, 10 min), and the supernatant was collected. Plasmid DNA was precipitated with 7.5 M NH_4Ac (200 µL) and isopropanol (700 µL), flash frozen in liquid N_2 and centrifuged at high speed (12,000 x g, 4 °C, 20 min). The pellet was then rinsed with 1 mL 70% ethanol, and re-suspended in TE buffer (10 mM Tris·HCl pH 8.0, 1 mM EDTA, 50 µg/mL RNAseA).

2.2.4 Large scale plasmid preparation

Large scale plasmid preparations were carried out using a commercially available kit (Qiagen), which is based on the alkaline lysis method (Birnboim, 1983). Briefly, an overnight bacterial culture was centrifuged (3,000 x g, 4 °C, 5 min) and subjected to a lysis protocol. Following the lysis, the contents were centrifuged at high speed (10,000 x

g, 4 °C, 30 min) to remove cellular debris. A subsequent centrifugation of the supernatant (10,000 x g, 4 °C, 15 min) was added to remove residual debris. The resulting eluate was combined with 0.7 volumes of isopropanol, centrifuged (10,000 x g, 4 °C, 30 min), and the pellet was rinsed with 70% ethanol and left to dry. The DNA pellet was re-suspended with sterile TE (pH 8.0) and quantified by fluorometry.

2.2.5 DNA electroelution

DNA fragments were recovered from agarose gel slices using an electroeluter (International Biotechnologies Inc.) essentially as instructed by the manufacturer. The gel slice was loaded onto the electroelutor, pre-filled with 0.5x diluted TBE buffer (45 mM TrisBorate, 1 mM EDTA). Current (90V, constant voltage) was passed through the gel slice drawing the negatively charged DNA toward the anode to a V-shaped well containing a salt solution (7.5 M NH_4Ac, 0.1% bromophenol blue) used to retain the DNA eluted from the gel slice. After an appropriate amount of time (estimated from guidelines), 600 µl of salt solution was drawn from the V-shaped well and mixed with 700 µl of isopropanol, flash frozen in liquid N_2, and centrifuged at high speed (12,000 x g, 4 °C, 30 min). The supernatant was carefully discarded and the pellet rinsed with 70% ethanol, dried, and re-suspended in an appropriate amount of TE.

2.2.6 Phenol/chloroform extraction

Phenol/chloroform extractions of nucleic acids were carried out as described in Sambrook *et al.* (1989). Plasmid DNA or RNA samples were brought to 500 µL with

sterile ddH_2O. A mixture of 500 µL of PCI (phenol (buffer-saturated, pH 8) /chloroform / isoamylalcohol; 25:24:1) was added to the sample, the tube was vortexed and then centrifuged at high speed (12,000 x g, RT, 2 min). The upper aqueous layer was removed and retained, and the procedure repeated. The aqueous layer was then mixed with 500 µL CI (Chloroform/ isoamylalcohol; 24:1), vortexed, and centrifuged (12,000 x g, RT, 2 min), with the upper aqueous layer again being removed and retained. A final concentration of 0.3 M NaAc and 2 volumes of absolute ethanol were added to the extract, which was flash frozen, and centrifuged at high speed (12,000 x g, 4 ^{o}C, 30 min). The resulting pellet was rinsed with 95% ethanol, then 70% ethanol, and re-suspended in sterile ddH_2O.

2.2.7 Fluorometry

Plasmid concentrations were quantified using a Hoefer fluorometer (TKO 100) according to the manufacturer's instructions. The machine is adjusted to '0' with TNE buffer (10 mM Tris·HCl (pH 7.4), 1 mM EDTA, 200 mM NaCl, 1 mg/mL Hoechst 33258 dye) and to '1000' with TNE containing 1 µg/µL of a calf-thymus DNA standard (Fisher). Readings from the plasmid samples were then related to the DNA standard.

2.2.8 DNA Sequencing

DNA sequencing was performed using a commercially available kit (T7 Sequencing kit, Pharmacia Biotech) as instructed, which is based on the dideoxy method

developed by Sanger (Sanger *et al.*, 1977). Otherwise, samples were sent to the Central facility at MOBIX (McMaster University) for DNA sequencing.

2.2.9 Two-hybrid system

2.2.9.1 Yeast culture and growth conditions

Protocols outlining the growth and maintenance of yeast were obtained from Ausubel *et al.* (1990). Yeast cultures were grown and maintained using YEP(D) solid plates and liquid cultures with the addition of glucose (2% v/v; from a 20% stock solution). Yeast transformed with plasmid(s) were grown in synthetic complete (SC) media (or on plates), which consist of the minimal essential components required for yeast growth, though lacking amino acids for which the yeast are auxotrophic. In cases where one, or more, of the amino acids were not present in the pre-formed SC media, stock solutions of those amino acids were supplied exogenously to the media at three times their required concentration. The yeast were grown and maintained at 30 ^{o}C.

2.2.9.2 Two-hybrid assay

The two-hybrid assay was developed as a means to investigate protein-protein interactions *in vivo* (Fields and Song, 1989). It was developed based on the observation that transcription factors contain functionally separable, modular domains (eg. DNA binding, transcriptional activation). As shown in Figure 2.2.9.2A, the DNA binding (DB) domain fragment is sufficient to localize the protein to a cis-acting binding site, while the transcriptional activation (TA) domain can activate expression of the target gene.

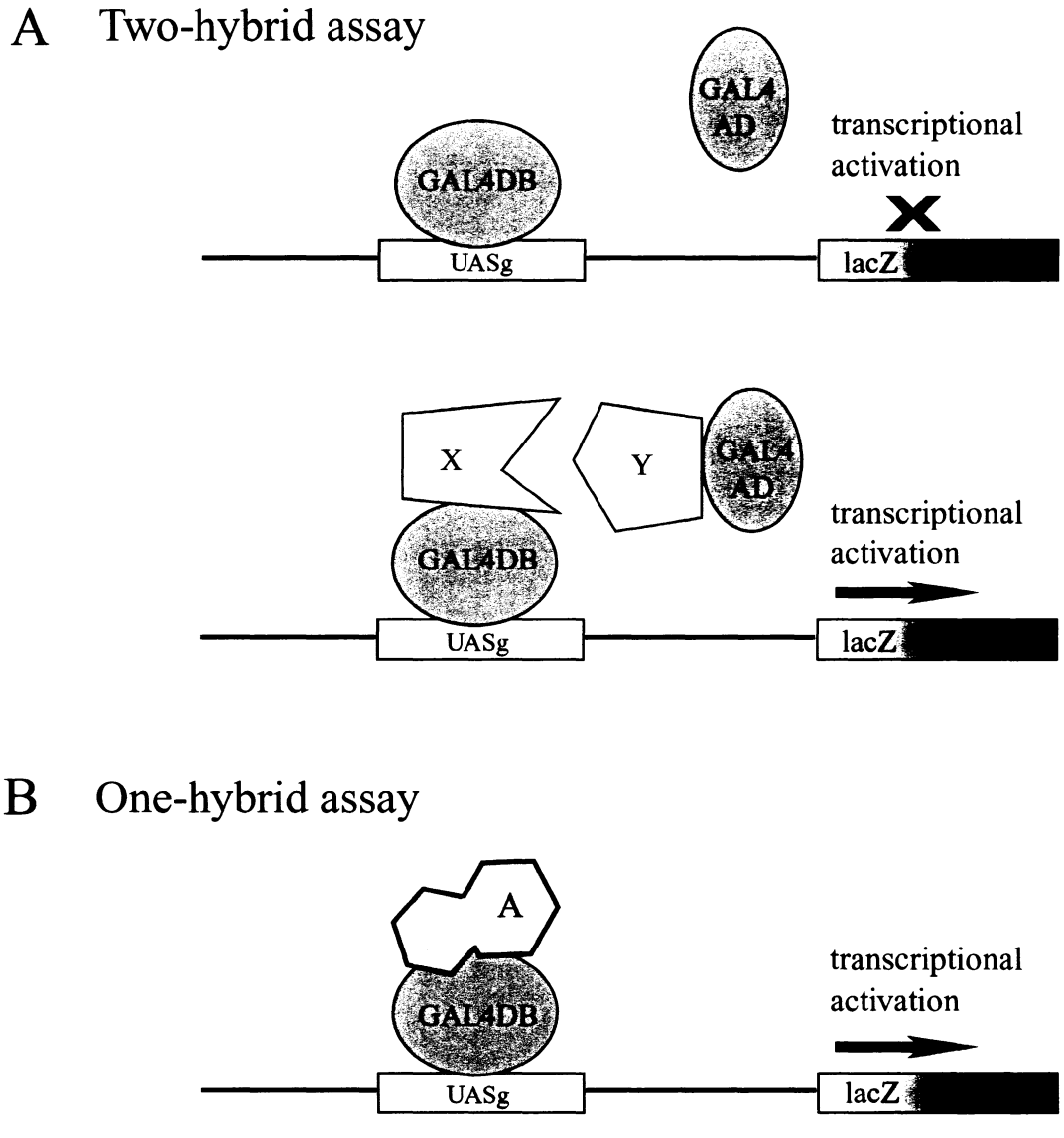

Figure 2.2.9.2. Two-hybrid system. A. Schematic diagram of the two-hybrid assay; used to detect protein-protein interactions in yeast (Fields and Song, 1989). Proteins 'X' and 'Y' are fused to the GAL4 DNA binding domain and GAL4 activation domain, respectively. A non-covalent interaction between X and Y results in functional reconstitution of the GAL4 transactivator, resulting in expression of the lacZ reporter gene. UASg; upstream activation sequence containing GAL4 responsive elements. **B.** One-hybrid system. Activation of the lacZ reporter can also occur in the absence of the GAL4 activation domain construct; the protein fused to the GAL4 DNA binding domain ('A') possesses an activation domain.

However, although these domains can function separately, expression of the downstream reporter gene requires that both the DNA binding and transcriptional activation domains are reconstituted through non-covalent interactions of protein fusions of these respective domains, since the activation domain has to localized to the corresponding upstream promoter sequence. Both the presence and relative strength of an interaction can be inferred from the amount of reporter gene expression that is quantified (Estojak *et al.*, 1995). Alternatively, if the protein (or polypeptide) tethered to the DNA binding construct itself has a functional transcriptional activation domain, expression of the reporter gene can also occur through a single fusion (Figure 2.2.9.2B).

2.2.9.2.1 Qualitative yeast overlay assay

A qualitative assessment of the two-hybrid assay can be obtained through a yeast overlay assay, which is used as a rapid means for determining an interaction (Bohen and Yamamoto, 1993). It is an *in situ* reaction that monitors the cleavage of a chromogenic substrate (Xgal) by β-galactosidase, on immobilized yeast colonies, resulting in blue colour formation. Briefly, agarose (0.5% w/v) was dissolved in an appropriate amount of 0.5 M $NaPO_4$ (pH 7.0) buffer, allowed to cool slightly, and mixed with SDS (0.1%), DMF (2%), and Xgal (0.02% w/v). The molten mixture was placed directly onto streaked yeast colonies, and placed at 37 ^{o}C. Blue colour formation is typically seen in less than 30 min, but plates were also left overnight in order to detect relatively weak interactions. Differences relating to the intensity of the blue colour that formed within this assay were readily distinguished.

2.2.9.2.2 Quantitative yeast β-galactosidase assay

Quantitative β-galactosidase assays were also used to measure the relative differences in reporter gene expression within the two-hybrid assay, permitting the use of numerical values as an indicator of the relative strength of an interaction. These assays were carried out as outlined in Ausubel *et al.* (1990). Briefly, yeast cultures (5 mL) were grown (in their appropriate auxotrophic media) overnight to saturation. The following day, it was diluted into fresh media (1:5 dilution) and grown to late log phase (OD_{600} ~ 1.0), and harvested by centrifugation at high speed (12,000 x g, 10 sec). The supernatant was removed and replaced with 1 mL Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, adjusted to pH 7.0). Three successive cycles of freeze/thaw (liquid N_2/30 ^oC water bath) were used to lyse the yeast, and the resulting debris was removed by centrifugation (12,000 x g, 5 min). β-galactosidase activity was initiated with the addition of 400 μL ONPG (4 g/mL; made in Z buffer), with the reaction proceeding between 0.5 to 4hr. ONPG is a chromogenic substrate that produces a yellow colour when cleaved, thus allowing for absorbance measurements to monitor colour formation at OD_{420}. After manually observing an adequate amount of yellow colour formation (between 0.2 and 0.7 absorbance units at OD_{420}), the reaction was stopped with (200 μL) 1 M $NaHCO_3$. Any background debris that contributed to the reading at OD_{420} was accounted for with an additional absorbance reading at OD_{550}. β-galactosidase activity units were determined using the formula:

$$U = 1000 [OD_{420} - 1.75*(OD_{550})]/ t*v*OD_{600}$$

where U = arbitrary units,
OD_{420} = O-nitrophenyl product
OD_{550} = background debris
OD_{600} = cell density
t = time (min)
v = volume (mL)

2.2.9.3 Yeast transformation

Yeast transformations were based on an optimized LiAc protocol developed by Elble (1992). Briefly, yeast cultures were grown to saturation, centrifuged at high speed (12,000 x g, 10 sec), and the supernatant discarded. The transformation reaction(s) were added directly to the yeast pellet and consisted of up to 0.5 µg plasmid, 10 µg ssDNA, followed by the addition of 0.5 mL of plate solution (40% w/v polyethyleneglycol (PEG_{3350}), 100 mM LiAc, 10 mM Tris·HCl pH 7.5, 1 mM EDTA). The tubes were briefly vortexed and left overnight at RT. The following day, the yeast were harvested (12,000 x g, 10 sec), and all but 100 µL of the plate solution was removed. The yeast pellet was then re-suspended using the remaining plate solution, and spread on synthetic complete plates lacking the appropriate amino acid(s). Transformed colonies appeared after 2-3 days (at 30 °C).

2.2.9.4 Protein isolation from yeast

The protocol for the isolation of protein from yeast is outlined in Ausubel *et al.* (1990). Briefly, saturated yeast cultures (5 mL) were centrifuged (3,000 x g, 4 °C, 5 min),

with the corresponding pellet washed twice with cold PBS (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl) and re-suspended in RIPA buffer (50 mM Tris·HCl pH 7.2, 150 mM NaCl, 0.1% SDS, 0.5% DOC, 0.5 mM PMSF, protease inhibitor cocktail). The extract was then transferred to a microfuge tube and an equal volume of acid-washed glass beads (425-600 μ, Sigma # 68772) was added and then vortexed for intervals of 8 x 30 sec, with intervening segments on ice. The resulting debris was then removed by high speed centrifugation (12,000 x g, 4 ^{o}C, 10 min), and the supernatant was transferred to a fresh tube. The contents were either stored at –70 ^{o}C, or prepared for SDS-PAGE and Western blot analysis following quantification of the protein concentration (see 2.13.1).

2.2.10 In vitro binding assays

2.2.10.1 In vitro transcription/translation reactions

'Coupled' or 'uncoupled' *in vitro* transcription/translation reactions using rabbit reticulocyte lysates were carried out according to manufacturer's instructions (Promega). The term 'coupled' refers to the presence of both transcription and translation phases within a single reaction mixture, made available through a commercially available kit (Promega). Alternatively, 'uncoupled' reactions are generated from separate transcription and translation reactions. Transcription reactions with 1 μg plasmid, containing transcription buffer, 10 mM ATP, CTP, TTP, 1 mM GTP, 100 mM DTT, 60 U RNasin, 45 U RNA POL (SP6 or T7), were incubated for 2hr at 37 ^{o}C. The RNA was then precipitated using phenol/chloroform extraction, and flash frozen in liquid N_2 and

centrifuged (12,000 x g, 4 °C, 20 min). The resulting pellet was re-dissolved with 25 µL sterile ddH_20 containing 60 U RNasin. Tubes containing 2 µL aliquots are then used for translation reactions. Translation reaction mixtures are provided separately, and used as instructed (Promega). The plasmids for this procedure were prepared by phenol/chloroform extraction, and concentration(s) determined by fluorometry. Proteins were synthesized in the presence of a radio-labeled amino acid (L-[^{35}S]-methionine), allowing for visualization by autoradiography.

2.2.10.2 Expression and purification of bacterial fusions

pMAL-2c (New England Biolabs) is an IPTG-inducible bacterial expression plasmid utilizing the cytosolic maltose-binding protein (MBP) as an amino-terminal fusion to mutants constructed in VP16 (Shaw *et al.*, 1995). An overnight culture (5 mL) of each construct was used to inoculate a fresh 50 mL flask and grown to an OD_{600} of 0.6, and induced with 1 mM IPTG for 2hr at 30 °C. Cells were then harvested (3,000 x g, 4 °C, 5 min) and re-suspended in 4mL of column buffer (20 mM Tris·HCl pH 7.4, 1 mM EDTA, 200 mM NaCl, 1 mM DTT, 1 mM PMSF). The cells were lysed using a probe sonicator (kindly provided by Dr. R. Gupta, McMaster University) for eight times 30 sec intervals at 4 °C, with intervening segments on ice. The extracts were resolved by centrifugation at high speed (12,000 x g, 4 °C, 10 min). The resulting supernatant was mixed with 50 µl amylose beads (maltose polymer complexed to a matrix, 1:1 slurry in PBS) for 4 hr (4 °C) and washed with column buffer. Expression levels were then compared by SDS-PAGE and staining with Coomassie brilliant blue.

The expression and purification of the GST fusion proteins was similar to the MBP fusions. Briefly, the GST constructs were transformed into $BL21_{(DE3)}$ bacterial cells, grown to an OD_{600} of 0.6, and induced for 90 min at 37 °C with 0.1 mM IPTG. Bacterial pellets were re-suspended using NETN buffer (20 mM Tris·HCl pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM PMSF, protease inhibitor cocktail) and subsequently purified using glutathione-Sepharose 4B resin (also prepared as a 1:1 slurry in PBS), or a column (GSTrap FF; Amersham) as instructed by the manufacturer.

2.2.10.3 In vitro pull-down assays

Pull-down binding assays were conducted to detect specific protein-protein interactions *in vitro*. The binding assays utilizing the MBP fusions have already been described in Shaw *et al.* (1995), and are analogous to the protocol involving the GST fusions. GST fusion proteins were purified by mixing an appropriate amount of bacterial extract (as determined by the amount of fusion protein following the purification, ~1 μg) with 50 μl (1:1 beads/PBS) of glutathione-Sepharose 4B beads, for 4hr at 4 °C on a rocking platform. The beads were harvested by low speed centrifugation (750 x g, 1 min) and washed with IPAB (20 mM HEPES pH 7.9, 150 mM KCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 0.1% NP-40, 1 mM PMSF, protease inhibitor cocktail). The beads were then re-suspended with 250 μL IPAB containing 20 mg/mL BSA and an appropriate amount of *in vitro* translated, radio-labeled protein (as indicated in the figure legends), and incubated for 2 hr (4°C) with continuous rocking. A specific interaction results from the translated protein remaining bound to the beads via the fusion protein. The beads

were isolated by low-speed centrifugation (750 x g, 1 min), and the supernatant discarded. Washes were repeated 3x with 1 mL IPAB (containing 20 mg/mL BSA), and twice with 1 mL IPAB. Following the washes, 2x SDS-PAGE loading buffer was added to the mixture in order to elute the bound proteins from the beads, which were separated by high-speed centrifugation (12,000 x g, 1 min). The supernatant was then run on SDS-PAGE and visualized by autoradiography.

2.2.10.4 Co-immunoprecipitation

Co-immunoprecipitation assays were essentially conducted as previously described (Meertens *et al.*, 1998). Briefly, cells were transiently transfected with the appropriate plasmid(s) and harvested with NP40 buffer (50 mM Tris·HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM PMSF, protease inhibitors). Extracts containing 500 µg of total cellular protein were pre-cleared with 25 µL (1:1 slurry) proteinG-Sepharose for 4 hr (continuous rocking) at 4 °C. The beads were removed by centrifugation (12,000 x g, 4 °C, 1 min) and the supernatant collected. The antibody was added to the supernatant for 4-16 hr (4 °C), followed by 25 µL of proteinG-Sepharose beads for 4 hr (4 °C). The mixture was then harvested at low speed (750 x g, 1 min), washed three times with 1 mL of IP1 (50 mM Tris·HCl pH 7.5, 500 mM NaCl, 0.1% NP-40, 0.05% DOC), and twice more with IP2 (50 mM Tris·HCl pH 7.5, 0.1% NP-40, 0.05% DOC). Following the washes, the beads were re-suspended in SDS loading buffer, then resolved on SDS-PAGE and detected by Western blot analysis.

2.2.11 Cell culture

2.2.11.1 Cell culture maintenance

Cell lines were maintained with Dulbecco's Modified Eagle Media (DMEM) supplemented with 1% penicillin/streptomycin, 1% L-glutamine, and either 10% fetal bovine serum (FBS) or 10% calf serum (CS) as indicated for each cell line (refer to section 2.1.3.3). The cells were maintained on 10cm plates at the appropriate temperature and at 5% CO_2. Confluent cells were split by removing media and rinsing the cells with pre-warmed (37 ^{o}C) PBS, followed by the addition of 1 mL of 1x trypsin solution, and left until the cells started detaching from the plate (typically ~1 min). Upon removal of the trypsin solution, the plates were subjected to mechanical agitation and re-suspended in 10 mL of growth media, and split to an appropriate dilution.

2.2.11.2 Transient transfections

All transfections were carried out on 6-well plates. The day preceding the transfection experiment, each plate well was seeded with approximately 5 x 10^6 cells, so as to reach 70-80% confluence the following day. Lipofectamine reagent (Invitrogen) was used for all transfections as instructed by the manufacturer, with the following optimizations: A total of (up to) 2 μg of total plasmid DNA was added to 100 μL DMEM which was then mixed with an additional 100 μL of DMEM containing 4 μL of Lipofectamine and left for 30 min at RT. After the addition of 800 μL of DMEM to the mixture and placement onto each well, the cells were incubated with the transfection

mixtures for 4 hr (37 ^{0}C). Following the incubation, the transfection mixture was replaced with fresh growth media, and again after 24 hr. The cells were harvested after 40-48 hr.

2.2.11.3 Cell Lysis

2.2.11.3.1 Preparation of cells for Western blot

In order to prepare mammalian cell extracts for Western blot analysis, cells were washed three times with cold PBS and harvested with NP-40 buffer (50 mM Tris·HCl pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM PMSF, protease inhibitor cocktail), transferred to a microfuge tube and placed on ice. The tubes were subsequently vortexed at maximum speed for 3 x 10 sec, and sonicated for 3 x 10 sec, followed by centrifugation (12,000 x g, 4 ^{o}C, 5 min) to remove cellular debris. The total protein within each extract was then quantified (see 2.13.1) prior to the addition of SDS loading buffer.

2.2.11.3.2 Preparation of cells for reporter gene analysis

Transiently transfected cells used for reporter gene analysis were washed three times with cold PBS, and incubated with 0.4 mL Reporter lysis buffer (RLB; Promega). The extracts (buffer and cells) were removed from the plate with a cell scraper, placed in a microfuge tube and kept on ice. Samples were vortexed 2 x 10 sec, and centrifuged at high speed (12,000 x g, 4 ^{o}C, 2 min), with the resulting supernatant being placed in a new tube and stored at –70 ^{o}C. In all cases, a *luciferase* gene was used within reporter constructs to monitor activity (deWet *et al.*, 1987), and luciferase assays were carried out with a luminometer (Lumat LB9507; EG&G Berthold) according to manufacturer's

instructions. The amount of extract utilized corresponded to readings within the linear activity range of the machine. Briefly, 10 µl of extract was placed in a 13 x 100 mm plastic tube (Sarstedt) and placed within the luminometer, programmed to receive 100 µl of luciferin reagent (470 µM luciferin, 270 µM coenzymeA, 530 µM ATP, 33 µM DTT, 20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA). The resulting fluorescence, in the form of RLUs (relative light units), was normalized to the total protein concentration (within the extract) for all experiments. The error within each data set was determined by calculating the standard deviation.

2.2.12 Virus Assays

2.2.12.1 Virus stocks and maintenance

Virus stocks were amplified from confluent cells that were infected with an MOI of 0.1, using a minimal volume of DMEM for 1-2 hr. The media was then removed and replaced with growth-serum containing media. Where necessary, viruses were grown in the presence of 3 mM hexamethylene bisacetamide (HMBA) in order to boost the viral titer (Preston and McFarlane, 1998). Following the appearance of cytopathic effects (2-4 days), the cells were harvested by centrifugation (750 x g, 4 ^{o}C, 5 min) and the pellet was re-suspended in 10 mL of growth-containing media. The re-suspended cells were subjected to three cycles of freeze/thaw using liquid N_2, and cell debris was removed by centrifugation (750 x g, 4 ^{o}C, 5 min). The supernatant was then aliquoted into cryovials for storage at -70 ^{o}C. Viral titres were determined by plaque assay.

2.2.12.2 Plaque assays

Serial dilutions of viral stocks from infected cells were made in DMEM, with 500 µL being used to infect confluent cells in 6-well plates for 2 hr at 37 ^{o}C. Following absorption, the viral inoculum was replaced with growth media supplemented with 0.05% human gamma globulin (HGG). After 48-72 hr, the media was removed and the cells were fixed and stained with 0.1% crystal violet (in ethanol solution) at RT for 30 min. The stain was then removed and the plates were rinsed with water and left to dry, and counted over a range of dilutions to yield the number of plaque forming units (PFU).

2.2.12.3 Virus complementation assays

A virus complementation assay was developed in order to investigate the ability of a mutant of VP16 that is compromised in its interaction with vhs to rescue the growth of VP16-null viruses 8MA or 8MAΔSma. It was hypothesized that unregulated vhs activity (through the lack of VP16 binding) would result in the destruction of viral genes and prevent a productive infection, resulting in an inability to isolate the corresponding virus with the VP16 mutant. Similar assays have also been used for other HSV proteins (Rice and Lam, 1994, Wanas *et al.*, 1999, Whitbeck *et al.*, 1999). Therefore, based upon the ability of 8MA and 8MAΔSma to propagate on a cell line that provides VP16 in *trans*, it was reasoned that this could be further extended to VP16 that was expressed in transiently transfected cells. By comparing the wild-type and mutant VP16 transfected constructs within this context, the relative amount of complementation required for sustaining a productive infection can be measured by the number of plaques that are

formed. Consequently, the PFUs were used as an indicator for the amount of virus growth. The VP16 complementation assay utilized here was carried out in two-steps: First, Vero cells (non-complementing for 8MA or 8MAΔSma) were transfected with 2 μg of VP16-expressing plasmids (or the corresponding vector control). After 24 hr, media was removed and the cells were infected with virus at an MOI of 5. The infected cells were subsequently left for 48-72 hr, until cytopathic effects were visible. Virus-containing extracts were collected from the cells by scraping into the existing supernatant and collecting the entire extract into 15 mL Falcon polypropylene tubes and placing them at –20 °C. In the second phase, the virus-containing extracts were used to infect 16-8 cells. Since the first segment of the complementation assay involved utilizing VP16 generated from transiently transfected plasmids, subsequent rounds of replication would lack the ability to propagate in a non-complementing cell line, and thus form plaques, since VP16 was not be present. Therefore, only infectious virions that are present in the extract can grow in the VP16-expressing 16-8 cells. The extracts generated from the non-complementing cells were subjected to three rounds of freeze/thaw cycles (using liquid N_2) in order to infect a confluent monolayer of 16-8 cells. Growth media was then removed from confluent 16-8 cells, and 0.5 mL of the prepared extract was placed on the cells for 1 hr with occasional rocking, followed by removal of the media and replacement with fresh media (containing 0.05% HGG). The cells were incubated at 37 °C for 48-72 hr until plaques appear, after which the viral titres were calculated as previously described.

2.13 Western blots

2.13.1 Protein concentration

Protein concentration was quantified through a BioRad protein assay kit, based on the Bradford method (Bradford, 1976). A BSA standard curve was generated and used as a reference for the amount of protein within extracts.

2.13.2 Western blot analysis

Equivalent amounts of total protein within extracts were prepared in SDS loading buffer and loaded onto a 0.75 mm-spaced SDS-PAGE (Laemmli, 1970), along with pre-stained molecular weight protein markers (high range MWM; GibcoBRL). An appropriate amount of nitrocellulose (Amersham) was obtained, pre-incubated in ddH_2O, and then in transfer buffer (25 mM Tris, 150 mM glycine, 20% (v/v) methanol). The nitrocellulose was then placed with the SDS-PAGE gel in a protein transfer apparatus (BioRad) and run at fixed amperage (100 mA), for 16 hr at RT. Following the transfer, the nitrocellulose was placed in a blocking solution of 5% non-fat milk powder (Carnation) in TBST buffer (10 mM Tris·HCl pH 7.5, 150 mM NaCl, 0.05% Tween20), for 1 hr with continuous rocking. Subsequently, an appropriate amount of primary antibody (diluted in TBST, containing 1% milk fat) was added to the nitrocellulose, for 1 hr at RT, followed by three washes for 15 min with TBST. Secondary antibody (appropriate amount diluted in TBST, containing 1% milk fat), was then added for 45 min at RT, followed by three washes for 15 min using TBST and an additional 5 min

with TBS (10 mM Tris·HCl pH 7.5, 150 mM NaCl). Antibody detection was carried out using a commercially available ECL kit (Amersham) as instructed.

2.2.14 Protein-DNA interaction

2.2.14.1 Preparation of oligonucleotide probe

Radioactively labeled oligonucleotide DNA probes for use in electrophoretic mobility shift assays were prepared as previously described (Miyata *et al.*, 1996). Briefly, 20 pmol of complementary oligonucleotide strands were annealed, and engineered to contain 5' overhang segments that could subsequently be filled in with the Klenow fragment of DNA polymerase I in a reaction containing 33 μM each dTTP, dCTP and dGTP, and 100 μCi α-^{32}P-dATP. The reaction was allowed to proceed for 15 min (RT) and the volume was then brought to 100 μL with TE (pH 8.0). Unincorporated nucleotides were separated from the labeled DNA probe by elution through a Sephadex G50 spin column by low speed centrifugation (2,000 x g, 4 min) (Sambrook *et al.*, 1989). The Sephadex G50 columns were formed with 1 mL of Sephadex G50 resin (Pharmacia) equilibrated in TE (pH 8.0) and placed in a 1cc syringe plugged with sianylized glass wool, and washed twice with 100 μL TE (pH 8.0) via centrifugation (2,000 x g, 4 min). The radioactivity incorporated into the probe was measured by liquid scintillation spectrometry. The specific activity of probe generally exceeded $5x10^6$ cpm/pmol.

2.2.14.2 Electrophoretic mobility shift assay (EMSA)

The electrophoretic mobility shift assay used here to investigate protein-DNA interactions *in vitro* was based on the protocol developed by Garner and Revzin (1981) and Fried and Crothers (1981), and refined specifically with respect to VIC by Werstuck and Capone (1989a). Essentially, 20 µL binding reactions contained buffer D (20 mM Hepes pH 7.9, 50 mM KCl, 0.5 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 0.05% NP-40), 10 µg BSA, 4 µg DNA competitor (2:1 ssDNA: poly (dIdC·dIdC)), and 0.2 pmol radio-labeled probe. Reactions were maintained for 25 min at 28 ^{o}C, and terminated with 2x gel loading buffer, and resolved on 4% PAGE. Gels were pre-run for 4hr (230 V, 4 ^{o}C) in 0.25 x TBE. The gels were then dried and autoradiographed in order to visualize complex formation.

Chapter 3. Characterization of the VP16/vhs interaction

HSV1 VP16 is involved in at least two essential roles in viral pathogenesis: i) transcriptional activation of viral IE genes, and ii) virion morphogenesis, in which an interaction with another viral protein, the virion host-shutoff protein (vhs) has been shown to play a role (Lam *et al.*, 1996). This chapter presents the results of a structural and functional analysis of the interaction between VP16 and vhs.

At the start of this thesis, work from our laboratory in conjunction with Dr. Smiley's group, had shown that viral proteins VP16 and vhs interact within infected cells (Smibert *et al.*, 1994). The initial mutational studies indicated that the regions important for binding to vhs overlapped with other known interactions of VP16, which implied the potential for 'crosstalk' between the various interacting partners. Moreover, although it was hypothesized that VP16 would regulate vhs function due to a vast excess of VP16 over vhs within the incoming virion, the biological relevance of this interaction had not yet been elucidated (Smibert *et al.*, 1994). Therefore, the goal of my research was to differentiate vhs binding from other aspects of VP16 function, including the various protein-protein and protein-DNA interactions within the VP16-induced complex (VIC), as a means to better understand aspects of VP16 binding to vhs. In addition, the novel interaction between VP16 and vhs also served as an opportunity to explore a potential role for VP16 in virus assembly and pathogenesis.

Mutagenesis experiments were used in order to isolate a specific region of importance within VP16 with respect to its interaction with vhs. Since it was not clear at the time if the various binding regions within VP16 would overlap, we expanded upon

previous mutagenesis work to localize the binding domain for vhs in greater detail. We surmised that this strategy, which would eventually lead to the generation of site-specific mutants, would allow us to differentiate between competing functional aspects of VP16. In addition, we also sought to better understand the relative importance or biological relevance of the interaction with vhs within the context of HSV. At the time these experiments were undertaken, structural information with regard to VP16 did not exist, so residues (or regions) within VP16 required for vhs binding, VIC formation, and the viral assembly were deduced based on the primary sequence of the protein. The results presented here provide a detailed mutational analysis of the interaction between VP16 and vhs, further delineate aspects of VP16 structure and function, and show that the interaction between VP16 and vhs is critical in maintaining the viral lytic cycle.

3.1 Role of selective positively charged residues in binding to vhs

Since the initial mutational profile of the residues involved in VP16 binding to vhs coincided with those required for VIC assembly, it was possible that the interactions required for VIC assembly could overlap those required to bind vhs (Smibert et al., 1994). The mutational data compiled to that point, had indicated the potential for some overlap involving interactions within VP16. Given the lack of structural or functional data with respect to the role of single amino acid residues within VP16 in mediating protein and DNA interactions, studies were undertaken in order to differentiate between these various interactions within a relatively small region of the protein (Shaw et al., 1995). The various site-specific mutants that were constructed for these experiments were

chosen for three reasons: i) VP16 appears to be enriched for positively charged residues, as in its orthologs, suggesting that these residues may be involved in mediating various interactions (Moriuchi *et al.*, 1993, Purewal *et al.*, 1994, Elliott and O'Hare, 1995), ii) Charged residues are more likely to appear on protein surfaces and thus may be involved in at least some aspect of an interaction (Jones and Thornton, 1996, Sheinerman *et al.*, 2000), and iii) The regions encompassing residues 141-178 and 335-370 were previously shown to be important for both VIC assembly and binding to vhs (Werstuck and Capone, 1989a, Werstuck and Capone, 1989b, Smibert *et al.*, 1994). This study was among the first to utilize site-directed mutagenesis to investigate the role of specific residues in contributing to interactions by VP16 (Hayes *et al.*, 1993, Wu *et al.*, 1994, Shaw *et al.*, 1995).

Site-specific mutants corresponding to positively charged residues within VP16 (shown schematically in Figure 3.1A) were obtained from P. Shaw (Shaw *et al.*, 1995), within the context of the amino-terminal 404 residues of VP16 and were subsequently cloned as fusions to the GAL4 AD domain. These plasmids were then transformed into PCY2, harboring a GAL4 DNA binding domain fusion of the Apa/Sma fragment (spanning residues 179-344) of vhs in the yeast two-hybrid system, in order to assess the *in vivo* binding of each of the VP16 site-directed mutants with vhs. This segment of vhs encodes a polypeptide that has previously been shown to be sufficient to bind VP16 (Smibert *et al.*, 1994). As demonstrated in Table 3.1, all of the mutants interact with vhs within the two-hybrid system, as determined by β-galacatosidase overlay assays. In addition, the VP16 mutants were also used to assess binding to the amino-terminal 380

Table 3.1. Altered charged residues in VP16 retain binding to vhs. [a]

Plasmids	β-galactosidase activity		
	DB	DB-vhs	DB-HCF
AD	-	-	-
AD-VP404	-	+	+
AD-VP404(R155A)	-	+	+
AD-VP404(R162A)	-	+	+
AD-VP404(R164A)	-	+	+
AD-VP404(R169A)	-	+	+
AD-VP404(C176A)	-	+	+
AD-VP404(R360A)	-	+	+
AD-VP404(R366A)	-	+	+
AD-VP404(R368A)	-	+	+
AD-VP404(K370A)	-	+	+

a. Two-hybrid assay of VP16 harbouring the indicated site-specific mutants fused to the GAL4 activation domain, and either vhs or HCF-1, in yeast PCY2. DBvhs; the vhs (Apa/Sma fragment) fused to the GAL4 DNA binding domain, DB-HCF; the amino-terminal 380 residues of HCF-1 fused to the GAL4 DNA binding domain. Activity was determined by overlay assay. '+' indicates an interaction (blue colour formation in < 30 min), while '-' resulted from no colour formation after an overnight incubation.

residues of HCF-1, in which there was no difference in binding observed with any of the mutants (Table 3.1). This was somewhat unexpected since several of the mutants reported in Shaw *et al.* (1995) were unable to bind VCAF-1 (HCF-1) using EMSA based microaffinity binding assays. The apparent differences between the two-hybrid and the *in vitro* binding assays may be due to differences in the binding stringency of these two methods (Estojak *et al.*, 1995).

In vitro pull down binding assays were also used to assess binding between each of the mutant VP16 proteins and vhs. Maltose binding protein (MBP)-VP16 fusions consisting of the Sal fragment of VP16 (residues 5-411), were used in pull-down assays with an *in vitro* translated, radio-labeled vhs protein in order to demonstrate specific binding between the mutant VP16 proteins and vhs. As detailed in the methods section, the fusions were bound to beads and incubated with radio-labeled vhs. As with the two-hybrid results, the radio-labeled vhs was retained with each of the mutant VP16 fusion proteins, but not with the MBP control (Figure 3.1C). The expression profile for the MBP-VP16 constructs (Figure 3.1B) confirms that equivalent amounts of each fusion were utilized in the binding assays. The results indicate that all of the mutants constructed here, were able to interact with vhs *in vitro* with pull-down assays and *in vivo* using the two-hybrid system, though several of these mutants were variously affected for binding to DNA, Oct-1/DNA and VCAF-1 (Shaw *et al.*, 1995). As a result, we were able to further uncouple vhs binding from protein and DNA interactions required for VIC assembly.

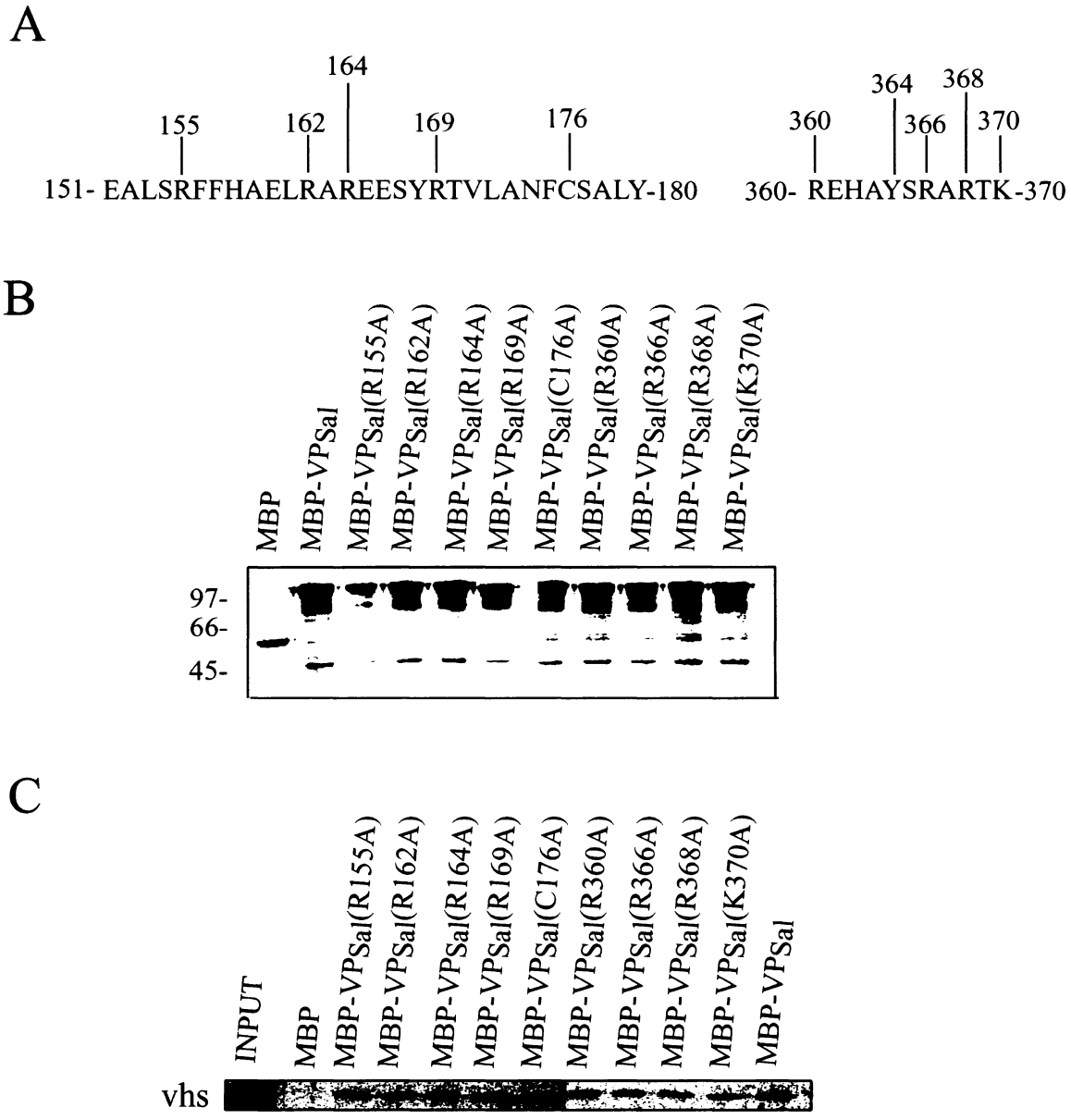

Figure 3.1. Charged residues in VP16 that affect VIC still bind vhs. A. Schematic of VP16 residues changed to alanine. **B.** $VP16_{Sal}$(encompassing residues 5-411 and the various indicated mutants thereof) was fused to the maltose binding protein (MBP) for *in vitro* binding assays. Fusions were purified and analyzed by SDS-PAGE and Coomassie staining. Molecular weight markers are indicated to the left (in kDa). **C.** *In vitro* pull-down assays with MBP-VP16 fusions and *in vitro* translated, ^{35}S-labeled vhs. The input lane corresponds to the total used for binding.

3.1.1 Discussion

While site-directed mutagenesis was useful in further establishing differences in VIC assembly and vhs binding, these experiments do not preclude the possibility that vhs may have an overlapping binding site within VP16 to block VIC formation through, for instance, steric hindrance. Given that the determinants for VIC formation within VP16 are closely interspersed within a small region(s) of the primary sequence of VP16, additional experiments are required to elucidate those differences. Other studies have also shown that the protein and DNA interactions mediated by VP16 are closely intertwined, with adjacent residues possessing binding determinants for distinct functions (Shaw *et al.*, 1995, Lai and Herr, 1997). A common binding element for vhs within VP16 that is also critical for VIC and transcriptional activation, therefore, can also not be ruled out. In summary, the fundamental theme of the experiments presented here, and by others, is that site-directed mutagenesis will be required to uncover the importance of specific interactions maintained by VP16. In addition, although the mutants utilized in the experiments described here did not abrogate binding to vhs, they were nevertheless useful in demonstrating that there likely was not a gross disruption in protein structure, offering additional evidence that these mutants were structurally and functionally informative. Subsequent experiments within this thesis sought to precisely define a region within VP16 that is necessary for vhs binding.

3.2 Localization of a vhs binding region within VP16

An understanding of the importance of the interaction between vhs and VP16, in the context of HSV pathogenesis, requires that residues involved in the interaction be mapped in sufficient detail to differentiate between the specific functions of VP16. An initial characterization of the interaction by deletion mutagenesis determined the regions between residues 141 to 178 and 335 to 369 to be important (Smibert *et al.*, 1994). However, those regions were also shown to be important for mediating various aspects of protein-protein and protein-DNA interactions within VIC (Werstuck *et al.*, 1989a). Since the localization of specific residues were deemed to be critical to promoting our understanding of the functional relevance of this interaction, additional detail in this regard was sought through further carboxyl-terminal deletion mutagenesis. It also appeared likely that the alteration of single amino acid residues would eventually be required to adequately map the residues involved in the interaction, since a relatively small region was found to mediate multiple interactions (Shaw *et al.*, 1995, Lai and Herr, 1997). Deletion mutants from the carboxyl-terminus of VP16 where chosen, as opposed to internal deletions since: i) In the absence of structural data, and without knowing the structure and function relationships between the various regions of VP16, carboxyl-terminal deletions were thought to be less disruptive to overall protein structure (Poon and Roizman, 1995), and ii) the majority of residues found to be important for protein-protein interactions in VIC had already been shown to lie between residues 335 to 388 using site-specific mutants (Hayes and O'Hare, 1993, Wu *et al.*, 1994, Shaw *et al.*, 1995, Lai and Herr, 1997). Therefore, the goal of these experiments was to delineate residues

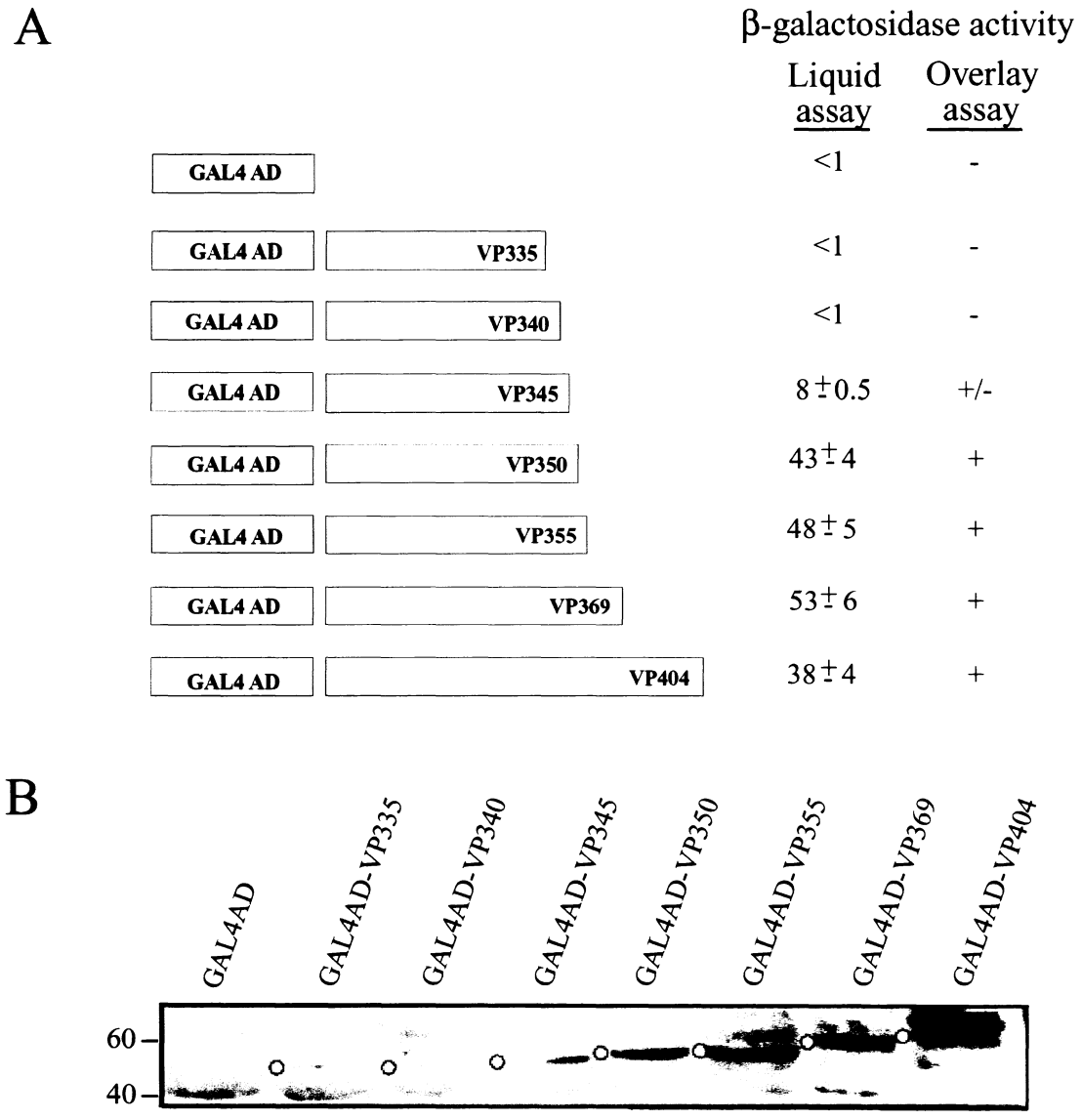

Figure 3.2. Localization of a vhs binding domain in VP16 using the two-hybrid assay. Schematic representation of VP16 carboxyl-terminal deletion mutants fused to the GAL4 activation domain used in a two-hybrid assay with the GAL4 DNA binding domain vhs fusion. β-galactosidase reporter expression was assessed by quantitative (liquid) and qualitative (overlay) assays. Values for liquid assays were obtained from duplicates of three independent isolates (+/- SD). Overlay assay activities are denoted by '+' for an interaction, '+/-' for a (relatively) weak interaction, and '-' for no interaction; as judged by the amount of blue colour formation. **B.** Western blot of extracts containing GAL4AD-VP16 constructs. Fusions were detected with a VP16 polyclonal antibody directed against the $VP16_{Sal}$ fragment corresponding to residues 5-411 (diluted 1:1000). Molecular weight markers are represented schematically (in kDa). Circles are present to clarify the position of each fusion.

important for binding vhs from other VP16 interactions, using additional deletions, such that single amino acid mutagenesis could subsequently be applied.

The two-hybrid assay has been used extensively over the last decade to isolate and characterize protein-protein interactions, in addition to employing mutagenesis strategies to localize important binding regions (Toby and Golemis, 2001). We sought to isolate a minimal region within VP16 that was sufficient to interact with vhs, by further expanding upon previous mutagenesis studies, through the creation of carboxyl-terminal deletion mutants within this context. Mutants were generated at five amino acid intervals at residue 355, through to 335, and were fused to the GAL4 activation domain. VP16 deletions terminating at residues 404 and 369 were previously shown to bind vhs, while a further deletion to amino acid 335 did not (Smibert *et al.*, 1994). As shown in Figure 3.2, qualitative and quantitative β-galactosidase assays using the GAL4 activation domain fusions of VP16 deletions, terminating at residues 355, 350 and 345, displayed binding to vhs when co-transformed with a GAL4 DB binding domain vhs construct in the two-hybrid system, though the deletions to residues 340 and 335 did not. The results indicate that residues between 340-345 were critical for binding; though a binding determinant lies between residues 345 to 350, since those residues restore the level of binding relative to the wild-type protein (compare activities of 345, 350 and 404; Figure 3.2). Therefore, while the region between residues 340 and 350 contains a binding determinant for an interaction with vhs, within the two-hybrid system, and amino acids 1- 345 of VP16 are sufficient to interact with vhs. A Western blot was also conducted to ensure that the fusions were being expressed to similar levels. The constructs corresponding to deletions

at residues 404, 369, 355 and 350 show equivalent levels of expression, while those of 345, 340 and 335 appear to be diminished (Figure 3.2). The differences in the level of binding between mutants of VP16 terminating at residues 340, 345 and 350 and vhs, may therefore be due to differences in their expression levels or relative stability of the VP16 deletions. Thus, the differences in expression levels have to be taken into account in order to infer differences in binding. It is not clear if the deletions corresponding VP335, VP340, VP345 were in fact sufficiently expressed within the two-hybrid system, or were simply not detected within this specific experiment. Difficulties encountered in generating the data have precluded us from resolving that question. A direct comparison involving all of the VP16 fusions in binding to vhs would therefore require that equivalent amounts of VP16 were present. Nevertheless, these results indicate that a binding determinant within VP16 that is upstream of residue 350 is critical for an interaction with vhs.

Additionally, *in vitro* binding assays with *in vitro* translated ^{35}S-labeled vhs (Apa/Sma fragment; residues 179-344), and VP16 deletions fused to GST (glutathione-S-transferase) were carried out. The purpose of the experiment was to assess the binding specificity of each VP16 mutant to vhs, and to confirm the two-hybrid data (Phizicky and Fields, 1995). Since the fusions differed significantly in their expression, protein expression was gauged using SDS-PAGE and Coomassie staining (Figure 3.2.1). Thus, appropriate amounts of extract for each fusion protein was purified, in order to produce equivalent amounts of fusion for each binding reaction. As shown in Figure 3.2.1, the fusion constructs corresponding to full-length VP16, VP404, 355, 350 all retained vhs.

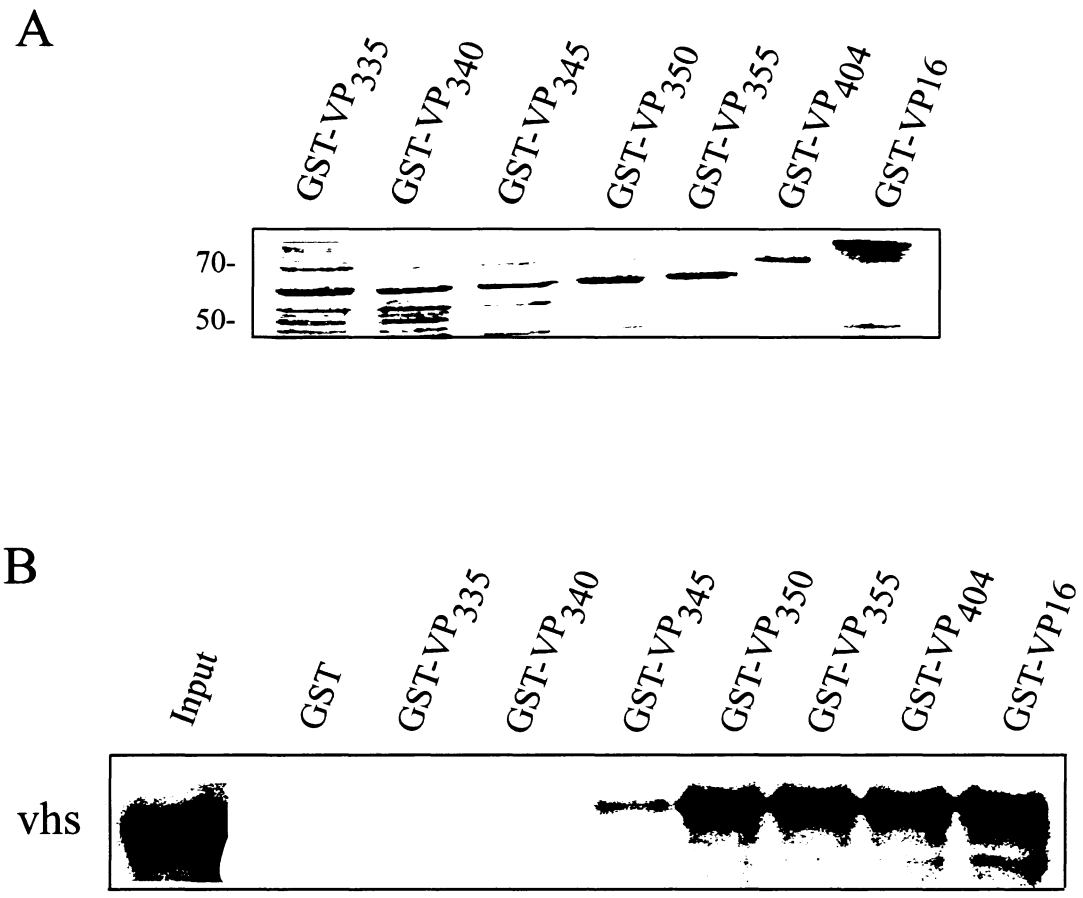

Figure 3.2.1. In vitro mapping of a region within VP16 that interacts with vhs. A. Expression and purification of GST-VP16 fusions on SDS-PAGE and visualized by Coomassie staining. Each fusion is labeled with respect to its terminal residue. Molecular weight markers are indicated to the left (in kDa). **B.** An *in vitro* translation, ^{35}S-labeled vhs (polypeptide corresponding to the Apa/Sma; residues 179-344) was incubated with each VP16 fusion in a pull-down binding assay. Bound vhs was separated by SDS-PAGE and visualized by autoradiography. The left lane represents 30% of the input translation used for each assay.

The VP16 deletion mutant to residue 345 also retained binding, though it was significantly diminished compared to the other truncated proteins. The fusions corresponding to the mutants terminating at residues 340 and 335, as well as the GST control, did not interact with vhs (Figure 3.2.1). Taken together, the data from the *in vitro* binding assays, as with the two-hybrid analysis, indicates that the amino terminal 345 residues of VP16 are sufficient to bind vhs, and that a binding determinant for vhs is localized between residues 340 to 350 of VP16. Furthermore, since two distinct protein modules (GAL4AD and GST) produced similar results, it is unlikely that either moiety is involved in modulating the interaction.

3.2.1 Discussion

A region encompassing amino acids 340 to 350 of VP16 is necessary for an interaction with vhs. A number of mutagenesis and peptide-inhibition studies have also established, however, that the interactions involving VIC are specifically localized to a region between residues 360 and 390 (Greaves and O'Hare, 1989, Greaves and O'Hare, 1990, Wu *et al.*, 1994, Simmen *et al.*, 1997). Thus, it would appear that from the mutagenesis studies conducted thus far, that the residues important for binding vhs are distinct from those involved in the assembly of VIC. The initial experiments indicating that vhs could block VIC assembly, when pre-incubated with VP16, may therefore not rely on direct competition for a binding site, but rather, be a consequence of steric hindrance due to an adjacent binding site (Smibert *et al.*, 1994). Furthermore, the recent availability of the core structure of VP16 (Liu *et al.*, 1999a, see Figure 1.4.5) has

provided an essential context through which to elucidate the functional importance of specific residues in vhs binding. The region encompassing residues 333 to 344 form a α-helix, with the remaining residues up to amino acid 350 forming a structured loop region. The residues involved in VIC assembly, between amino acids 360 and 390, were not present in the solved structure, presumably due to disorder within the crystal (Liu *et al.*, 1999a). As noted by the authors, the VIC binding region is likely to remain flexible in its unbound state, while adopting a stable conformation upon binding to other proteins or DNA. It is interesting to note the distinctions between vhs binding and VIC formation with respect to residues involved within VP16; the ability to bind vhs and the determinants for viral assembly are retained within the core structure of VP16, but not the in segments required for VIC formation. Thus, the flexible segments of the protein may have evolved in order for VP16 to adopt a mechanism through which it functions as a potent regulator of viral gene expression, although other aspects of VP16 function may have previously existed. This notion is particularly intriguing given that transcriptional activation by VP16 is not essential for virus propagation *in vitro* (Ace *et al.*, 1989, Poon and Roizman, 1995, Smiley and Duncan, 1997). Although residues 340 to 350 of VP16 contain a critical binding determinant for the binding to vhs, these results do not preclude other regions within VP16 from having a role in binding to vhs, for instance through the contribution of residues 141-178 of VP16, as previously suggested (Smibert *et al.*, 1994).

3.3 Role of single amino acid residues within VP16 for binding to vhs

In order to expand upon the deletion mutagenesis experiments, single amino acid substitutions were generated by alanine-scanning mutagenesis within the region spanning residues 340 to 350 of VP16. It was believed that these mutants would allow us to explore in greater detail the structural and functional implications of the interaction with vhs, within the context of the full-length protein, by attempting to limit the impact upon the overall structure of the protein. This strategy would also allow other functions attributed to VP16 to remain intact, assuming that their functions did not overlap.

Work from our laboratory (Shaw *et al.*, 1995) and others (Hayes *et al.*, 1993, Wu *et al.*, 1994, Lai and Herr, 1997), has established that a relatively small region of VP16 is responsible for maintaining the various protein and DNA contacts necessary for the formation of VIC. These studies reveal a surprising level of complexity among the interaction networks of VP16, indicating that even adjacent residues are responsible for distinct functional interactions (Shaw *et al.*, 1995, Lai and Herr, 1997). Consequently, the goal of selectively disrupting the interaction with vhs, in the context of other functional aspects of VP16, would likely require the mutagenesis of specific residues. Moreover, the role of single amino-acid residues in binding to vhs would be particularly useful in light of the solved structure of the core VP16 protein, and also with respect to VP16 orthologs, which retain a significant amount of homology between residues 340 and 345 (see Figure 4.0). An analysis of the residues between 340 and 350 with respect to VP16 function, though present to some extent, has to date not been comprehensively studied (Lai and Herr, 1997, Liu *et al.*, 1999).

A

Plasmids	β-galactosidase activity Overlay assay	
	AD	AD-VP404
DB	-	-
DB-vhs (A/S)	-	+
DB-vhs (A/S) (W321A)	-	-

B

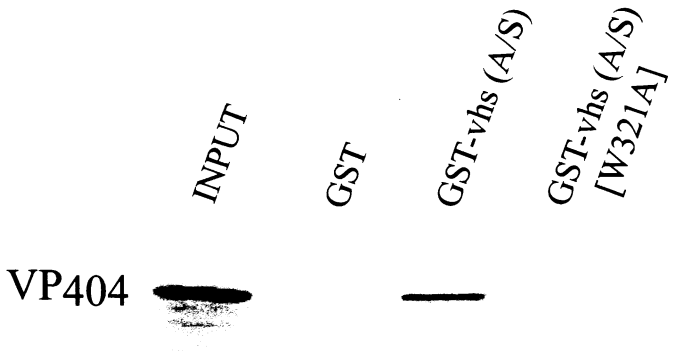

Figure 3.3.1. A single point mutation in vhs abrogates binding to VP16. A. Two-hybrid assay between VP16 and vhs plasmids in PCY2 and analyzed by an overlay assay. The vhs constructs fused to the GAL4 DNA binding domain correspond to the Apa/Sma fragment; which encodes a polypeptide spanning residues 179-344 of vhs, which is sufficient to interaction with vhs (Smibert *et al.*, 1994). AD; GAL4 activation domain. An overlay assay interaction is denoted '+' (blue colour detected in < 30 min), while the '-' indicates the lack of an interaction after an overnight incubation. **B.** Pull-down binding assays with equivalent amounts of wild-type and mutant vhs-linked GST fusions, were incubated with in vitro translated, ^{35}S-labeled VP16. The amino-terminal 404 residues of VP16 is represented as VP_{404}. Input refers to 30% of the total translation used for each assay.

3.3.1 A single amino acid is critical for interaction with vhs

The two-hybrid system is useful for analyzing protein interactions *in vivo*, including the characterization of the role of single amino acid residues in modulating those interactions (Fields and Sternglanz, 1994, Toby and Golemis, 2001). In fact, a single amino acid residue within a minimal domain fragment of vhs (W321) was shown to be critical for interaction with VP16, using both the two-hybrid system and *in vitro* binding assays (Schmelter *et al.*, 1996). Since this interaction was disrupted only within the context of a minimal binding domain within vhs (Schmelter *et al.*, 1996, see Figure 1.5), additional experiments were subsequently employed here to demonstrate that the mutant could not interact with VP16 as part of a larger fragment of vhs (Figure 3.3.1). These results confirm the importance of the tryptophan 321 residue of vhs in binding to VP16.

To assess the role of single amino acids in VP16 for binding to vhs, a series of alanine-scanning mutants between residues 340 to 355 of VP16 were constructed within the context of its amino-terminal domain (residues 1-404). This domain is sufficient to mediate various protein-protein and protein-DNA interactions, but lacks the AAD required for transcriptional activation. Each of the site-specific mutants was fused to the GAL4 activation domain (AD), co-transformed with the DNA binding domain fusion of vhs (Apa/Sma fragment; residues 179-344), or HCF-1 (Kelch domain; residues 1-380), for two-hybrid analysis. The two-hybrid assays were analyzed by β-galactosidase overlay assay. As shown in Table 3.3, all of the mutants, with the exception of leucine 344 (L344A), were able to interact with vhs at levels indistinguishable from the wild-type

Table 3.3.1. Analysis of VP16 mutant binding to vhs using the two-hybrid system. [a]

Plasmids	β-galactosidase activity Overlay assay		
	DB	DB-vhs	DB-HCF
GAL4AD	-	-	-
GAL4AD-VP404	-	+	+
GAL4AD-VP404 (R341A)	-	+	+
GAL4AD-VP404 (A342G)	-	+	+
GAL4AD-VP404 (K343A)	-	+	+
GAL4AD-VP404 (L344A)	-	-	+
GAL4AD-VP404 (D345A)	-	+	+
GAL4AD-VP404 (S346A)	-	+	+
GAL4AD-VP404 (Y347A)	-	+	+
GAL4AD-VP404 (S348A)	-	+	+
GAL4AD-VP404 (S349A)	-	+	+
GAL4AD-VP404 (F350A)	-	+	+
GAL4AD-VP404 (T351/352A)	-	+	+
GAL4AD-VP404 (S353A)	-	+	+
GAL4AD-VP404 (P354A)	-	+	+
GAL4AD-VP404 (E355A)	-	+	+
GAL4AD-VP404 (Δ340-345)*	-	-	-
GAL4AD-VP404 (Δ345-350)*	-	-	-
GAL4AD-$VP_{335-355}$*	-	-	nd
GAL4AD-VP404 (Y364A)	-	+	-

a. Two-hybrid analysis of VP16 constructs with single amino acid substitutions in VP16, with either vhs or HCF-1, was carried out in yeast PCY2. GAL4DB-vhs; the Apa/Sma fragment of vhs fused to the GAL4 DNA binding domain. GAL4DB-HCF; the amino-terminal 380 residues of HCF-1. The GAL4 activation domain fusions of VP16 were constructed within the context of the amino-terminal 404 residues. '+' indicates an interaction (blue in <30 min), and '-' indicates no colour formation. DB; GAL4 DNA binding domain, VP335-355; 21 amino acid polypeptide spanning the indicated residues, nd; not determined. * indicates that expression could not be confirmed by Western blot.

protein (developing blue colour within 20min). The L344A mutant did not display any blue colour formation, even following an overnight incubation. On the other hand, all of the mutants, including the L344A mutant, were capable of interacting with HCF-1 (amino-terminal 380 residues) with similar efficiency. Since the binding determinant for HCF-1 is found between residues 361-364 of VP16 (Lai *et al.*, 1997, Simmen *et al.*, 1997, LaBoissiere *et al.*, 1999), these results were expected, though they additionally served as indicators that the VP16 mutants likely did not suffer from a gross disruption in overall structure. Alternatively, internal deletions between residues 340 to 345, and 345 to 350 did not bind vhs, and since protein expression could not be demonstrated either by binding to HCF-1 (Table 3.3) or by Western blot, those activities could not be confirmed. Similarly, a polypeptide fragment of VP16 encompassing residues 335 to 355 was fused to the GAL4AD, but did not interact with vhs (see Table 3.3).

In vitro binding assays were also used to characterize the role of various alanine mutants in maintaining an interaction with vhs, by constructing various mutants within the amino-terminal 404 residues of VP16 that were fused to GST (glutathione-S-transferase). The purified bacterial extracts of each fusion were co-incubated with *in vitro* translated, ^{35}S-labeled vhs (Apa/Sma fragment), and subjected to *in vitro* pull-down binding assays. As indicated in Figure 3.3.1.1, the results were generally correlative with the two-hybrid assays, with the exception of K343A, which lacked binding to vhs *in vitro*. Additionally, as in the two-hybrid assays, all of the VP16 site-specific mutants retained the ability to interact with HCF-1 *in vitro*. To ensure specificity of this assay, under the conditions used here, a known binding mutant for HCF-1 within VP16, Y364A

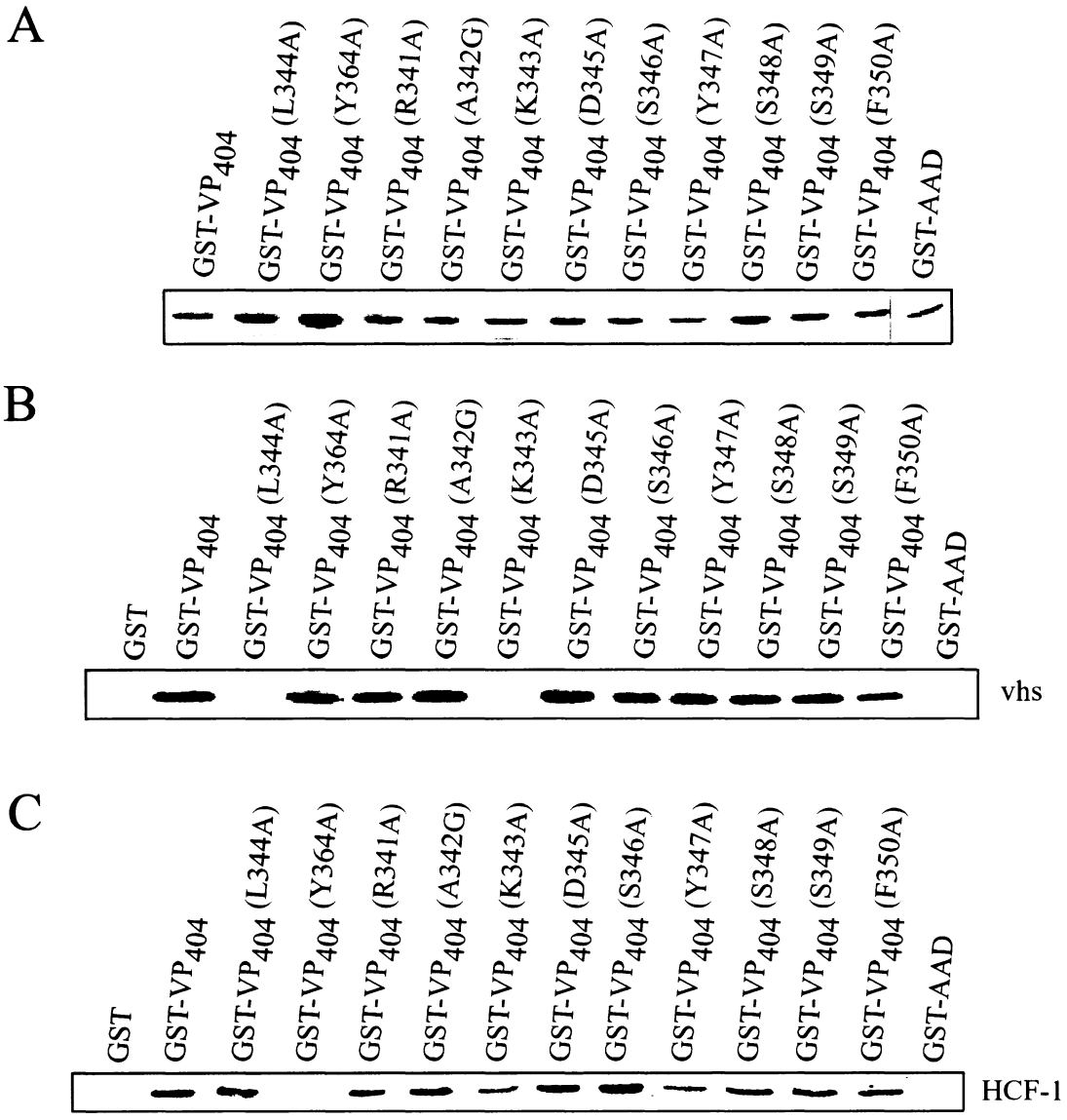

Figure 3.3.1.1. *In vitro* analysis of VP16 site-specific mutant binding to vhs and HCF-1. Pull-down assays with GST fusions to the amino-terminal 404 residues of VP16 (containing various site-specific mutants) were incubated with *in vitro* translated, ^{35}S-labeled vhs (Apa/Sma fragment; residues 179-344) (panel **B**) or *in vitro* translated, ^{35}S-labeled HCF-1 (amino-terminal 902 residues) (panel **C**). The binding assays were resolved by SDS-PAGE and and visualized by autoradiography. GST fusion protein expression for each of the VP16 constructs is demonstrated by SDS-PAGE and Coomassie staining (panel **A**). AAD; acidic activation domain (carboxyl-terminal 80 residues of VP16). The input lanes represent 30% of the total translation used for each binding assay.

(Freiman and Herr, 1997, LaBoissiere *et al.*, 1999), did not bind. These results are also consistent with previous mutagenesis experiments by Lai and Herr (1997), in which the R341A and D345A mutants did not affect binding to HCF-1. To ensure a direct comparison of each mutant with respect to the interactions between the VP16 mutants and vhs or HCF-1, each of the fusion proteins were expressed to similar levels as judged by Coomassie brilliant blue staining of their expression using SDS-PAGE (Figure 3.3.1.1). Since there were differences with the two-hybrid system with respect to the K343A mutant, it is not clear if that mutant, in fact, specifically contributes a binding determinant for vhs. The analysis is complicated by previous mutagenesis studies and structural studies indicating that the K343 side chain is buried toward the interior of the protein, co-ordinating a set of critical network of intramolecular contacts within VP16 (Lai and Herr, 1997, Liu *et al.*, 1999a). The K343A mutant possesses poor expression as a bacterial GST-fusion, which has been attributed to structural instability (Lai and Herr, 1997). The L344A mutant was also expressed poorly, though it is not clear if this residue is also involved in maintaining intramolecular protein interactions. The extent to which protein instability, as a result of these amino acid substitutions, plays a role in mediating the lack of binding to vhs is not known. As demonstrated in subsequent sections, the lack of stability (or expression) of L344A does not appear to be solely a function of its expression in bacteria (Figure 3.4.2) or due to a substitution with alanine (Figure 3.3.2).

Additionally, we investigated whether the AAD of VP16 could support binding to either vhs or HCF-1 using *in vitro* pull-down assays. The VP16 AAD is known to interact with a host of cellular transcription factors (refer to Figure 1.4.2.1) and at least one viral

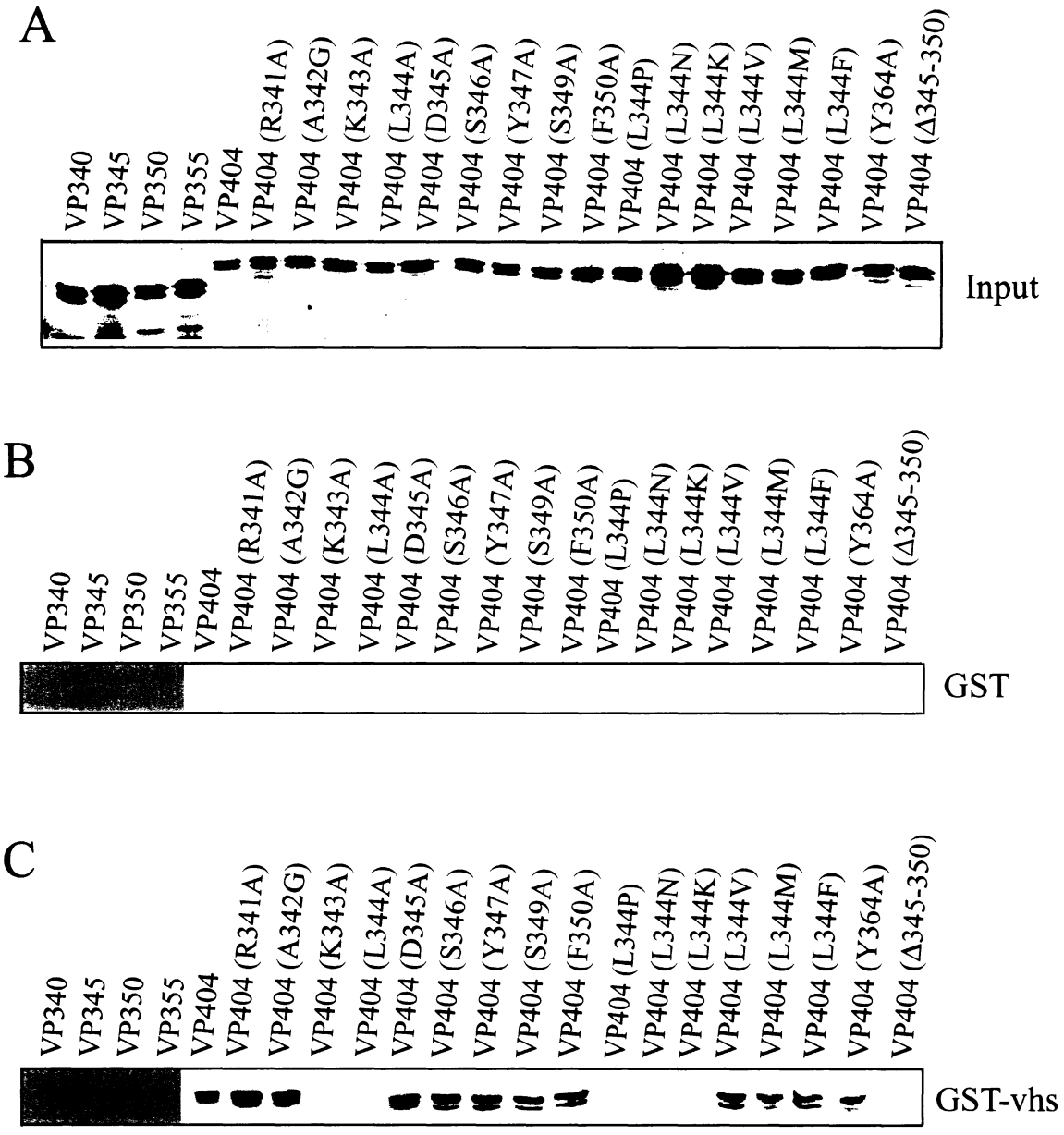

Figure 3.3.1.2. In vitro pull-down assays with GST-vhs and in vitro translated VP16 mutants. **A.** Input translations used for each binding assay are shown and represent 20% of the total amount of protein used for each assay. These carboxyl-terminal deletions (VP340,VP345, VP350, VP355) are also shown. The site-specific mutants within the background of the amino-termainal 404 residues of VP16. Each of the translations were incubated with purified, bacterially expressed fusions of a control GST (**B**), or with GST-vhs (Apa/Sma) (**C**) in pull-down assays. The VP16 carboxyl-terminal deletions (340 and 355) were subjected to a longer exposure for th autoradiogram since the interaction was not readily detected.

protein, VP22 (Elliot *et al.*, 1995). However, despite the presence of binding studies with respect to interactions between VP16 and vhs or HCF-1, a role for the AAD of VP16 in binding to these proteins has previously not been investigated. As demonstrated in Figure 3.3.1.1, a specific interaction between the AAD of VP16 with vhs and HCF-1 was not detected, thus making the amino-terminal domain of VP16 both necessary and sufficient to interact with vhs and HCF-1.

3.3.2 Bulky hydrophobic residues can replace L344 in binding to vhs

The specific structural requirements of the L344 residue (in VP16) in binding to vhs, was examined by additional site-directed mutagenesis. Substitutions of L344 with amino acids; Pro, Asn, Lys, Val, Ile, Phe, and Met, were chosen to investigate the relative importance of hydrophobicity and secondary structure in maintaining the interaction. Amino acids Val, Ile, Phe, Lys and Met were expected to restore the hydrophobic character of the Leu side-chain, while Pro and Asn were likely to disrupt the alpha helix structure (Chou and Fasman, 1978, Creighton, 1983). Met is also found in VP16 orthologs (see Figure 4.0).

L344 was replaced with the indicated amino acids in the context of the amino-terminal 404 residues of VP16 and fused to the GAL4 activation domain, for two-hybrid analysis. The constructs were co-transformed with either the GAL4 DNA binding domain (DB) fusion of vhs or HCF-1, using the constructs described previously, and transformed into PCY2. All of the mutants were able to interact with HCF-1, as judged by the β-galactosidase overlay assay, with an efficiency that was similar to the wild-type construct

Table 3.3.2. Hydrophobic residues do not disrupt the interaction with vhs. [a]

Plasmids	β–galactosidase activity Overlay assay		
	DB	DB-vhs	DB-HCF
AD	-	-	-
AD-VP404	-	+	+
AD-VP404 (L344A)	-	-	+
AD-VP404 (L344P)	-	-	+
AD-VP404 (L344K)	-	+	+
AD-VP404 (L344N)	-	+/—	+
AD-VP404 (L344I)	-	+	+
AD-VP404 (L344V)	-	+	+
AD-VP404 (L344F)	-	+	+
AD-VP404 (L344M)	-	+	+

a. Two-hybrid analysis of VP16 constructs with single amino acid substitutions at position 344 of VP16, with either vhs or HCF-1, was carried out in yeast PCY2. GAL4DB-vhs; the Apa/Sma fragment of vhs fused to the GAL4 DNA binding domain. GAL4DB-HCF; the amino-terminal 380 residues of HCF-1. The GAL4 activation domain fusions of VP16 were constructed within the context of the amino terminal 404 residues. '+' indicates an interaction (blue in <30 min), '+/-' indicates a relatively weak interaction (light blue after an overnight incubation), and '-' indicates no colour formation following an overnight incubation.

(Table 3.3.2). However, as previously established with the Ala mutant, the Pro substitution was unable to bind vhs, and the Asn mutant produced a relatively weak interaction with vhs. In addition, the *in vitro* binding assays were again used to corroborate the two-hybrid assays. As with the Ala and Pro mutants in the two-hybrid assay, binding to the Asn mutant was completely abolished using *in vitro* binding assays, while the Lys mutant was also significantly diminished in binding to vhs within this context (Figure 3.3.2). The fusions were expressed and purified to similar levels as judged by SDS-PAGE and Coomassie staining (Figure 3.3.2), although Ala, Pro and Asn, had lower expression levels compared with the other mutants. All of the mutants were still capable of binding HCF-1 *in vitro* (Figure 3.3.2). Taken together, these results generally indicate that, for the most part, hydrophobicity is an important component of L344 in maintaining binding to vhs. However, these results do not distinguish between L344 having a direct role in promoting an interaction with vhs, or indirectly, for instance, through the maintenance of protein stability or intramolecular interactions.

To further assess the specificity of the site-specific mutants of VP16 in binding to vhs, a series of reciprocal binding experiments were also conducted. The purpose of this assay was to determine the binding specificity for the various VP16 mutants under conditions where vhs would be present in large excess (opposite to the other *in vitro* binding assays), and to assess the functional integrity of each of the mutants as *in vitro* translations (refer to figure 3.4.1). A binding assay between the VP16 mutant translations and GST fusion of HCF-1 was not carried out due to difficulties in expression that fusion construct. Therefore, pull-down binding experiments were conducted with vhs

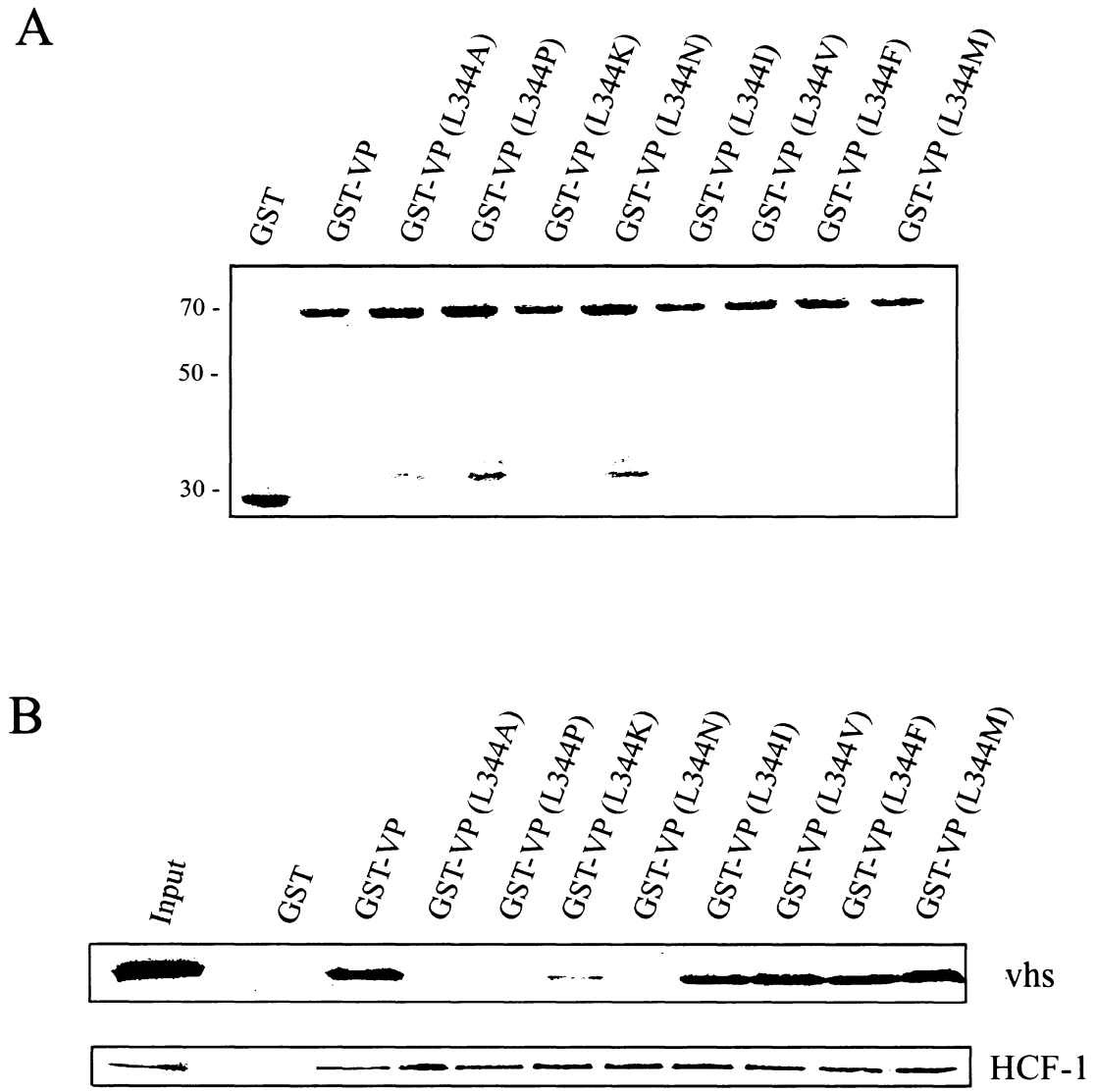

Figure 3.3.2. Role of specific amino acid alterations at VP16 L344 in binding to vhs and HCF-1. Pull-down *in vitro* binding assays with VP16 fused to GST and *in vitro* translated, ^{35}S-labeled vhs or HCF-1 (panel B). The binding assays were resolved by SDS-PAGE and visualized by autoradiography. The GST-VP16 fusions correspond to the amino-terminal 404 residues of VP16. vhs; Apa/Sma fragment corresponds to residues 179 to 344, HCF-1; amino-terminal 902 residues. Each fusion was expressed and compared by SDS-PAGE and Coomassie staining (panel **A**). A schematic position of the molecular weight markers are indicated to the left of the panel (in kDa). The input lane represents 30% of the total translation used for each assay.

(Apa/Sma) fused to GST, and co-incubated with the various *in vitro* translations of the mutant VP16 constructs. The *in vitro* translations of VP16 shown in Figure 3.3.1.2, represent 20% of the input protein used in the binding assays with GST (control) and GST-vhs (Apa/Sma). The results were analogous to previous findings, confirming that mutants K343A, L344A, L344P, L344N, and L344K were abrogated in binding to vhs, although there appeared to be reduced binding with both the L344N and L344K mutants. In addition, the mutant with an internal deletion between residues 345 to 350 was also unable to interact with vhs, suggesting that this region, in general, plays a critical role for the interaction. The carboxyl-terminal deletion mutants of VP16 were also translated *in vitro* and co-incubated with the GST-vhs fusion protein. Notwithstanding the fact that all of the carboxyl-terminal mutants of VP16 were diminished in binding to vhs within the context of this assay, the deletion to amino acid 345 (VP_{345}) of VP16 was capable of binding to vhs, and the mutant truncated at residue 340 (VP_{340}) did not. Surprisingly, the deletion to amino-terminal 355 residues (VP_{355}) was also severely diminished in binding to vhs. These results were unexpected in the sense that the level of binding with the various carboxyl-terminal deletions was similar to that of the deletions encompassing the amino-terminal 404 residues of VP16 within the other assays presented. The reason(s) for the reduced binding of the various deletions, but not the 404 deletions, are not clear but may be due to differences in their relative stability as *in vitro* translation products, as opposed to bacterial fusion proteins. Notably, all of the mutants appear to be translated with similar efficiency. Alternatively, the presence of a fusion within these constructs may serve to stabilize the proteins and (or) binding to vhs. Nevertheless, these results

provide additional confirmation that residues between 340 and 345 of VP16 are important for binding to vhs, as indicated by single amino acids that are altered within the context of the larger protein. Furthermore, a hydrophobic residue appears, generally, to be required to replace leucine at position 344 of VP16 in binding to vhs.

3.3.3 Discussion

A single residue within VP16 is critical for the interaction with vhs both *in vivo* and *in vitro*, as determined by the construction of a series of site-specific alterations of the residues between amino acids 340 and 350 of VP16. Notably, this region contains two invariant residues; R341 and K343 (Figure 4.0). Previous mutagenesis experiments have indicated a role for R341 in promoting DNA binding (Lai and Herr, 1997, Babb *et al.*, 2001), though it was not affected for VIC assembly (Lai and Herr, 1997). In addition, attempts by others to mutate K343 (to Ala) resulted in poor bacterial fusion expression, which was attributed to a critical structural role within the protein (Lai and Herr, 1997, Liu *et al.*, 1999a). Examination of the VP16 structure reveals that residues 340-344 form part of a α-helix, while residues 345 to 350 form part of a co-ordinated loop segment. Both R341 and D345 are solvent exposed, while the leucine (344) side-chain is only partially solvent exposed (Liu *et al.*, 1999a, Swiss PDB viewer; http://us.expasy.org). Therefore, the interaction between VP16 and vhs may occur, at least in part, through a 'hydrophobic' interaction; residues that lack significant hydrophobic character disrupt the interaction with vhs when substituted for position 344 of VP16. As a result, the interaction with vhs may serve to relieve entropic constraints surrounding its side-chain

(consistent with the presence of other bulky hydrophobics), and further maximize van der Waals contacts. Alternatively, a bulky hydrophobic side-chain at position 344 may also be required to maintain the structural integrity of the overall protein, given that the mutants that compromise the interaction with vhs also appear to correlate with the reduced expression by VP16, possibly as a result of greater instability. Therefore, an intact local structure surrounding L344, as opposed to specific contacts with vhs, would be important for sustaining the interaction with vhs. In addition, other aspects of protein structure, and in particular secondary structure, may also have a role in promoting an interaction. This is particularly evident since the substitution of L334 with Pro and Lys disrupt the interaction with vhs, despite maintaining some degree of hydrophobic character. An analysis of the hydrogen bond network involving L344 indicates that the L344 peptide amide likely forms a hydrogen bond with the A342 carbonyl and (or) the K343 side-chain, though the L344 carbonyl does not appear to participate in intramolecular bond formation (Swiss PDB viewer; http://us.expasy.org). The amide group of L344 could also switch to binding a carbonyl group from vhs, thus switching from an intramolecular to an intermolecular bond, which may serve to stabilize the interaction. Furthermore, the unmatched carbonyl of L344 could also be paired with an amino donor, such as the one present on the tryptophan side-chain of vhs (W321). Since a small modular domain within vhs is sufficient to bind VP16, and a single tryptophan residue at position 321 (W321) is critical for maintaining the interaction (Schmelter *et al.*, 1996), the pairing of these residues remains an intriguing possibility. An analysis of the VP16-binding domain within vhs indicates that it may form a β-sheet, possibly allowing

for the alignment of the leucine and tryptophan residues in a critical packing arrangement. Alternatively, the W321 side-chain could be inserted into a hydrophobic pocket within VP16, thus relaxing entropic constraints and forming additional van der Waals interactions (Creighton, 1983, Janin, 1995, Jones and Thornton, 1996, Brady and Sharp, 1997). It is unlikely that the maintenance of an alpha helix structure, with respect to the positioning of the L344 residue is sufficient to account for the interaction with vhs, since alanine still abrogates the interaction but is expected to maintain secondary structure (Chou and Fasman, 1978, Creighton, 1983). Moreover, it is difficult to conjecture about the need for maintaining a helical structure at position 344 of VP16, since all of the hydrophobic amino acids share a propensity for helix formation. The substitution of A342 with Gly should also disrupt helix formation, though this mutant also had no effect on binding to vhs.

Leucine residues contribute prominently in various interaction domains of proteins (Jones and Thornton, 2000). Within transcription factors, leucine residues are prominent in various structural or sequence motifs; such as the 'leucine-zipper' (Alber, 1992), and the 'LXXLL' nuclear receptor co-regulatory protein binding module (Heery *et al.*, 1997). Those motifs may modulate binding through the formation of a defined secondary structure (amphipathic helices); however, as in other protein interactions, a role for flanking amino acids in determining specificity and stability of the interaction may also be important (Hendsch and Tidor, 1999, He and Wilson, 2003). Therefore, while L344 plays a critical role in the interaction with vhs through a mechanism that appears to be partially dependent on the hydrophobic nature of that residue, and perhaps

on an intact secondary structure between residues 340 and 350 of VP16, the contribution of other residues toward the specificity or stability of this interaction should also not be overlooked.

3.4 Functional assays of VP16 site-directed mutants

3.4.1 Role of VP16 site-specific mutants in VIC assembly

Electrophoretic mobility shift assays (EMSA) are used to analyze protein-DNA complex assembly, through the formation of slowly migrating species bound to a radio-labeled dsDNA oligonucleotide probe (Garner and Revzin, 1981, Fried and Crothers, 1981). VP16-induced complex (VIC) formation can be utilized as a tool to assess the functionality of VP16 mutants with respect to their ability to function as transcriptional activators. Consequently, VIC assembly has been studied extensively utilizing diverse sources of VP16 protein, such as from bacterially expressed fusions, virus extracts, *in vitro* translated proteins, and nuclear or cellular extracts (apRhys *et al.*, 1989, Werstuck and Capone, 1989a, Katan *et al.*, 1990, Xiao and Capone, 1990, Kristie and Sharp, 1993, Shaw *et al.*, 1995, Lai and Herr, 1997, LaBoissiere *et al.*, 1997). Therefore, a comparison was required between the various sources of VP16 and HeLa nuclear extract (NE), in order to better characterize differences between the mutants generated here from those reported by others. A high affinity (octa+) TAATGARAT DNA element from the proximal sequence of the ICP0 promoter (Gerster and Roeder, 1988) was used to demonstrate a specific VIC complex. As demonstrated in Figure 3.4.1A, a greater amount of complex is formed with the bacterially expressed fusions or virus purified VP16 than

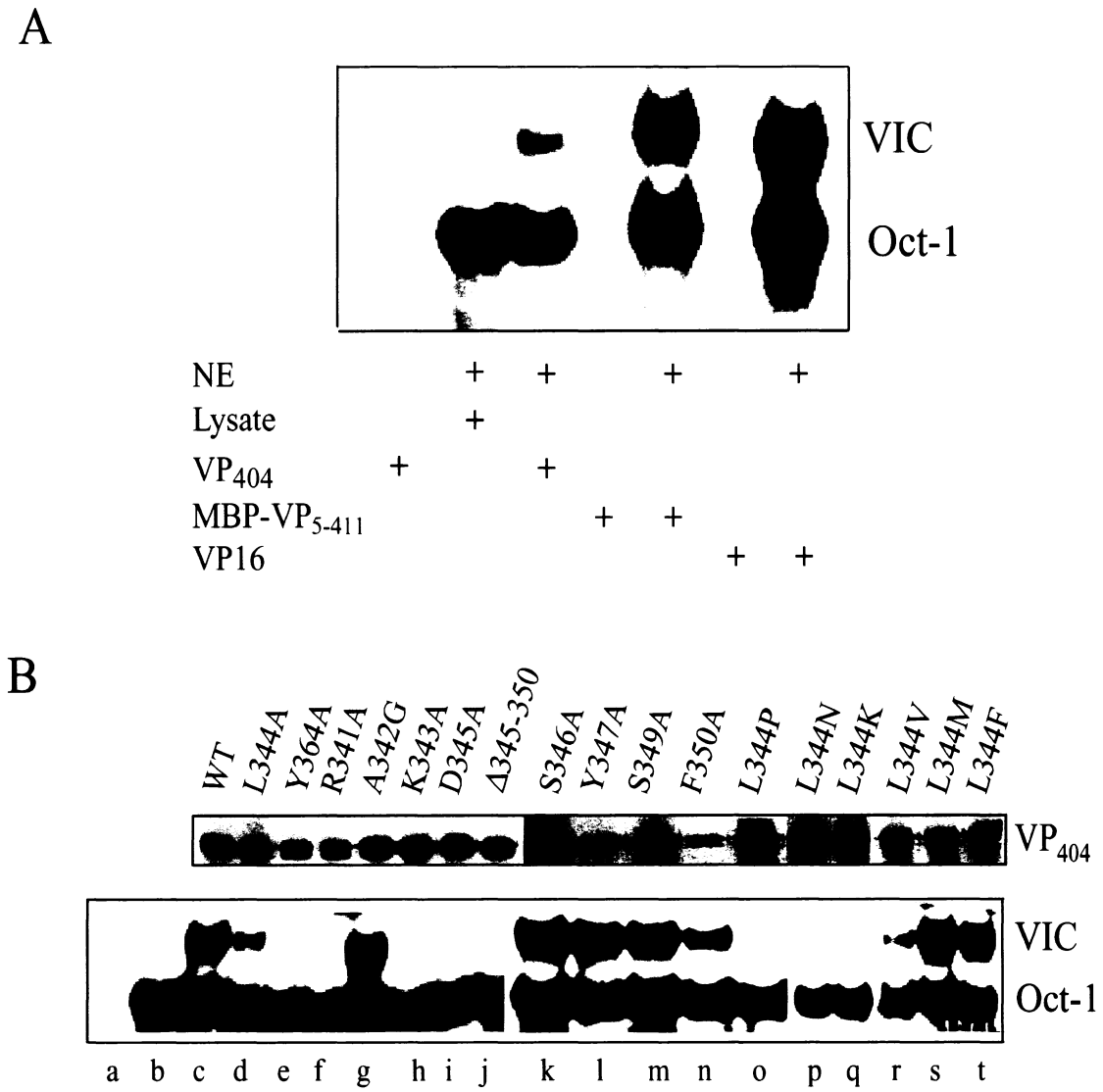

Figure 3.4.1. VIC formation by VP16 site-specific mutants. A. VIC formation was assessed by electrophoretic mobility shift assay using VP16 obtained from various indicated sources. Reaction mixtures contained 0.2pmol of radio-labeled octa+ TAATGARAT probe (Gerster and Roeder, 1988), run on 4% PAGE. The positions of VIC and Oct-1 are indicated to the right. Retic; rabbit reticulocyte lysate (2μL), VP404; *in vitro* translation in reticulocyte lysate of the amino-terminal 404 residues of VP16. MBP-VP_{5-411}; the 'Sal' fragment of VP16 (residues 5-411) fused to the maltose binding protein (1μg), VP16; 2μg of protein purified from HeLa cells infected with HSV1. NE; 10μg of HeLa nuclear extract. **B.** I*n vitro* translations (2μL; input shown in top panel) of VP16 truncations (VP_{404}) with the indicated site-specific alterations, were assayed for VIC as above. Lane a contains the wild-type VP16 (VP_{404}) translation without NE, while lane b contains NE with unprogrammed reticulocyte lysate.

with the *in vitro* translated VP16. These results indicate that the factors within the NE, which are required to form VIC, are present and form the expected complex using *in vitro* translated VP16. Moreover, factors within the NE required for VIC are not limiting within this assay.

Each of the site-specific mutants of VP16, described here in binding studies with vhs (and HCF-1), were also assayed for VIC formation, including the mutants between residues 340 to 350 and the various amino acid substitutions of L344. As shown in Figure 3.4.1B, the wild-type VP16 *in vitro* translation (corresponding to the amino-terminal 404 residues of the protein) did not form a complex in the absence of NE (lane a), while a specific interaction with the probe is obtained in lane c in the presence of the nuclear extract. The factor within the NE that interacts with the TAATGARAT probe (lane b), in the absence of VP16, has previously been determined to be Oct-1 (Stern *et al.*, 1989). The L344A mutant (lane d) was also capable of VIC complex formation, which is therefore consistent with the separation of its role in vhs binding and VIC assembly. The specificity of this assay was also assessed using the Y364A mutant, since it has previously been shown to be defective in binding to HCF-1 and thus unable to form VIC (LaBoissiere *et al.*, 1999). As expected, the Y364A mutant (lane e) did not form VIC. Additionally, consistent with the findings of Lai and Herr (1997), we have also shown that the R341A mutant was affected for complex formation and K343A was not able to form VIC. The inability of the K343A mutant to form VIC was rationalized with respect to its poor expression as a bacterial fusion, while the R341A mutant was important for its role in binding to DNA (Lai and Herr, 1997). The authors also mutated D345A, but

reported no effect on the ability to form VIC. Alternatively, under the conditions used here, D345A does not appear to generate VIC (lane i). Thus, the differences may reflect a more stringent requirement for complex formation created by the use of *in vitro* translates versus bacterial fusions (see Figure 3.4.1). The substitution of single amino acids between residues 346 and 350 (lanes k-n) did not appear to affect complex formation, though an internal deletion removing residues 345-350 within VP16 did (lane j). Moreover, the various L344 substitutions with Val, Met, and Phe did form efficient complexes (lanes r-t), but those with Pro, Asn and Lys did not (lanes o-q). It is interesting that the L344A was capable of forming VIC, while the L344P, L344N, L344K and Δ345-350 mutants could not; possibly indicating a general adverse effect of those mutations on the protein structure. However, all of the mutants retained an interaction with HCF-1, thereby indicating that each possesses some degree of structural integrity.

3.4.2 VP16 site-specific mutant transactivation

Transient transfection assays are also used to evaluate VP16 as a transcriptional activator (Campbell *et al.*, 1984, Werstuck and Capone, 1989b, Shaw *et al.*, 1995, Douville *et al.*, 1995, Lai and Herr, 1997). Therefore, each site-directed mutant, within the context of full-length VP16, was assayed for transactivation of an immediate-early promoter linked reporter in COS-1 cells (Figure 3.4.2). Most of the mutants, including K343A and L344A possess activities that are analogous to wild-type VP16 in activating reporter gene expression. The other mutants, including L344P, L344N and Δ345-350, appear to be somewhat attenuated. The reduced activity may reflect the fact that those

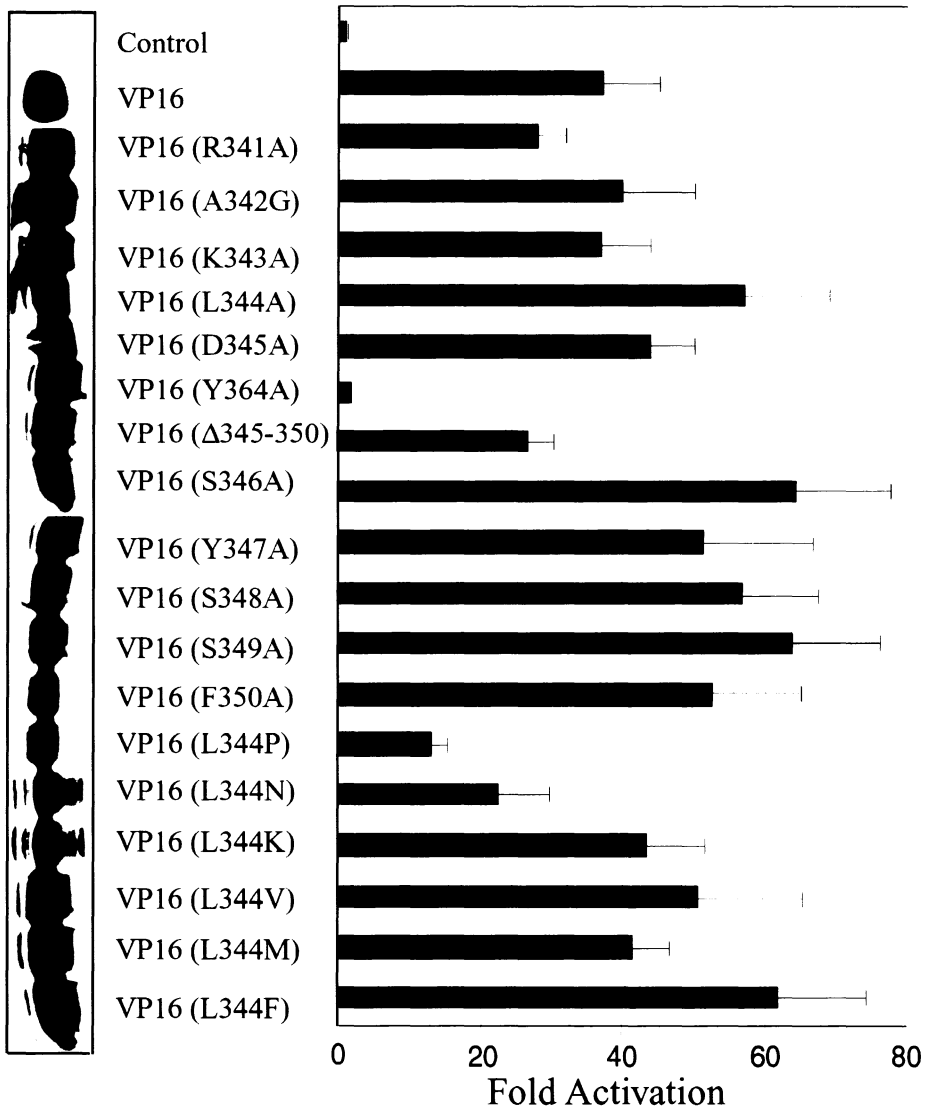

Figure 3.4.2. Transactivation by site-specific VP16 mutants. Site-specific mutants within VP16 were generated within pEVRF65 expression plasmids and transiently transfected (10ng) into COS-1 cells with 0.1μg of the α4-luc reporter gene. Activity data was obtained from duplicates of three independent experiments. Error was calculated using standard deviation. The activity obtained from the reporter construct in the presence of pEVRF0 empty vector ('control') was given a value of one unit. Expression levels for each construct was determined by Western blot analysis (anti-$VP16_{AAD}$; 0.1 μg/mL). To determine the level of expression for each VP16 construct 1μg of plasmid was tranfected for all of the mutants, except mutants K343A, L344A, L344P, L344N, Δ345-350 where 1.5μg was used.

mutants also suffer from an inability to form a VIC complex (Figure 3.4.1). Expression analysis indicates that the mutant proteins are produced to similar levels (Figure 3.4.2; left panel), indicating the differences in activity are not significantly due to differences in the amount of protein. The VP16 (Y364A) mutant does not interact with HCF-1 and is defective for transactivation (LaBoissiere *et al.*, 1999), effectively serving as a control for this assay. The apparent discrepancy between the EMSAs and transient transfections may be the result of differences in the level of stringency between *in vitro* and *in vivo* complex formation. The EMSA represents an equilibrium condition obtained from the formation of a specific complex, while complexes formed *in vivo* may be sufficient to allow for the accumulation of the reporter (luciferase) protein over the course of the transfection. Furthermore, all of these mutants (with the exception of Δ345-350 and Y364A) bind HCF-1 as determined by the two-hybrid system and *in vitro* binding assays, thus potentially allowing for VIC formation under less stringent conditions. Additionally, since R341A and D345A can promote complex assembly under the alternative conditions utilized by Lai and Herr (1997), the method used here to assess complex assembly may be less permissive for identifying VIC. In summary, these experiments demonstrate that VP16 (L344A) can form VIC and transactivate a reporter construct in transfected cells, thereby uncoupling vhs-binding within VP16, from its role as a transactivator of viral IE genes.

3.4.3 Virus complementation assays

At late times post-infection, VP16 downregulates vhs, thereby protecting late viral mRNAs from destruction (Lam *et al.*, 1996). VP16 also has an unspecified role in viral egress, which is required for virus maturation even in the absence of a functional vhs (Mossman *et al.*, 2001b). We sought to investigate the biological consequences of VP16 downregulation of vhs within the context of HSV1, by using a mutant of VP16 that selectively abrogates vhs binding, without affecting other known interactions with respect to VIC. The extent to which the specific lack of vhs binding to VP16 plays a role in the viability of the virus, or whether an interaction with vhs and a role in viral egress are linked, has not previously been demonstrated directly (Lam *et al.*, 1996, Mossman *et al.*, 2000b).

In contrast to wild-type HSV1, an 8MA virus (which lacks VP16) infection leads to an almost complete elimination of viral mRNA, indicating that unregulated vhs could not result in the accumulation of viral structural proteins necessary for virus assembly and propagation (Lam *et al.*, 1996). Our approach to investigate whether a specific interaction with vhs was a critical aspect of VP16 function, in the context of the virus, was to develop a complementation assay utilizing the VP16 null virus (8MA), and the vhs-binding mutant; VP16 (L344A). We hypothesized that the lack of vhs binding would result in unregulated vhs activity, thereby causing a block to virus growth. Alternatively, the complementation of a similar virus mutant, 8MAΔSma (which lacks both VP16 and

Table 3.4.3. Virus complementation assays.[a]

Transfection	Plaque assay on 16-8 cells		
	8MA (PFU/mL)	8MAΔSma (PFU/mL)	
Mock	0-4	20	(1)
	0-8	60	(2)
VP16	$2.5\text{-}3 \times 10^6$	$1\text{-}2 \times 10^3$	(1)
	$1.6\text{-}2 \times 10^4$	6×10^2	(2)
VP16 (L344A)	$0.8\text{-}9 \times 10^2$	$3.5\text{-}5 \times 10^2$	(1)
	$2.6\text{-}3 \times 10^1$	2×10^2	(2)

a. Vero cells were transiently transfected with 2μg of the indicated plasmids; pEVRF0 (mock), pEVRF65 (VP16) and pEVRF65 (L344A), followed by superinfection with 8MA or 8MAΔSma (MOI of 5). Extracts were then generated and titred on 16-8 cells by plaque assay. Two independent experiments (each in duplicate) were counted over a range of dilutions and expressed as PFU/mL.

vhs) by VP16 (L344A), was not expected to affect virus growth since vhs is not present to prevent the expression of viral structural proteins.

Virus rescue experiments are used extensively to characterize protein function within the context of the virus, and serve as a useful tool to investigate the properties of a viral protein (Smiley, 1980, Lam *et al.*, 1996). While rescue experiments are used to alter the genetic background of the virus, virus complementation assays are intended to evaluate the role of mutant proteins during a viral infection, through transient expression within cells prior to an infection (Rice and Lam, 1994, Wanas *et al.*, 1999, Whitbeck *et al.*, 1999). Specifically, the virus complementation assays devised here involve transiently transfecting cells with VP16 (or a mutant thereof), followed by a super-infection with either the 8MA or 8MAΔSma virus. Since a cell line that constitutively expresses VP16 (16-8 cells; Weinheimer *et al.*, 1992) is used to complement and propagate the virus, it was hypothesized that transiently supplied VP16 would also be sufficient to rescue this defect. Therefore, the virus complementation assays shown in Table 3.4.3, measure the relative ability of the VP16 mutant and wild-type proteins to maintain a virus burst following infection with 8MA on a non-complementing cell line. Results from four independent assays indicate that transfections with the wild-type VP16 protein produced a titre of ~$1x10^6$ PFU/mL, compared with ~$5x10^2$ PFU/mL for the L344A mutant, thus establishing that the wild-type VP16 was 3-4 orders of magnitude more effective at propagating infectious virus than the vhs binding mutant of VP16. This directly demonstrates that a critical defect in virus assembly can be attributed, specifically, to the disruption of the interaction between VP16 and vhs during a viral

infection. The reason(s) that the L344A mutant was able to complement 8MA beyond the empty vector control, however, is not clear, but could be indicative of a residual ability of the VP16 mutant to bind vhs. Consequently, vhs may not completely destroy viral mRNA during late times of an infection, thus allowing VP16 to complement the assembly defect during egress.

In order to examine the differences (or overlap) in the residues required for binding to vhs from those required to overcome the virus assembly defect in HSV, a similar set of virus complementation assays to those carried out with 8MA, were applied to 8MAΔSma. If vhs-binding is distinct from virus assembly, then the L344A mutant would be expected to effectively complement the growth of 8MAΔSma, to levels that are analogous to the wild-type protein. Alternatively, if vhs-binding did overlap with a component of virus assembly function within VP16, then the L344A mutant should not (or significantly diminished) complement virus growth. In comparing the titres from both the wild-type and mutant VP16 proteins (Table 3.4.3), the results indicate that ~$1x10^3$ PFU/mL for the wild-type VP16 and ~$3x10^2$ PFU/mL for the L344A mutant, demonstrating that VP16(L344A) was able to effectively complement 8MAΔSma. These results indicate that defect in virus assembly of 8MA is not linked to a vhs-binding mutant, since the mutant was only 3-4 fold (as opposed to 3-4 orders of magnitude, ~5000 fold with 8MA) lower in establishing plaque forming units compared to wild-type construct. Therefore, the difference in plaque formation between 8MA and 8MAΔSma can be attributed to the inability of VP16 (L344A) to bind vhs. Even though the existence of overlap in the functional aspects of vhs binding and virus assembly can not be ruled

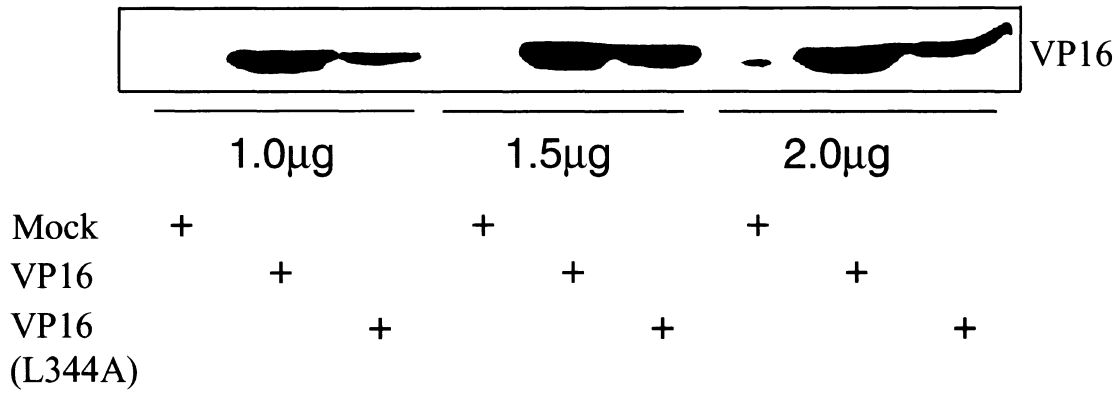

Figure 3.4.3. Western blot of VP16 transfected for virus complementation assay. Protein expression levels of the transfected plasmids were assessed by Western blot over a range of plasmid amounts, as indicated. The proteins were detected with a polyclonal antibody to the VP16 AAD (anti-VP16AAD; 0.1µg/mL).

out, prior mutagenesis data from Ace and colleagues (Ace *et al.*, 1989), and structural analysis of VP16 by Liu *et al.* (1999a), suggests that the residues involved in virus assembly are distinct from those required for VIC. Moreover, as determined within this thesis, the residues involved in virus assembly appear also to be distinct from those involved in binding to vhs. Therefore, the assays with 8MAΔSma would serve as a useful control experiment for 8MA, since both viruses only differ in the presence of a functional vhs protein. Hence, the relatively small difference in virus growth between the L344A mutant and wild-type VP16 with respect to 8MAΔSma may be due to the different level of protein expression within the transfected cells, or differences in immediate-early transactivation between the wild-type and mutant proteins. In order to verify that the L344A mutant is expressed in Vero cells and that the expression was similar to the level of wild-type VP16, we examined the expression within transfected cell extracts by western blot, over a range of concentrations. Although VP16 (L344A) has reduced expression within Vero cells relative to the wild-type protein (Figure 3.4.3), it is unlikely that the vast difference in titre generated between wild-type and mutant VP16 in 8MA is due to the difference in expression levels, since the titres obtained with 8MAΔSma reveal a relatively minor effect on virus growth. The differences in plaque formation within the complementation assays are also unlikely to be the result of differences caused by protein expression or levels of IE transactivation, since the input VP16 is present within the virus is obtained from the complementing (16-8) cell line.

3.4.4 Discussion

Although amino acids required for both vhs binding and formation of VIC are localized to a relatively small region within VP16 (~50 residues), VIC assembly and transactivation were uncoupled from residues required to bind vhs, as demonstrated by the mutants described herein. Therefore, given the close proximity of the residues involved in both functions (within the primary sequence), it would appear that the ability of vhs to block VIC assembly is likely due to the steric interference of the protein rather than competition for a common binding surface. Since vhs and HCF-1 do not compete for the same binding site within VP16, it is still possible that the interaction of HCF-1 with VP16, for instance at early times post-infection, may result in concomitant displacement of vhs from VP16 (Smiley *et al.*, 2001). As a result, even though the interaction between VP16 and vhs can be uncoupled from VIC, structural and functional 'crosstalk' may still exist. In addition, while the residues examined here did not affect binding to Oct-1 or HCF-1, the possibility that another factor, or post-translational modification of VP16 is required in VIC assembly also appears to be unlikely (Werstuck and Capone, 1993). In summary, the characterization of the VP16 L344A mutant, by uncoupling vhs binding from VIC and transactivation, provides a useful tool in the investigation of the VP16/vhs interaction during the course of an HSV1 infection.

Since both VP16 and vhs are pre-formed within the viral tegument, and VP16 is present in large excess over vhs, Smibert *et al.* (1994) hypothesized that VP16 would likely regulate vhs during an infection. Accordingly, in a series of subsequent experiments, Smiley and colleagues (Lam *et al.*, 1996, Mossman *et al.*, 2001b)

demonstrated that VP16 blocked vhs activity at intermediate and late times post-infection, thereby allowing the accumulation of viral late proteins in order to promote virus assembly (Lam *et al.*, 1996). The authors demonstrated that while a VP16 null-virus (8MA) produced a cessation in protein synthesis, the phenotype was overcome by the re-introduction of VP16 within the virus. The construction of a VP16/vhs double mutant (8MAΔSma) also indicated that vhs was responsible for the reduced protein synthesis in the VP16-null background. Moreover, the acidic activation domain of VP16 was also not required for the rescue, and infection of a cell line constitutively expressing VP16 was resistant to protein synthesis degradation by 8MA (Lam *et al.*, 1996). In further support of those conclusions, the isolation of a second-site revertant within 8MA, localized to an internal deletion within the vhs sequence, enabled the growth of 8MA on a non-complementing cell line (Mossman *et al.*, 2001b). The findings provided independent genetic evidence for the role of VP16 in regulating vhs, and demonstrated conclusively that VP16 downregulates vhs activity at late times post-infection. However, previous studies examining the interaction between VP16 and vhs, while elucidating the biological relevance of this interaction, were complicated by the fact that the amino-terminal region of VP16 possesses multi-functional domains. The various domains of VP16, required for VIC and virus assembly, were not sufficiently defined with respect to the interaction with vhs. Therefore, that aspect of VP16 function could not previously be characterized with respect to their role within HSV, leaving their precise structural or functional inter-relationship with respect to vhs binding and the downregulation of vhs at late times of an

infection unresolved. Accordingly, as demonstrated here, a direct interaction between VP16 and vhs is required for the downregulation of vhs activity.

The mechanism of VP16 mediated downregulation of vhs, however, remains unclear. An understanding of the functional aspects surrounding the downregulation of vhs by VP16 could also serve as a basis to explore the events that take place at early times post-infection, in which VP16 is likely to be prevented from binding to vhs (Smiley *et al.*, 2001). Two scenarios arise as the most likely means through which the regulation of vhs activity can occur. First, downregulation may involve competition or obstruction of the associated activity through a direct interaction of a region(s) required for vhs activity through competition with a co-factor (Feng *et al.*, 2001, Lu *et al.*, 2001a). The regions (or domains) of sequence similarity with Fen-1 nucleases (see Figure 1.5) lie outside the residues isolated within vhs that are important for binding to VP16, indicating that VP16 may not directly block vhs activity. However, VP16 may also compete for a co-factor binding site (eg. eIF4H; Feng *et al.*, 2001) within vhs, or promote an allosteric change in vhs structure in order to render it inactive. Determination of the structural juxtaposition of the VP16 binding site, with respect to the activity domains of vhs, may be required to explore this possibility. Second, VP16 may re-localize vhs to another sub-cellular compartment and/or into the viral assembly pathway, potentially rendering it inactive. VP16 is found to localize to the nuclear periphery at late-times post-infection, which also may serve as a site for the assembly of tegument proteins in virion formation (Ward *et al.*, 1996, Inglis, 1998, Kato *et al.*, 2000). Thus, HSV may have developed the means to both block vhs activity and package it into the virion simultaneously. The fact

that viral gene expression and entry into the viral assembly pathway is not required for a vhs mediated block in cellular translation does not necessarily preclude sequestration as a means to regulate vhs activity, particularly if VP16 does not directly bind to the active region(s) of vhs (Lam *et al.*, 1996). The elucidation of the mechanism of VP16 downregulation of vhs will presumably be required in order to exploit this interaction as a target for a viral inhibitor.

Vhs orthologs (with the exception of HSV2 vhs) within other alphaherpesviruses lack a conserved VP16 binding motif (Schmelter *et al.*, 1996, Sato *et al.*, 2002). Moreover, VP16 orthologs while possessing a Met residue at position 344, lack the adjacent serine rich region to residue 350 (Liu *et al.*, 1999a, and Figure 4.0). Moreover, the vhs orthologs (not including HSV2) are not packaged within viral particles; which may be indicative of the differential strategies used by these viruses during a lytic (or latent) infection (Hinkley *et al.*, 2000). Therefore, regulation by VP16 may not only differentiate the alphaherpesviruses with respect to promoting and sustaining their lytic infection cycle, but also with respect to immune evasion. In the absence of vhs, HSV1 is more immunogenic, possibly due to its inability to modulate cellular responses within the cell leading to increased recognition by the immune system (Geiss *et al.*, 2000, Samady *et al.*, 2003). Since a role for vhs in neuropathogenesis is implied by the presence of vhs orthologs in other alphaherpesviruses, but not beta- and gammaherpesviruses (Smith *et al.*, 2002), it may be important to clarify the similarities or differences with respect to the function of both VP16 and vhs in other alphaherpesviruses, as an additional means to understand the interplay between these viruses and their target cells.

3.5 Future Directions

The VP16 binding region within vhs, comprising a small modular domain, does not correlate with its activity as a viral mRNAse (Jones *et al.*, 1995, Schmelter *et al.*, 1996). However, it is possible that VP16 affects vhs function by altering the interaction of another factor that is critical for its activity (Feng *et al.*, 2001, Lu *et al.*, 2001b). Alternatively, VP16 may localize vhs to a region(s) within the cell, thereby blocking its activity at late times, in addition to potentially packaging it into the viral tegument (Read *et al.*, 1993, Lam *et al.*, 1996). Unpublished studies from our laboratory involving a GFP (green fluorescence protein)-vhs fusion protein, was used to demonstrate re-localization of the fusion to the nuclear periphery in the presence of a co-transfected VP16 (Inglis, 1998). On the other hand, the mechanism(s) surrounding the dissociation of VP16 and vhs at early times post-infection also remain unresolved. HCF-1 may displace vhs from VP16, allowing for localization of VP16 to the nucleus (LaBoissiere *et al.*, 1999), or the interaction may be regulated by phosphorylation. The phosphorylation status of vhs is known to change upon entry into the cell (Smibert *et al.*, 1992, Read *et al.*, 1993). VP16 is also phosphorylated at residue 375 by casein kinase II (CKII), which is necessary for VIC formation (O'Reilly *et al.*, 1997). It is possible that amino acid 375 is structurally juxtaposed with residues encompassing amino acids 340 to 350. Indeed, a consensus CKII target sequence is found at Ser320 of vhs (Schmelter *et al.*, 1996). The elucidation of the biochemical mechanism(s) and events surrounding the interaction between VP16 and vhs during the course of an infection should prove to be invaluable in the pursuit of an antiviral target against HSV.

Chapter 4. Investigation of VP16 transactivation

VP16 mediated transcriptional activation of viral immediate early genes serves as a paradigm for eukaryotic gene expression (Herr, 1998). The work presented in this chapter, essentially followed from a novel finding from our laboratory establishing that deletion constructs of VP16, appended to the DNA binding domain of GAL4, could activate the expression of a reporter gene in yeast (Popova *et al.*, 1995). This phenotype appeared to be particularly robust in yeast, but only marginal in mammalian cells; however, the boundaries for both activities were equivalent within both systems. We sought to further investigate this phenotype, since we believed that it might lead to a better understanding of the mechanism of VP16 transcriptional activation. The rationale for pursuing a putative transcriptional activation domain within the amino-terminal region of VP16 was based on the following observations: First, the region(s) within VP16 found to possess the activation phenotype in yeast correlated to those found to be important for binding vhs. Vhs was found to block VIC formation when pre-incubated with VP16 (Smibert et al., 1994). Second, truncated VP16 (lacking its AAD) proteins appeared to be capable of activating transcription of viral immediate early genes. Viruses in which the AAD of VP16 was deleted were not only viable, but also retained a significant ability to activate viral IE genes (Ace *et al.*, 1989). Third, an analysis of VP16 using transcription assays indicated the truncated protein had considerable activity in the activation of target genes (Cleary *et al.*, 1993, Wu *et al.*, 1994). Fourth, VP16 orthologs (see Figure 4.0), which activate transcription of their respective target genes in some cases through the formation of a VIC-like complex, do so without a distinct VP16-like

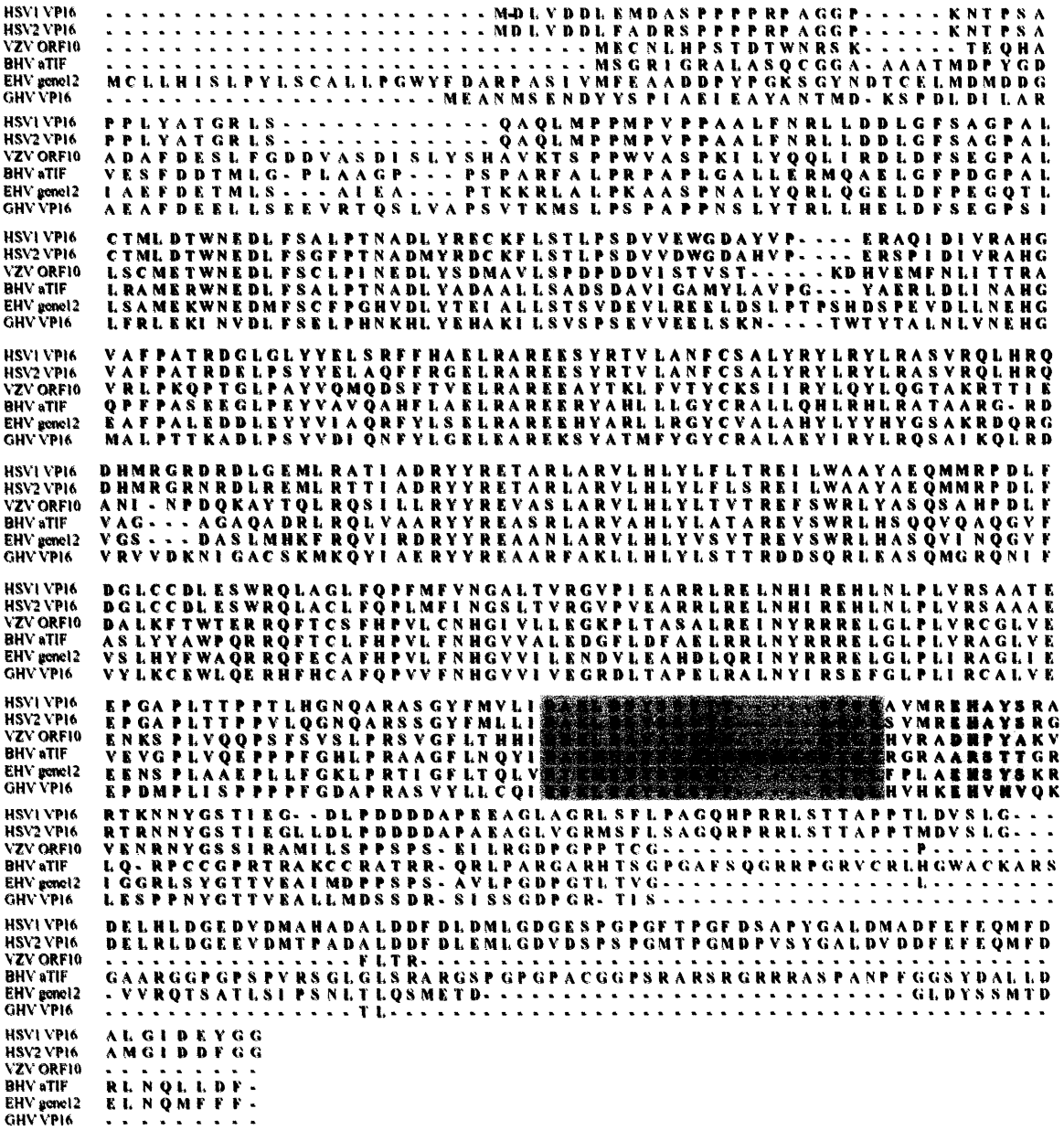

Figure 4.0. Amino acid sequence alignment of VP16 orthologs. ClustalW (version 1.82) (Higgins, 1994) generated amino acid sequence alignments of VP16 and various orthologs (www.ebi.ac.uk/clustalw). Sequences were obtained from the NCBI database using the following accession numbers: HSV1; PO6492, HSV2; P23990, VZV; CAA27893,EHV1; NP045229, BHV-1; P30020, GHV-1; AAA03149. Residues 340-355 of HSV1 VP16 are highlighted in green, while the HCF-1 binding motif is shown in orange. The minimal VIC forming domain of VP16 is shown with a light grey background, and the AAD is shown with a light yellow.

AAD (Greaves and O'Hare, 1991, Carpenter and Misra, 1992, Moriuchi *et al.*, 1993, Purewal *et al.*, 1994, Elliott and O'Hare, 1995). Therefore, we hypothesized that further elucidation of a putative internal activation domain within VP16 might reveal a novel aspect of IE gene expression, or uncover a previously unrecognized mechanism through which the AAD and an internal segment of VP16 could functionally interact. We were also interested in understanding the basis for the difference in the magnitude of activation between yeast and mammalian cells, which we surmised could also lead to a better understanding of VP16 function and its regulation.

In order to investigate the functional importance of the amino-terminal region in maintaining an activation phenotype in yeast and mammalian cells, we constructed additional carboxyl-terminal deletion mutants of VP16. Subsequently, site-directed mutants were also subsequently created to examine the potential role of specific residues in contributing to the activity, since we hypothesized that a detailed mutagenesis study would sufficiently allow for the differentiation of the various aspects of VP16 function. This chapter presents the results of an investigation of an internal segment within VP16 in promoting transcriptional activation, by delineating a region important for activation in yeast from mammalian cells. Ultimately, this investigation led to the discovery of a fundamental new role for HCF-1 in VP16 mediated transcriptional activation.

4.1 Analysis of charged residues for the VP16 activation phenotype in yeast

Two regions responsible for the ability of truncated VP16 to activate the expression of a reporter gene in yeast, when appended to the DNA binding domain of

Table 4.1. Selected charged residues within VP16 do not affect the activation phenotype in yeast.[a]

Plasmid	β–galactosidase activity Overlay assay
DB	-
DB-VP404	+
DB-VP404 (R155A)	+
DB-VP404 (R162A)	+
DB-VP404 (R164A)	+
DB-VP404 (R169A)	+
DB-VP404 (C176A)	+
DB-VP404 (R360A)	+
DB-VP404 (R366A)	+
DB-VP404 (R368A)	+
DB-VP404 (K370A)	+
DB-VP404 (Y364A)	+

a. One-hybrid assay of the VP16 mutants fused to the GAL4 DNA binding domain in yeast PCY2. In all cases, an interaction was determined to occur by overlay assay; yielding blue colour formation in < 30 min. All of the mutants were constructed within the context of the amino-terminal 404 residues of VP16.

GAL4, were isolated through mutagenesis studies (Popova *et al.*, 1995). Those mutants also coincided with regions thought to be sensitive to VIC formation and the interaction with vhs (Werstuck and Capone, 1989a, Werstuck and Capone, 1989b, Smibert *et al.*, 1994).

The site-specific mutants constructed here were initially chosen in order to systematically investigate the role of charged residues for VIC assembly (Shaw *et al.*, 1995). The investigation of these residues with respect to the activation phenotype, was also undertaken, based on the following rationale: First, VP16 orthologs are also rich in positively charged (arginine) residues, particularly within those regions found to be important for function (refer to Figure 4.0). Second, a novel protein interaction in yeast (or mammalian cells) could potentially overlap with residues involved in mediating VIC assembly. Third, the relationship between this phenotype and the interactions within VIC, and with vhs, were unclear. Fourth, these mutants provided a comparison of the requirement of specific residues in the activation phenotype in yeast and mammalian cells with those necessary for VIC assembly.

As a result, each of the mutants were incorporated into the amino-terminal 404 residues of VP16 fused to the DNA binding domain of GAL4, within a yeast two-hybrid vector, and transformed into *S. cerevisiae* PCY2. As demonstrated in Table 4.1, a one-hybrid assay demonstrates that there was no difference in the level of activity between wild-type and mutant residues, using a qualitative β-galactosidase overlay assay. These results indicate that the predominantly positive residues mutated between regions corresponding to amino acids 141 to 178 and 335 to 369, do not affect the activation

phenotype of the VP16 fusions appended to the GAL4DB in yeast. Although these mutants disrupt protein- and DNA- interactions within VIC, including the interaction with the Oct-1/DNA complex, HCF-1 (or VCAF-1) and the cis-acting TAATGARAT DNA element (Shaw *et al.*, 1995), there was no effect on the transactivation phenotype or binding to vhs (section 3.1). Interestingly, each mutant, while variously affected for specific interactions within VIC, was able to sustain VIC and transcriptional activation in transiently transfected cells. This supported the notion that overlapping compensatory interactions within VIC allow for complex assembly and transactivation (Shaw *et al.*, 1995). Taken together, these results further serve to exclude various residues from being involved in the VP16 transactivation phenotype in yeast, while also validating the overall structural and functional integrity of each mutant with respect to VP16 function for the other assays outlined in Shaw *et al.* (1995).

4.1.1 Discussion

The segments spanning residues 141-178 and 335-369, or residues among VP16 orthologs, may contain a number of conserved residues with VP16 that coincide with their transcriptional activation domains, therefore, the results do not exclude the possibility that VIC formation overlaps with the activation phenotype of the truncated VP16. To the extent that the site-specific mutants tested here are the indicators of interactions within VIC, a correlation between residues required for VIC assembly with those of the activation phenotype of VP16 in yeast was not found. However, since only a limited number of mutants were generated, and VIC was not abolished, the potential for

such a coincidence remains a possibility. Positively charged residues within VP16 and have been shown to be involved in both protein and DNA interactions (Shaw *et al.*, 1995, Lai and Herr, 1997). To that end, Popova *et al.* (1995) have speculated that the interactions within VIC may overlap with a putative co-activator binding site within yeast. Oct-1 and HCF-1 orthologs are not present in yeast; however, a protein mimicking those proteins within yeast, in binding to VP16, cannot be ruled out.

4.2 Differentiation of the activity in yeast and mammalian cells

Transcriptional activation domains can be studied and characterized through the construction of fusions with heterologous DNA binding proteins (Ptashne and Gann, 1997). The carboxyl-terminal acidic activation domain of VP16, when appended to the GAL4 DNA binding domain, is an exceedingly potent transcriptional activator in both yeast and mammalian cells (Sadowski *et al.*, 1988). However, the amino-terminal region of VP16, which is responsible for mediating protein- and DNA- interactions within VP16, also transactivates reporter constructs in yeast and mammalian cells when appended to the GAL4 DNA binding domain (Popova *et al.*, 1995). This activity was localized to two regions, encompassing residues 141-178 and 335-369 of VP16, which were previously been shown to possess other functionally important regions of VP16 (Werstuck and Capone, 1989a, Werstuck and Capone, 1989b, Smibert *et al.*, 1994, Shaw *et al.*, 1995). While the activity of the truncated VP16 fusion is only marginally active in mammalian cells, its activity in yeast was comparable to GAL4-AAD, thus implying that the activity was functionally relevant. The purpose of the following experiments was to

uncover to differences in activity found within yeast and mammalian cells through mutagenesis studies, in order to provide further clues as to the role of specific residues within VP16 for the transactivation phenotype, so that we could begin to uncover the potential functional significance of this activity.

4.2.1 Localization of an important activation region within VP16 for activating in yeast

We proceeded with additional carboxyl-terminal deletions, as opposed to additional internal deletions, in an effort to minimize disruption to the overall structure of VP16 (Poon and Roizman, 1995, Liu *et al.*, 1999a), and since the region between residues 335 to 369 have been shown to be directly involved in VP16 complex assembly (Greaves and O'Hare, 1989, Greaves and O'Hare, 1990, Wu *et al.*, 1994, Simmen *et al.*, 1997). Utilizing both qualitative and quantitative β-galactosidase assays of the yeast one-hybrid system (Figure 4.2), we show that the amino-terminal 350 residues of VP16 fused to the GAL4 DNA binding domain was capable of potent activation (similar to the wild-type constructs; VP_{404} or VP_{369}), though a further deletion to residue 345 completely abolished this activity. The activities of previously characterized fusions, carboxyl-terminal deletions to residues 335, 369 and 404 of VP16, were consistent with the results presented here (Popova *et al.*, 1995). Since the protein fusions were expressed to similar levels, as judged by western blot analysis from yeast extracts (Figure 4.2), an activity determinant for the yeast transactivation phenotype was determined to exist between residues 345 to 350 of VP16. This region is comprised almost entirely of polar residues (three serine, one tyrosine), which, interestingly, is also found in a number of other

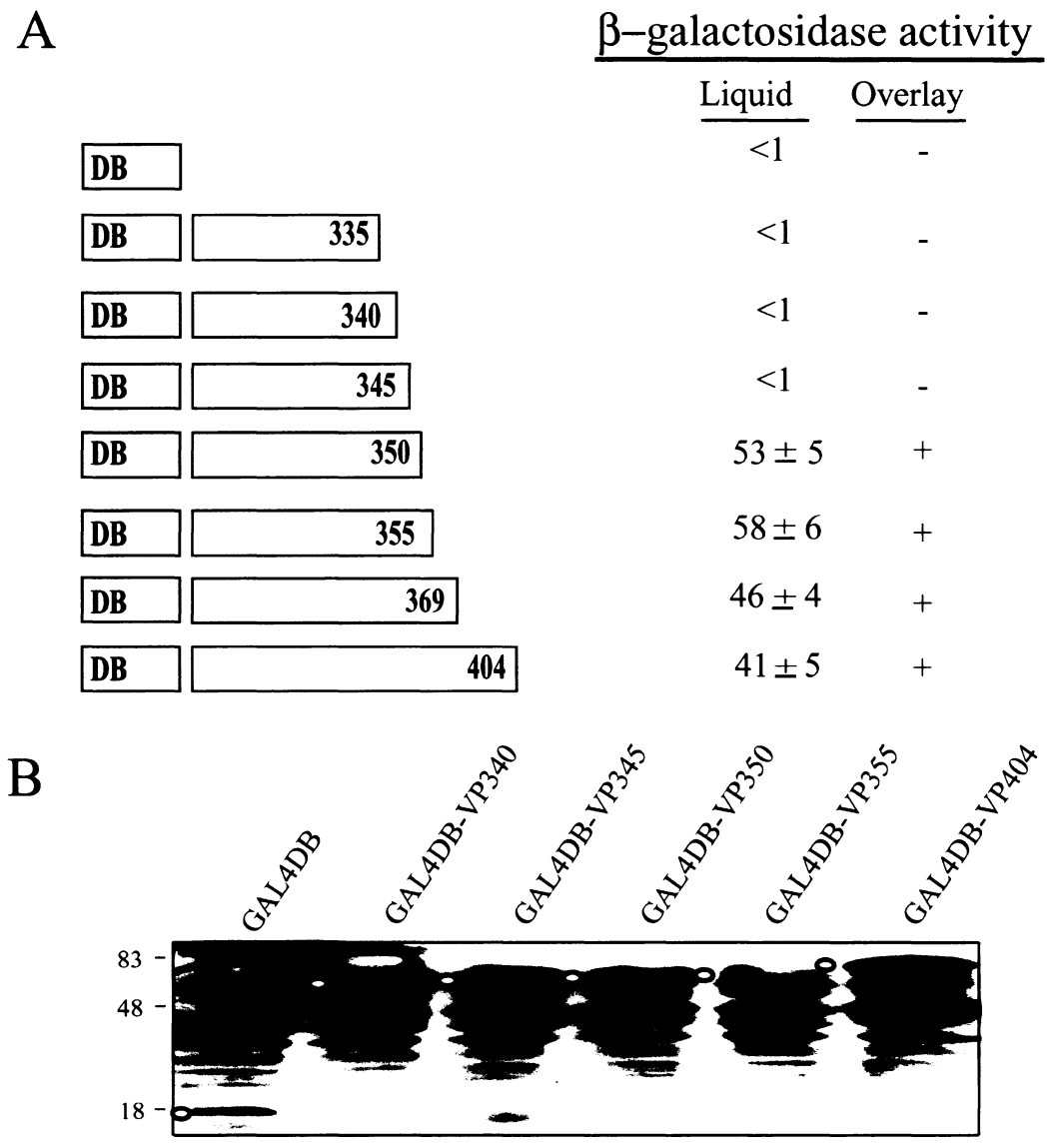

Figure 4.2. Mapping the residues in VP16 involved in the yeast transactivation phenotype. **A.** One-hybrid assay of carboxyl-terminal deletions of VP16 fused to the GAL4 DNA binding domain. Liquid assays from three independent transformation in yeast PCY2 (+/- SD), and overlay assays were used to assess activity. Activities using the overlay assayed were obtained in less than 30min, while the rest of the plates were left overnight without colour formation. The activities of VP335, VP369, and VP404 have previously been shown by Popova *et al.* (1995), which were analogous to the results presented here. **B.** Western blot analysis of GAL4 DB-VP16 fusion proteins generated from yeast extracts. The GAL4 DNA binding domain specific antibody (RKC51; 0.25 μg/mL) to detect expression. Molecular weight markers are represented schematically to the left (in kDa). The GAL4 DNA binding domain fusions are indicated by white circles.

Table 4.2. Single amino-acid substitutions in VP16 do not alter its activation phenotype in yeast[a].

Plasmids	β-galactosidase activity Overlay assay
GAL4DB	-
GAL4DB-VP404	+
GAL4DB-VP404 (R341A)	+
GAL4DB-VP404 (A342G)	+
GAL4DB-VP404 (K343A)	+
GAL4DB-VP404 (L344A)	+
GAL4DB-VP404 (D345A)	+
GAL4DB-VP404 (S346A)	+
GAL4DB-VP404 (Y347A)	+
GAL4DB-VP404 (S348A)	+
GAL4DB-VP404 (S349A)	+
GAL4DB-VP404 (F350A)	+
GAL4DB-VP404 (T351/352A)	+
GAL4DB-VP404 (S353A)	+
GAL4DB-VP404 (P354A)	+
GAL4DB-VP404 (E355A)	+
GAL4DB-VP404 (Δ340-345)*	-
GAL4DB-VP404 (Δ345-350)*	-
GAL4DB-$VP_{335-355}$*	-
GAL4DB-VP404 (Δ1-5)	+

a. GAL4 DNA binding domain fusions of VP16 possessing site-specific mutations between amino acids 340 and 355 were transformed into PCY2 yeast and analyzed by one-hybrid assay. '+' indicates blue colour formation (< 30min), while '-' represents no colour formation after an overnight incubation. * indicates that the expression of those fusions could not be confirmed by Western blot. VP404; amino-terminal 404 residues of VP16, VP335-355; a 21 amino acid polypeptide fragment encompassing the indicated residues of VP16. Δ corresponds to an internal deletion of the indicated residues.

transactivation domains (Hunter and Karin, 1992). An attempt to generate an autonomous activation domain comprising residues 335 to 355 appended to the GAL4 DNA binding domain, however, was not successful (Table 4.2; $VP_{335-355}$); possibly due to polypeptide instability since its expression was not detected. Moreover, given that a segment between residues 141-178 may also be involved in mediating this activity, the presence of an intact (core) protein may be required for this activity.

An investigation into the role of specific residues within this region was also attempted through the construction of single amino acid substitutions. Alanine-scanning mutagenesis of the residues between 340 and 355, resulted in activities that were indistinguishable from the wild-type construct (Table 4.2), as determined by qualitative yeast overlay assay. Although single amino acid substitutions do not appear to affect activity, it is possible that alteration of a single residue is not sufficient to disrupt the overall activity. Therefore, a structural element within VP16, as opposed to a specific amino acid(s), likely mediates the activation phenotype. The amino-terminal 350 amino acids constitute the VP16 core region, possessing a stable structure (Liu *et al.*, 1999a), and single amino acid substitutions (with alanine) would not be expected to adversely affect local protein structure (Morrison and Weiss, 2001). Notably, homogenous stretches of polyamino acids are known to affect transcriptional activation, though the mechanism for this is unclear (Gerber *et al.*, 1994). The possibility that multiple residues within VP16 are necessary for its phenotype in yeast, which can compensate for the alteration of a single residue, are also consistent with those findings.

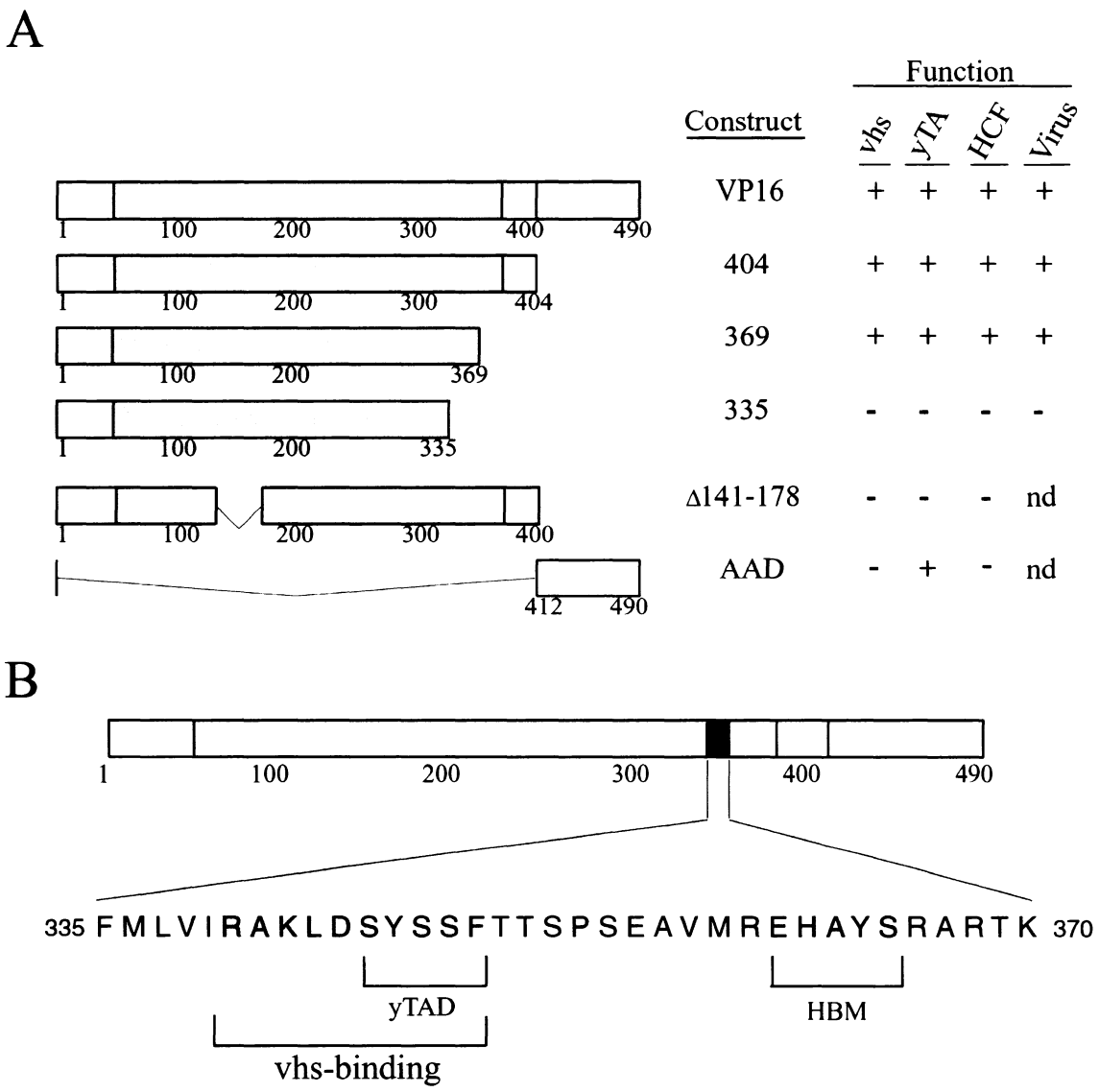

Figure 4.2.1. Summary of VP16 mutagenesis data. A. Summary of the activities attributed to various deletion mutants of VP16 described to date. The results were compiled from previous studies (Smibert *et al.*, 1994, Popova *et al.*, 1995, Faught, 1996) and from this thesis. Deletion mutants 369 and 335 are highlighted in blue, in order to draw attention to the differences in their function. **B.** Schematic of the VP16 region between residues 335 and 370. As determined within this thesis, the residues highlighted in green are important for the yeast transactivation phenotype while those highlighted in both green and yellow are important for binding to vhs. Residues important for binding to HCF-1 (Freiman and Herr, 1997) are highlighted in orange. HBM; HCF-1 binding domain, yTAD; yeast transactivation phenotype, AAD; acidic activation domain, virus; complementation of HSV1 assembly defect in both 8MA and 8MAΔSma, nd; not determined.

4.2.2 Comparison of the activation by truncated VP16 fused to GAL4DB in yeast and mammalian cells

An important aspect of the activity of the truncated VP16 protein with respect to its ability to transactivate reporter gene expression in yeast is that a similar, albeit very modest, activity was also present within mammalian cells. Therefore, a comparison of the activity of the mutants generated within the yeast system with those used in mammalian cells was carried out in order to delineate the regions of VP16 within both systems that are responsible for each respective transactivation phenotype.

Consistent with the previous findings of Popova *et al.* (1995) (see Figure 4.2.1), GAL4 DNA binding domain fusions of truncated VP16 proteins terminated at residues 369 and 404 possessed modest activation of a co-transfected reporter gene in transiently transfected mammalian cells, compared to the empty vector control (GAL4DB). However, additional carboxyl-terminal deletions to residues 340 to 355 appear to, at best, be only marginally active compared to the empty vector GAL4DB control plasmid (Figure 4.2.2). The lack of activity was not due to an absence of the fusion proteins, since western blot analysis demonstrated the expression of each of the newly constructed mutants (Figure 4.2.2; left panel). Also consistent with Popova *et al.* (1995), was that the activity of the VP_{369} fusion is consistently higher than the VP_{404} fusion. VP_{404} demonstrates lower activity, even overlapping with the other deletions labeled as inactive. The reason(s) for the decreased activity with VP_{404} fusion (compared to VP_{369}), or the large variability in activity with the other constructs tested is not clear. The magnitude of the activity of VP_{404} is in line with previous studies (Popova *et al.*, 1995).

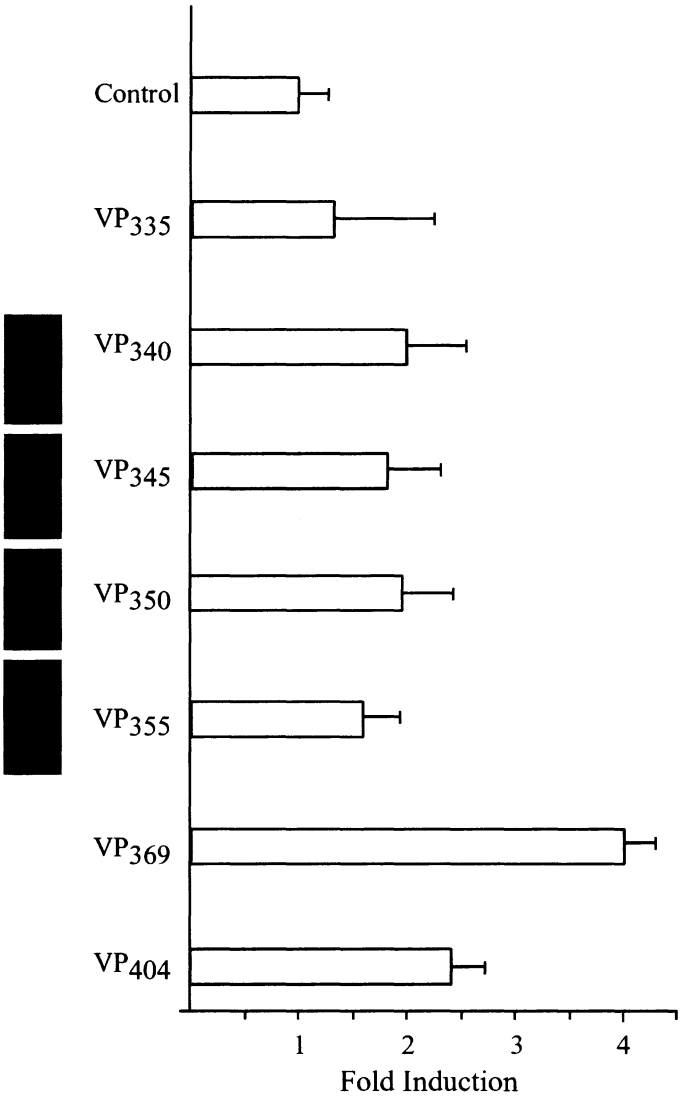

Figure 4.2.2. GAL4DB-VP16 deletion mutant activation in mammalian cells. The carboxyl-terminal deletions of VP16 were fused to the GAL4 DNA binding domain within a mammalian expression vector and transiently transfected into COS-1 cells (0.25μg) along with a GAL4 responsive reporter plasmid; 5xGAL4-*luc* (0.5μg). The activity is presented as fold induction relative to the GAL4 DNA binding domain ('control'), alone in combination with the reporter plasmid, given a value of one. The data for each activity was compiled from three independent tranfections of duplicates. The error within the resulting activities was calculated by standard deviation. The expression of the newly constructed fusions was demonstratedby Western blot analysis using a VP16 polyclonal antibody against VP16 residues 5 to 411 ('Sal'; 1:1000), and is indicated to the left of each corresponding activity.

Therefore, while residues 345 to 350 were determined to be important for the GAL4DB-VP16 transactivation phenotype in yeast, the region encompassing residues 355 to 369 appears to retain a determinant for activity in mammalian cells, indicating a divergence between the activities in yeast and mammalian cells.

4.2.3 Discussion

VP16 has long been regarded as a potent viral transactivator as a result of its AAD, thus making the novel finding of an activation domain within the amino-terminal region of VP16 appended to the GAL4 DNA binding domain in (both) yeast and mammalian cells particularly intriguing. Furthermore, additional evidence for an activation domain was taken from *in vitro* transcription experiments utilizing a truncated VP16 protein (Wu *et al.*, 1994), and from VP16 orthologs; which function as transactivators without an apparent AAD (Elliot, 1994, Misra *et al.*, 1995, Moriuchi *et al.*, 1995). For example, VZV ORF10 is capable of forming a functional VIC complex on a TAATGARAT element and promoting transcriptional activation (Moriuchi *et al.*, 1993), though it lacks an AAD. Of course, the possibility that VP16 activation phenotype in yeast did not have any bearing on the function of VP16 within mammalian cells, or within the context of a HSV infection, could also not be excluded. In fact, recent studies utilizing a truncated VP16 in yeast within the context of VIC, found that the AAD is required in order to activate transcription from a TAATGARAT promoter construct (Wilson *et al.*, 1997). These results raise the possibility that within the context of VIC, an activation domain within the amino-terminus of VP16 may be masked, or require the

AAD, or be promoter specific, or even be completely irrelevant. Accordingly, the VP16 AAD is extremely potent when appended to the GAL4DB, but appears to be muted within the context of VIC (Douville *et al.*, 1995, Hagmann *et al.*, 1997).

Nevertheless, the carboxyl-terminal deletions of VP16 indicate that the critical residues responsible for the transactivation phenotype in yeast are localized to amino acids 345 to 350. A striking feature of those residues, and those further downstream to amino acid 353, is the preponderance of polar amino acids. Notably, regions rich in serine/threonine residues, as well as glutamine, proline and acidic residues are involved in transcriptional activation (Tjian and Maniatis, 1994, Blau *et al.*, 1996). Serine/threonine rich activation domains, for the most part, have been shown to activate gene expression as a result of upstream cellular signaling mechanisms that modulate their activity predominantly via phosphorylation (Hunter and Karin, 1992, Holmberg *et al.*, 2001). However, this is probably not the case for VP16 since it appears to be phosphorylated exclusively downstream of residues 345 and 350 (O'Reilly *et al.*, 1997). Furthermore, since the site-specific mutants to alanine did not alter activity, it is unlikely that transactivation would be regulated through the modification of a single residue. Alternatively, homopolymeric amino acid expansions are known to be involved in transcriptional activation (Gerber *et al.*, 1994), possibly making this region the target of a negative regulatory response in mammalian cells that may not be present within yeast.

Several possibilities still remain regarding a mechanism for the yeast transactivation phenotype of VP16, which have yet to be explored. The amino-terminal region of VP16 (without its AAD) interacts with the TATA-binding protein (TBP)

(Tansey and Herr, 1995). The interaction between VP16 and TBP is not sufficient to support activation in transiently transfected HeLa cells, though it may be able to support activated transcription in yeast, since the GAL4-TBP is sufficient to transactivate reporter gene expression in yeast (Chatterjee and Struhl, 1995). The recruitment of TBP may overcome the need for additional TAFs in yeast, or bypass their requirement, making it a source of the differential activity found between yeast and mammalian cells (Walker *et al.*, 1996). It would be interesting to see if the mutant profile of the active GAL4-VP16 fusions correlates with binding to TBP. Another notable difference between yeast and mammalian systems lies in the fact that yeast contains a chromosomally embedded GAL1 promoter sequence, while reporter gene activity in mammalian cells takes place from a co-transfected plasmid.

In addition, it would not be surprising to find that VP16, as with other transactivators, possesses multiple activation domains. Transcription factors such as Oct-1 (Cleary *et al.*, 1993), SP1 (Courey and Tjian, 1988) and even the AAD of VP16 (Cress and Triezenberg, 1990, Regier *et al.*, 1993), contain multiple activation domains that interact with distinct co-activator proteins (Lemon and Tjian, 2000, Naar *et al.*, 2001). Diverse activation domains could also be used to mediate promoter specific activation (Emami *et al.*, 1995, Gaudreau *et al.*, 1999), or in order to promote functional synergy (Emami and Carey, 1992, Chang and Gralla, 1994, Merika and Thanos, 2001). Ultimately, the characterization of another activation domain within VP16 has the potential to lead to the elucidation of an important regulatory mechanism, even though a

functionally relevant activation domain within VP16 may have become redundant with the addition of the potent AAD.

4.3 HCF-1 co-activation of VP16

A complex array of protein interaction networks is responsible for mediating transcriptional activation within cells (Hochheimer and Tjian, 2003). As previously shown, experiments investigating the differences in transactivation activity of a truncated VP16 protein fused to the GAL4 DNA binding domain between yeast and mammalian cells, concluded that the amino-terminal 355 residues of VP16 are sufficient to activate reporter gene expression in yeast when fused to the GAL4DB. The activity in mammalian cells was subsequently localized to a region between residues 355 and 369. Since the region encompassing residues 355 to 369 of VP16 lacks an apparent transcriptional activation domain, it was possible that the activity was mediated through an interacting partner, such as a transcriptional co-activator. The only known interacting partner of the amino-terminal region of VP16, in mammalian cells, is HCF-1 (Wilson *et al.*, 1993, Freiman and Herr, 1997). The possibility that HCF-1 was implicated in VP16 activation was given even greater consideration since recent findings in our laboratory (Wong, 2002) and others (Lu *et al.*, 2000, Luciano and Wilson, 2002), demonstrated that HCF-1 contains an autonomous activation domain when appended to the GAL4 DNA binding domain. In addition, HCF-1 also appears to bind a growing list of transcription factors, further validating it as a putative co-regulatory factor. Specifically, HCF-1 co-activates GABP (Vogel and Kristie, 2000), LZIP (Lu *et al.*, 1997, Luciano and Wilson, 2000) and

Table 4.3. The amino-terminal 369 residues of VP16 are sufficient to interact with HCF-1[a].

Plasmids	β-galactosidase activity Overlay assay	
	DB	DB-HCF
GAL4AD	-	-
GAL4AD-VP_{369}	-	+
GAL4AD-VP_{404}	-	+

a. Two-hybrid assays were conducted in yeast PCY2. The amino-terminal 380 residues of HCF-1 was fused to the GAL4 DNA binding domain. VP369 and VP404 refer to the amino-terminal 369 and 404 residues of VP16, respectively. Both were fused to the GAL4 activation domain. '+' indicates an interaction (blue colour in < 30min), while '-' indicates that no colour formation occurred following an overnight incubation.

also full-length VP16 (Luciano and Wilson, 2002). In pursing the possibility that HCF-1 could be involved in mediating the transactivation phenotype of GAL4-VP16 in mammalian cells, HCF-1 was subsequently co-incubated with the amino-terminal 369 residues of VP16 in a two-hybrid assay to establish a potential interaction (Table 4.3). Given that the amino-terminal 369 residues of VP16 (GAL4-VP$_{369}$) are sufficient to interact with HCF-1, we wanted to investigate this interaction with respect to its activation phenotype of VP16 in mammalian cells. As is demonstrated further here, a functional role for HCF-1 in VP16 mediated activation suggests a more complex framework in which to contemplate VP16 transcriptional activation.

In order to assess the ability of endogenous HCF-1 to contribute to the activation phenotype of VP16 in mammalian cells, a site-specific mutation was constructed at residue 364 (Y to A) within GAL4-VP$_{369}$. This mutant has previously been shown to abrogate binding to HCF-1 (LaBoissiere *et al.*, 1999), and is part of the recently described HCF-1 binding motif (HBM) found between residues 361 to 365 (**EHAYS**) of VP16 (Freiman and Herr, 1997, Lu *et al.*, 1998). Therefore, COS-1 cells were transiently transfected with the GAL4 reporter plasmid and various GAL4DB-VP16 constructs, including GAL4DB-VP$_{369(Y364A)}$. GAL4DB-VP$_{369}$ activates reporter gene expression by 4-5 fold, consistent with previous results (Popova et al., 1995), while GAL4DB, GAL4DB-VP$_{335}$, and GAL4DB-VP$_{369\ (Y364A)}$ were inactive (Figure 4.3A). The difference in activity cannot be attributed to lack of expression of the Y364A mutant since it is expressed to comparable (or even greater) levels than the wild-type construct (Figure 4.3B). While these experiments implicate endogenous HCF-1 in mediating VP16

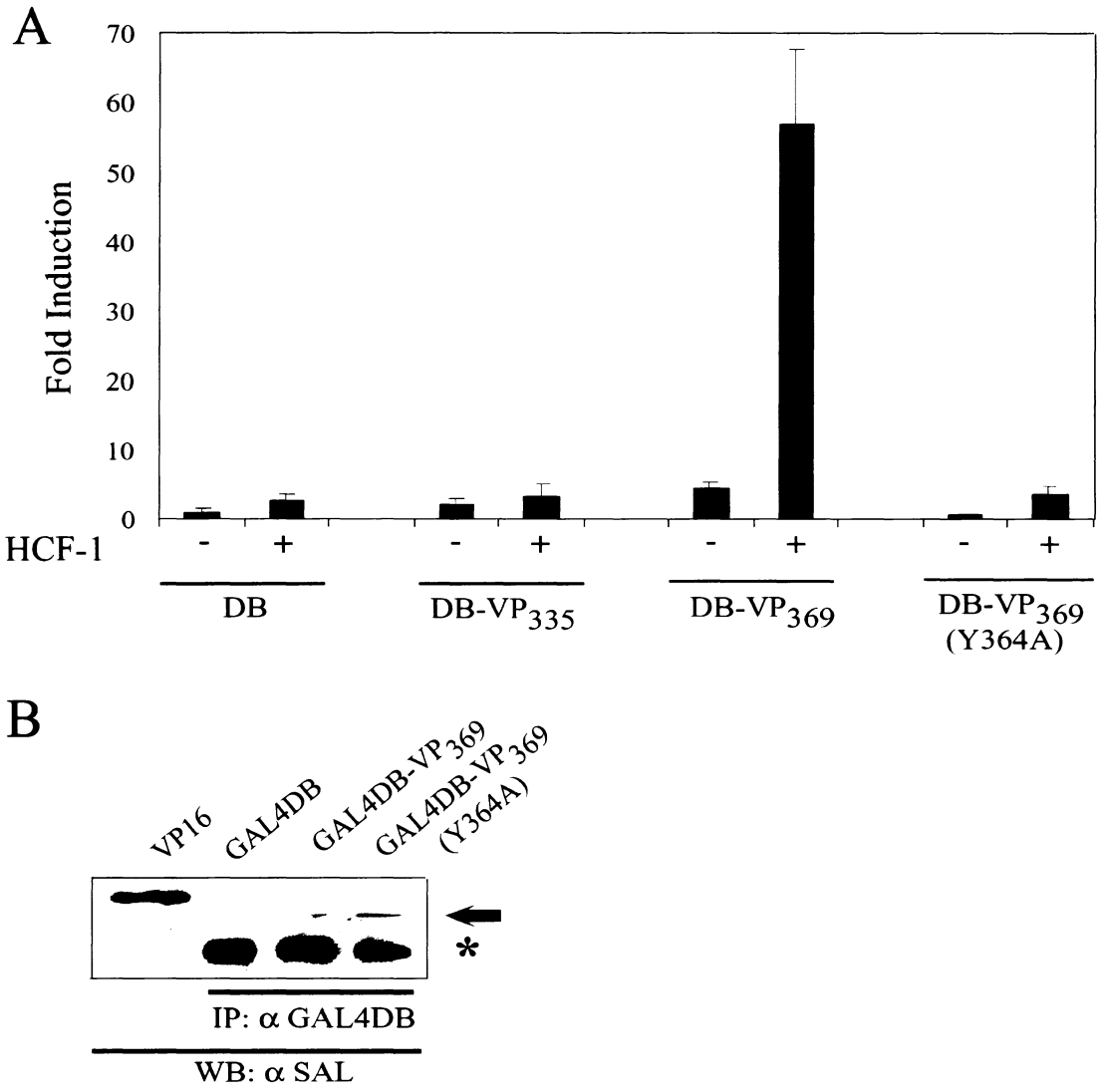

Figure 4.3. HCF-1 is a co-activator of VP16. A. COS-1 cells were transiently transfected with GAL4 fusions (0.25μg), 5xGAL-luc reporter (0.5μg), and pCGNHCF (1.25μg). VP335 and VP369 represent carboxyl-terminal deletions within VP16 to the indicated amino acid. VP16 (Y364A) has previously been shown to abolish binding to HCF-1 (LaBoissiere et al., 1999). Three independent experiments in duplicate were used to generate the activities. Error was calculated by standard deviation. The activities were made relative to the DB control, which was given an activity unit of one. **B.** An immunoprecipitation (IP) assay was carried out with 2.5μg anti-GAL4DB, and the Western blot with anti-$VP16_{SAL}$ (1:1000), a polyclonal antibody directed against residues 5-411 of VP16. The VP16 control sample was obtained from HeLa cells infected with HSV1. The arrow denotes the position of the VP369 fusions, and the asterisk indicates a non-specific band. Both of these experiments (A and B) were conducted by P. Bilan.

activation, we also examined the effect of exogenously added HCF-1. As also shown in Figure 4.3A, the addition of HCF-1 significantly potentiates GAL4-VP$_{369}$ activity (80 fold compared to 5 fold), but not that of GAL4DB, GAL4DB-VP$_{335}$ or GAL4DB-VP$_{369(Y364A)}$. Together, these results suggest that HCF-1 is a co-activator of the GAL4DB-VP$_{369}$ activation phenotype in COS-1 cells, and provides a rationalization for the presence of an activation domain within the amino-terminal region of VP16. These results also demonstrate that HCF-1 is capable of significant co-activation of VP16 as a GAL4DB fusion when added exogenously, suggesting that the level of endogenous HCF-1 within COS-1 cells is sub-optimal for this activity. Notably, HCF-1 also activates the reporter construct approximately six-fold with all of the other GAL4 DNA binding domain constructs, including the empty vector control (Figure 4.3A). The source of this independent activation is not presently clear, though an interaction with another factor, or the presence of a cryptic binding element within the reporter plasmid that is responsive to HCF-1, as possibilities. An analysis of the potential significance of this activation is discussed later in the context of subsequent results.

To better understand and characterize HCF-1 co-activation, a series of mutagenesis experiments in our laboratory (Wong, 2002), and others (Luciano and Wilson, 2002) have localized a isolated region within HCF-1 that is capable of transactivation when appended to a heterologous DNA binding domain, which corresponds to the 'acidic' domain (see Figure 1.4.4). Unpublished work from our laboratory has also found a potential transactivation domain within the amino-terminal basic domain of HCF-1 (D. Piluso, pers. comm.). As such, we sought to characterize

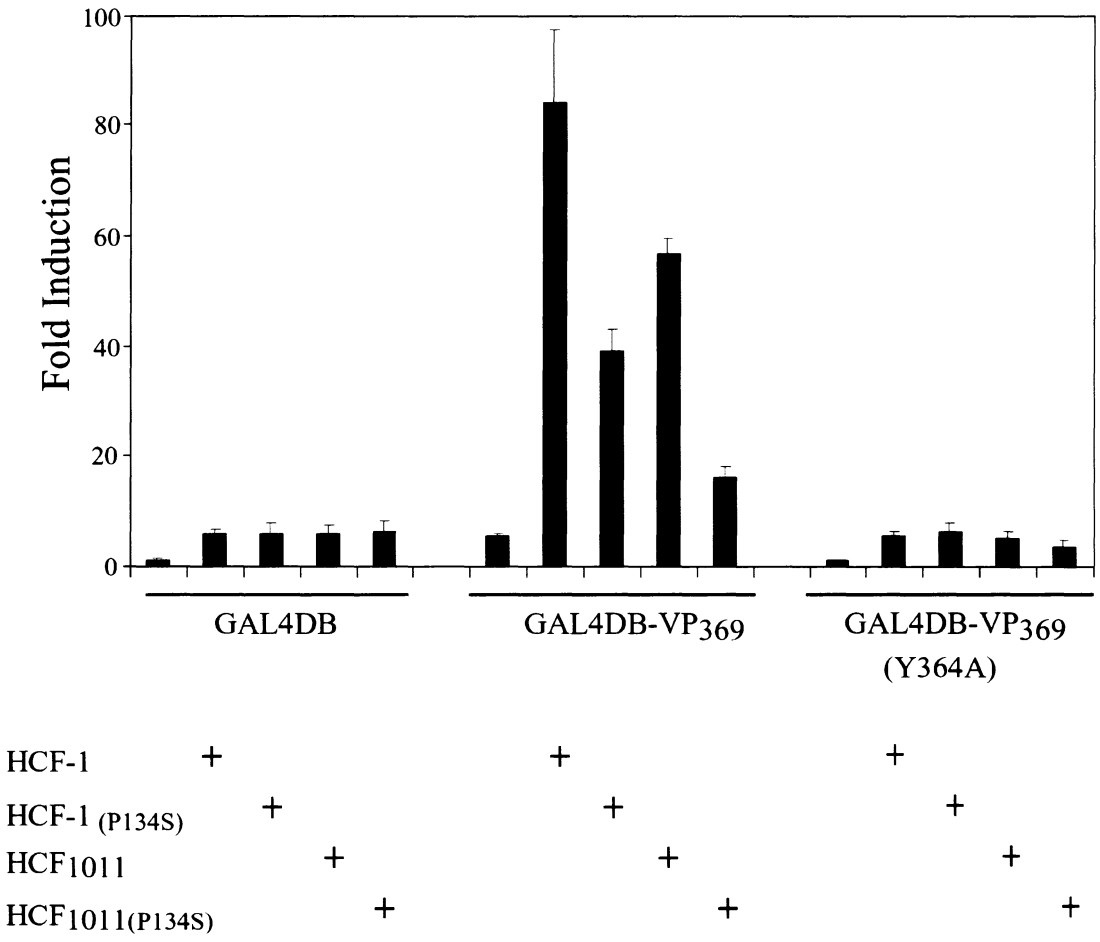

Figure 4.3.1. Mutants of HCF-1 can co-activate VP16. Various mutants of HCF-1 were utilized to map the region(s) within HCF-1 that contribute to the co-activation of VP16. HCF-1 (or mutants thereof; 1.25μg) were transiently transfected with GAL4 DNA binding domain fusions to VP16 (0.25μg) and a GAL4 responsive reporter plasmid (5xGAL4-*luc*, 0.5 μg). The corresponding empty vector control for HCF-1, along with the reporter plasmid, was given an activity unit of one such that the activities of the other fusions could be represented as fold induction relative to it. Activites were collected from duplicates of three independent transfections. Error was determined by standard deviation. HCF_{1011}; amino-terminal 1011 residues of HCF-1.

further the region(s) in HCF-1 responsible for co-activating GAL4DB-VP369. Furthermore, the utilization of GAL4DB-VP16 fusions in this context is useful in terms of providing a model system in which to examine region(s) that contribute to the co-activation function of HCF-1. Therefore, as demonstrated in Figure 4.3.1, various mutant constructs of HCF-1 were co-transfected with GAL4DB-VP_{369} in COS-1 cells. The truncated HCF-1 constructs, despite not having a NLS, have been shown to independently localize to the nucleus (Wysocka *et al.*, 2001b). The HCF-1 mutant corresponding to the amino-terminal 1011 residues has significant activity compared to wild-type HCF-1 (80-fold vs. 50 fold), suggesting that the amino- terminal region of the protein possesses significant activation potential, in that context. Additionally, and somewhat surprisingly, the P134S mutant also retained the ability to co-activate GAL4DB-VP_{369}. The P134S mutant does not interact with VP16 or form VIC within *in vitro* binding assays (Goto *et al.*, 1997, Wilson *et al.*, 1997), although others have demonstrated a weak interaction may be present under alternate circumstances (LaBoissiere and O'Hare, 2000). The P134S mutant, in the context of full-length HCF-1, was nearly as effective as the wild-type protein in activating reporter gene expression (65 fold; Figure 4.3.1).

As with the full-length HCF-1 construct, all its derivatives activated reporter gene 5xGAL-pGL3$_{SV40}$ expression by approximately 6-fold (Figure 4.3.1), but not on an analogous reporter construct lacking only the SV40 basal promoter (pGL3 Basic) (P. Bilan, pers. comm.). Consequently, HCF-1 activation of reporter gene expression may be occurring through a component of the SV40 early promoter, which contains SP1 and

other transcription factor binding sites (Courey and Tjian, 1988, Wildeman, 1988). Therefore, HCF-1 or its derivatives co-activate a truncated VP16 protein fused to a heterologous DNA-binding domain in mammalian cells, through what appears to be both a binding dependent- and independent- mechanism. The 'binding dependent' mechanism results in HCF-1 co-activation of VP16. The 'binding-independent' mechanism may occur by establishing synergy between VP16 and adjacent transactivator, for instance within the SV40 promoter, which then potentiates VP16 activity. The specific contribution(s) of each mechanism to the activity cannot be differentiated based on the experiments shown here; however, the simultaneous use of both mechanisms is probably the most likely scenario.

4.3.1 Discussion

Based upon its recognition of VP16, HCF-1 may serve as a key initiator of the viral lytic cycle; thus giving it a critical role in HSV biology. VP16 recognition by HCF-1 may serve as an indicator (or sensor) for the biological state of the cell, allowing the virus to gauge the suitability of proceeding with a productive viral infection (Wysocka *et al.*, 2001b, Wysocka and Herr, 2003). The function of a sensor during an infection may be to optimize the production of virus, or to allow for more efficient evasion of host antiviral defense mechanisms. Therefore, HCF-1 could act as a determinant for establishing a lytic or latent infection, in addition to being a key regulator in the reactivation from latency (Kristie *et al.*, 1999, Wysocka and Herr, 2003). In proliferating cells, HCF-1 is abundant and found in the nucleus where it carries out its role in HSV gene expression (Wysocka *et*

al., 2001b), whereas in neurons it is sequestered in the cytoplasm; thus unable to support VP16 transactivation. Interestingly, HCF-1 is induced to localize to the nucleus in conjunction with signals that cause reactivation of HSV from its latent state (Kristie *et al.*, 1999). Moreover, the levels of Oct-1 and GABP also increase in neurons in response to reactivation stimuli, which in conjunction with HCF-1, provides an interesting model in which to investigate gene expression mechanisms upon reactivation from latency (Kristie *et al.*, 1999). Perhaps the involvement of HCF-1 in immediate-early gene expression, in the absence of VP16, can offer some perspective as to the activation of viral immediate-early genes and subsequent virus replication during latency. If HCF-1 functions as a sensory protein during an HSV1 infection, its role with respect to VP16 activation and the balance achieved in determining the lytic versus the latent cycle, would be critical to future studies of HSV pathogenesis.

4.4 HCF synergizes with VP16 in the activation of HSV IE genes

The discovery of a role for HCF-1 in the co-activation of VP16, prompted us to investigate HCF-1 in VP16 mediated transactivation of viral IE genes. An independent functional role for HCF-1 in HSV1 gene expression also stems from other findings indicating that: i) HCF-1 interacts with cellular transcription factors, including SP1 (Gunther *et al.*, 2000) and GABP (Vogel and Kristie, 2000), both of which are directly involved in HSV1 gene expression, ii) HCF-1 co-localizes with HSV DNA at early times following an infection, in the absence of VP16 (LaBoissierre and O'Hare, 2000), and iii) HCF-1 homologs HCF-2 and ceHCF, are diminished in their ability to carry out

transactivation of viral IE genes, though they are unaffected for complex assembly with VP16 (Lee and Herr, 2001). At the outset of this work, a functional role for HCF-1 with respect to viral IE gene expression had not previously been explored, though it has recently been demonstrated (Luciano and Wilson, 2002). HCF-1 is shown here to affect viral immediate-early gene expression through at least two mechanisms: First, it is capable of activating the expression of a reporter gene linked to the promoter element for ICP4 in the absence of VP16. Second, it potentiates VP16 activity on an IE gene reporter construct in transiently transfected cells. Therefore, in addition to a newly established role for HCF-1 in promoting transcriptional activation within the context of VIC (Luciano and Wilson, 2002), these results demonstrate that HCF-1 is capable of synergizing with VP16 in the activation of viral IE genes, in part via a mechanism that appears to be independent of binding to VP16.

4.4.1 HCF-1 synergizes with VP16 in the activation of an immediate-early promoter

HCF-1 has only recently been shown to function as a potential transcriptional co-activator to a variety of transcription factors (Lu *et al.*, 1998, Vogel and Kristie, 2000, Gunther *et al.*, 2000, Luciano and Wilson, 2002, Wysocka *et al.*, 2003). Thus, VP16 activation of an immediate-early promoter construct in transiently transfected cells was used as the model system to determine whether HCF-1 functions as a co-activator within the VP16 induced complex, thus providing additional regulation of HSV1 gene expression. The ICP4 immediate-early (α 4) promoter region contains cis-acting response elements for VP16, SP1 and GABP (Triezenberg *et al.*, 1988b), and was used here to

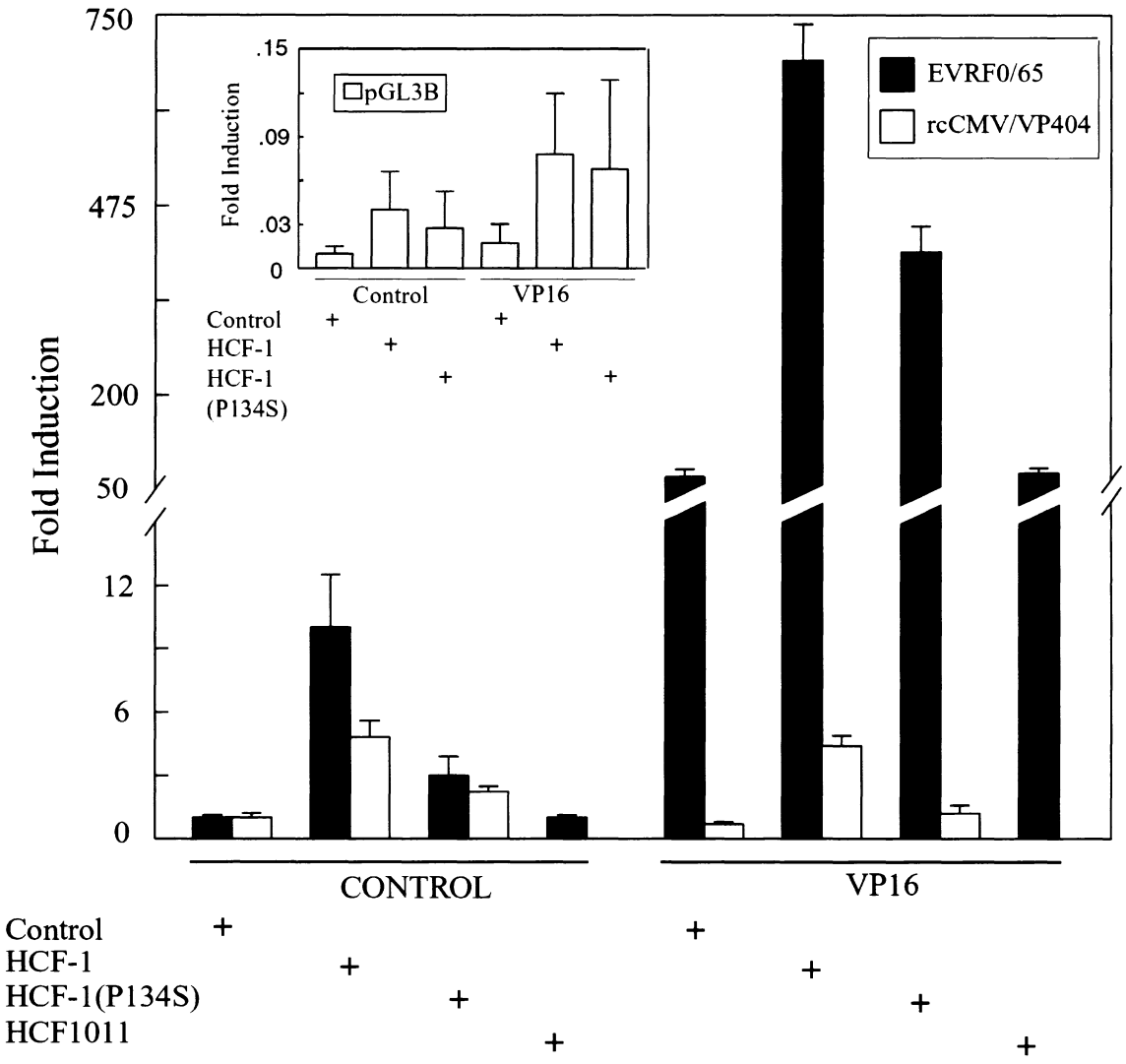

Figure 4.4.1. HCF-1 is involved in the activation of an HSV IE gene. The role of HCF-1 in the activation of an ICP4-linked reporter gene was investigated. Cos-1 cells were transiently transfected with VP16 (10ng of pEVRF65, 0.5μg of pRC/CMV-VP404), HCF-1 (1.8μg with VP16, 1.4μg with VP404) and the reporter plasmid containing the ICP4 promoter element α4-*luc* (0.1μg). The inset represents the results from an analogous set of experiments done with the control reporter plasmid pGL3-Basic. Control samples for VP16 , VP404 and HCF-1 utilized pEVRF0, pRC/CMV and pCGN vectors, respectively. The control samples with respect to HCF utilized the pCGN vector. The fold induction was calculated with respect to pEVRF0/pCGN/α4-*luc* sample, which was given a relative activity unit of one. The activities were compiled from duplicates of at least three independent experiments. Error was calculated by standard deviation. HCF_{1011}; amino-terminal 1011 residues of HCF-1.

investigate the role of HCF-1 in VP16 mediated viral gene expression. As demonstrated in Figure 4.4.1 (solid bars), the addition of both the wild-type and P134S mutant of HCF-1 to VP16, in COS-1 cells, significantly potentiates VP16 activation of the ICP4-linked reporter gene. However, in contrast to the full-length constructs, HCF_{1011} did not provide any synergy with VP16 in this context. Recall that in the experiments conducted with the GAL4 DNA binding fusions, HCF_{1011} was an effective co-activator of VP16 within that context. Therefore, the domain(s) required to promote synergy with VP16 is localized to the carboxyl-terminal region of the protein, which is consistent with the recent data presented by Luciano and Wilson (2002). These authors demonstrated conclusively that a carboxyl-terminal activation domain is present within HCF-1, and that VIC formation alone does not play a role in promoting activation by HCF-1. It is also possible that the sub-saturating amounts of VP16 used within these experiments may result in the majority of the VP16 binding to endogenous HCF-1, which would presumably minimize the effect of exogenously added HCF-1 on complex formation.

Both wild-type and P134S full-length constructs, but not HCF_{1011}, are also able to enhance luciferase expression significantly above background even in the absence of VP16. Since HCF-1 is not known to bind DNA (Kristie *et al.*, 1993), this activity may be occurring in conjunction with other factors within the ICP4 promoter. Accordingly, the transcription factor binding sites for Sp1 and GABP are present within the proximal promoter regions of HSV immediate-early genes, and may provide targets for HCF-1. Moreover, the binding determinants for interactions between HCF-1 and Sp1 or GABP are present within the basic domain, thus making the activity of the P134S mutant

plausible. Alternatively, HCF-1 may function through another mechanism, including other transcription factors or through post-transcriptional gene regulation (Ajuh *et al.*, 2002). Experiments with the pGL3 BASIC reporter (lacking the entire α4 promoter) show that the activity of HCF-1 and VP16, or both, were specifically dependent upon the presence of the ICP4 promoter sequence (Figure 4.4.1; inset).

We also wanted to determine if the activity mediated by HCF-1 was dependent on the presence of the VP16 AAD. As demonstrated in the previous section, HCF-1 co-activates VP16, in the absence of its AAD when it was appended to the GAL4 DNA binding domain. We hypothesized that an autonomous activation domain within HCF-1 may contribute to transactivation within VIC, even in the absence of its AAD, due to its localization to the promoter element within VIC. Therefore, transient transfections were carried out in COS-1 cells with an amino-terminal 404 residue mutant of VP16 (RC/CMV-VP_{404}) and HCF-1. Although the truncated VP16 protein is capable of promoting complex formation as efficiently as the full-length protein, HCF-1 synergy of VP16 activation of reporter gene expression does in fact require an intact AAD to be present within VP16 (Figure 4.4.1; open bars). These results are also consistent with those recently reported by Luciano and Wilson (2002), whose work indicates that the VP16 AAD is critical to HCF-1 co-activation. Differences in HCF-1 co-activation of VP16 as a GAL4 DNA binding domain fusion, or as a component of VIC may be due to orientation dependent effects or as a result of differences in promoter context (Hochheimer and Tjian, 2003). Indeed, these differences (GAL4 fusions versus VIC) are also observed in yeast, where a putative autonomous activation domain is present within

a truncated VP16 protein but is not observed within the context of VIC (see section 4.2). Taken together, these results indicate that HCF-1 acts synergistically with VP16 through a HSV IE regulatory sequence to activate its expression. The synergy created between HCF-1 and VP16 was shown to be dependent on both the carboxyl-terminal region of HCF-1 and the AAD of VP16, as well as an intact promoter region of the ICP4 gene. The requirements outlined here for HCF-1 dependent co-activation of VP16 within VIC, differ from those involving VP16 fused to the GAL4 DNA binding domain, though both systems appear to indicate that potentiation by HCF-1 may occur independently of binding to VP16, possibly through additional promoter-bound factors.

4.4.2 HCF-1 potentiates VP16 activation of a heterologous promoter

To further explore the possibility that the potentiation of VP16 by HCF-1 was due to various cis-acting elements within the ICP4 promoter (eg. GABP or SP1), as opposed to the TAATGARAT elements, oligonucleotides corresponding to the TAATGARAT sequence were generated and cloned upstream of the SV40 early promoter in the pGL2 reporter plasmid. Three tandem TAATGARAT elements fused upstream of the SV40 early promoter in the pGL2 reporter plasmid, were co-transfected with a VP16 expression plasmid into COS-1 cells. The addition of VP16 resulted in a 6-fold activation of luciferase reporter activity (Figure 4.4.2), consistent with previous studies (Latchman *et al.*, 1989, Lillycrop *et al.*, 1993, Douville *et al.*, 1995). In the presence of wild-type HCF-1 or HCF-1$_{P134S}$, VP16 mediated activity was significantly enhanced, resulting in 70 and 60-fold activation of the reporter gene, respectively. Potentiation by the HCF-1$_{P134S}$

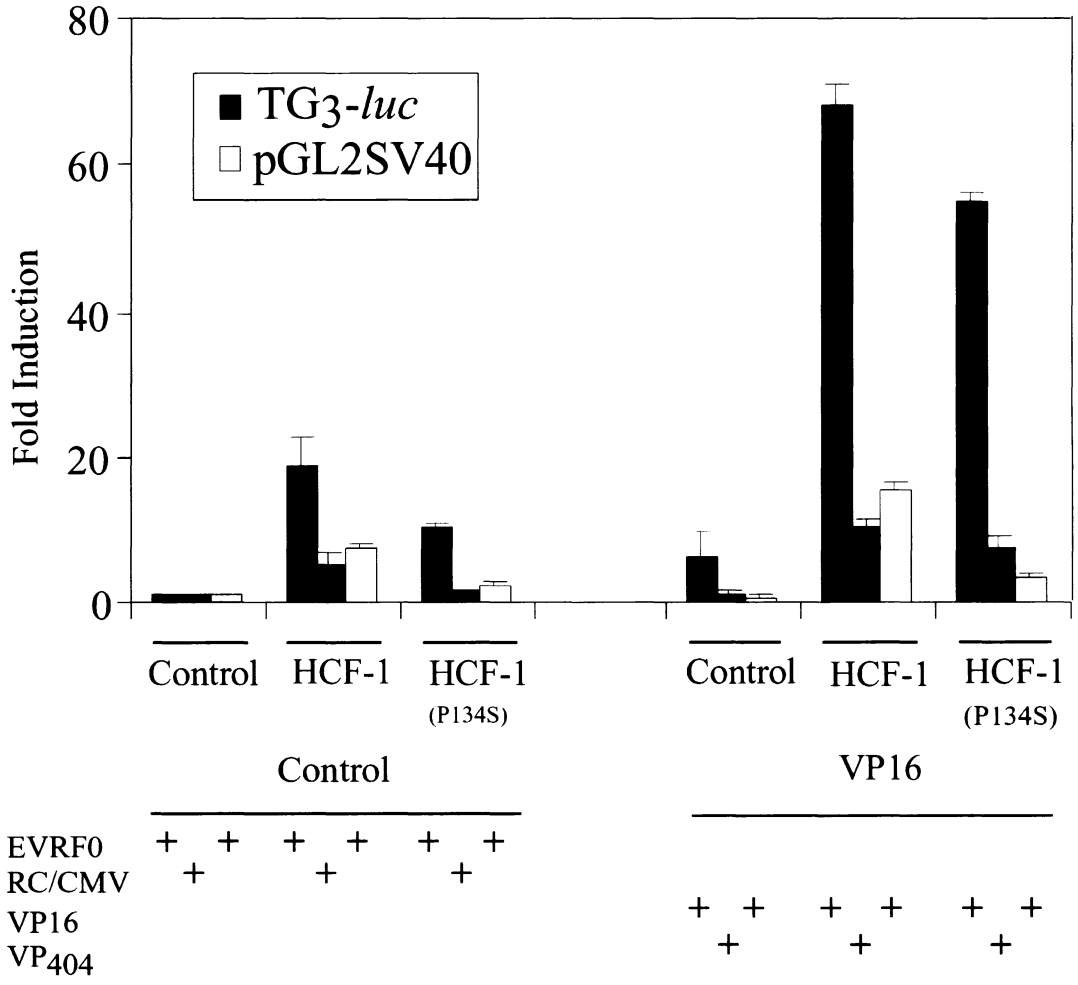

Figure 4.4.2. HCF-1 promotes synergy of VP16 activation on a heterologous promoter. COS-1 cells were transiently transfected with VP16 (10ng pEVRF65, 0.5μg pRC/CMV-VP404), HCF-1 (1.4μg) and TG3-*luc* (0.25μg), or their respective control plasmids. TG3-*luc* contains three tandem (5' to 3') ICP4 octa^{+} TAATGARAT elements upstream of the SV40 early promoter (pGL2-SV40). The pCGN empty vector plasmid was used as a control for HCF-1. The data was generated from duplicates of three independent experiments . Standard deviation was used to determine the amount of error present within each data set. The pEVRF0/pCGN/TG3-*luc* sample was given a relative activity of one, from which the fold induction was determined.

mutant further establishes that the synergy created between HCF-1 and VP16 likely occurs by a mechanism that is not entirely dependent upon HCF-1 binding to VP16, and therefore VIC formation. Additionally, as demonstrated in Figure 4.4.2, in the absence of VP16, both wild-type and mutant HCF-1 were able to activate reporter gene expression (18-fold and 10-fold, respectively). However, in the absence of the upstream TAATGARAT elements (ie. pGL2 reporter gene control), this activity was significantly reduced (8-fold and 2-fold, respectively). Therefore, it would appear that isolated TAATGARAT elements contribute, at least partially, to activation by HCF-1 in the absence of VP16. Since the TAATGARAT oligonucleotide elements contain overlapping octamer binding sites for Oct-1, in the absence of VP16, HCF-1 may function through Oct-1 (Wu *et al.*, 1994). Binding assays have indicated that HCF-1 interacts with Oct-1 (Mahajan and Wilson, 2000). Additionally, a portion of the HCF-1 activity is localized to the SV40 early promoter in pGL2 (10 fold activity over control for HCF-1, 3 fold for HCF-1$_{P134S}$; Figure 4.4.2). The SV40 early promoter contains numerous transcription factor binding sites, including several for SP1 (Courey and Tjian, 1988), consistent with the possibility that HCF-1 (and HCF-1$_{P134S}$) is capable of activation through these sites. As is also demonstrated in Figure 4.4.2, HCF-1 also requires both the VP16 (AAD) and cis-acting TAATGARAT elements in order to promote synergy with VP16. The fact that HCF-1 potentiation of VP16 requires an intact AAD within VP16 and the presence of TAATGARAT cis-acting elements, further denotes the central role for VP16 in mediating this activity.

4.4.3 Synergy provided by HCF-1 is observed in various cell lines

In order to rule out a cell-type specific effect in regard to the HCF-1 mediated synergy of VP16, transient transfections were conducted in other cell lines, including within HeLa and Vero cells. As demonstrated in Figure 4.4.3, the potentiation of VP16 by HCF-1 does occur in both of these cell lines, and is largely analogous to the activity observed in COS-1 cells (see Figure 4.3.1), through some notable differences were present. The amount of activation of the reporter gene in the absence of VP16, by HCF-1 and its P134S mutant, while quite robust within Vero cells (20- and 30-fold above the control, respectively), was only marginally detectable over the control (six and three fold, respectively) in HeLa cells. The greater potentiation of VP16 by HCF-1 in Vero cells could also be correlated with the VP16-independent HCF-1 activity, which was more pronounced in those cells. As was seen in other experiments, the level of VP16-independent HCF-1 activation appeared to be directly proportional with the amount of synergy that occurred.

Additionally, a useful tool in the analysis of HCF-1 function emerged with the discovery of a cell line (tsBN67) possessing a temperature-sensitive cell cycle defect attributed to HCF-1 (Goto *et al.*, 1997). This defect also coincides with abrogated VIC formation. As such, the effect of exogenously added HCF-1 on VP16 activation of IE gene expression within this mutant background was investigated. Using transient transfections at both the permissive (32 ^{o}C) and non-permissive (39.5 ^{o}C) temperatures of tsBN67 cells, we expected that the synergy promoted by HCF-1 would be lacking or severely diminished due to the absence of VIC within these cells. tsBN67 cells were

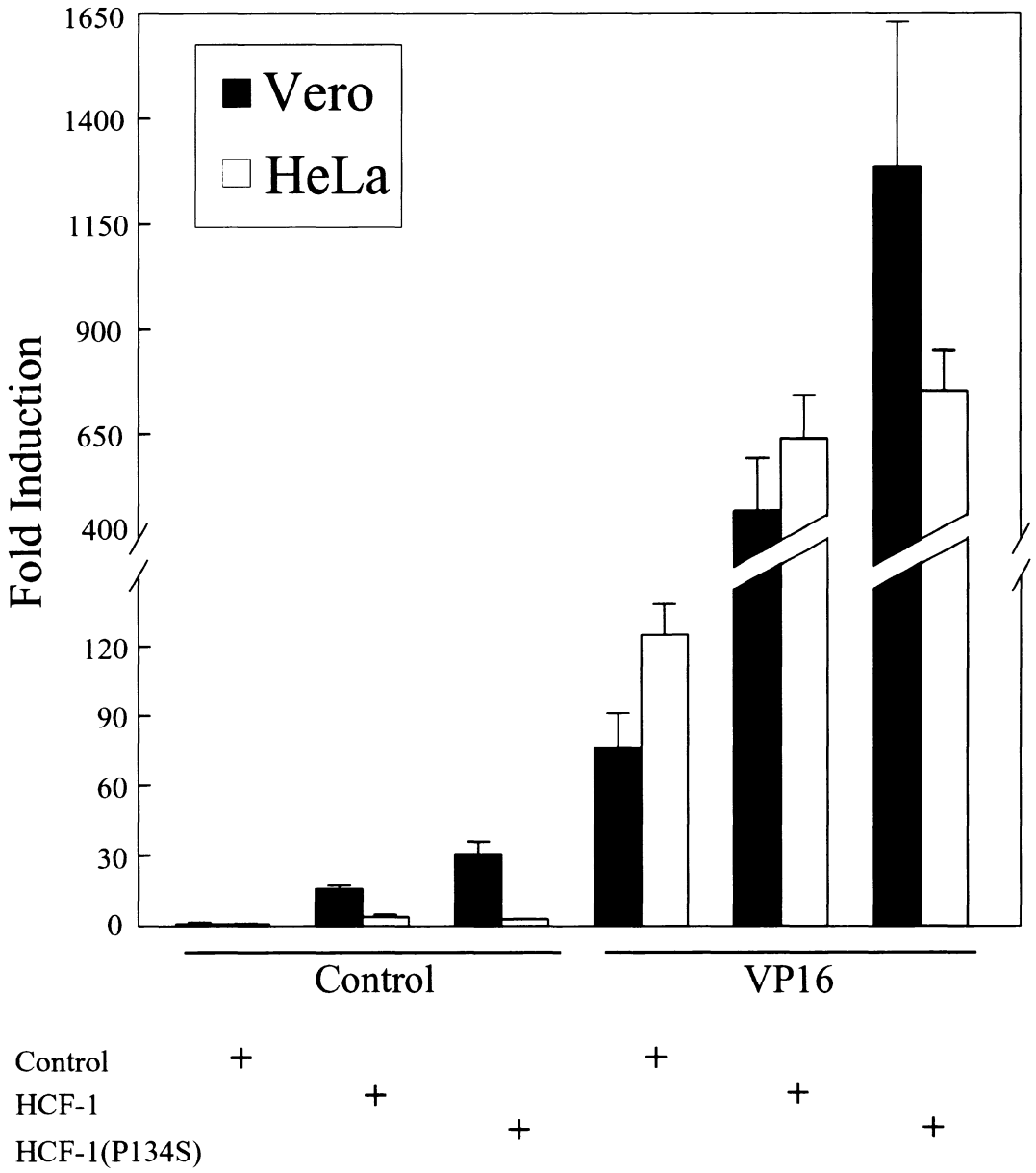

Figure 4.4.3. HCF-1 potentiates VP16 activation in diverse cell lines. HeLa and Vero cells were transiently transfected with VP16 (10 ng of pEVRF65) and HCF-1 (1.8 μg), or their corresponding control plasmids, along with α4-*luc* (0.1μg). Control plasmids used for VP16 and HCF were pEVRF0 and pCGN, respectively. Activities were compiled from duplicates of three independent experiments, with stardard deviation being used to determine the amount of error present. The activities of the combined control samples (pEVRF0/pCGN) was used, separately within each cell line, to determine the relative activity, which was made to be one unit.

transiently transfected with VP16 in the presence or absence of VP16. As shown in Figure 4.4.3.1, VP16 is still able to substantially activate reporter gene expression (34-fold) at the permissive temperature without HCF-1, and this activity is potentiated by both the wild-type (66-fold) and P134S mutant (105-fold) HCF-1 constructs. At the non-permissive temperature, activation of the reporter construct by VP16 was significantly reduced (17-fold), as mostly anticipated. Similarly, as shown by LaBoissierre and O'Hare (2000), the expression of the immediate-early genes and subsequent progression through the viral lytic cycle is relatively unaffected at the permissive temperature, and significantly diminished, though still apparent, at the non-permissive temperature.

Although the P134S mutant abrogates VIC assembly *in vitro*, some amount of binding occurs *in vivo*. In addition, LaBoissierre and O'Hare (2000) were also unable to demonstrate a significant effect on IE gene expression in the presence of an integrated copy of wild-type HCF-1 within tsBN67 cells, implying the presence of an additional unrecognized defect in these cells which may also impact upon the results reported here. Alternatively, transiently transfected tsBN67 cells may not fully reflect the temperature-sensitive phenotype, or the concomitant loss of the endogenous HCF-1$_{P134S}$ from the nucleus, preceding cell cycle arrest, which results in decreased activation of HCF-1 target genes (Wysocka *et al.*, 2001b). The residual activity may also have been present during the lag in the shift from the permissive to the non-permissive temperature. Interestingly, the ability of both wild-type and P134S mutant HCF-1 to activate reporter gene expression in the absence of VP16, particularly at the non-permissive temperature, was significantly diminished (if not lacking) (Figure 4.4.3.1). Since the tsBN67 cells are all

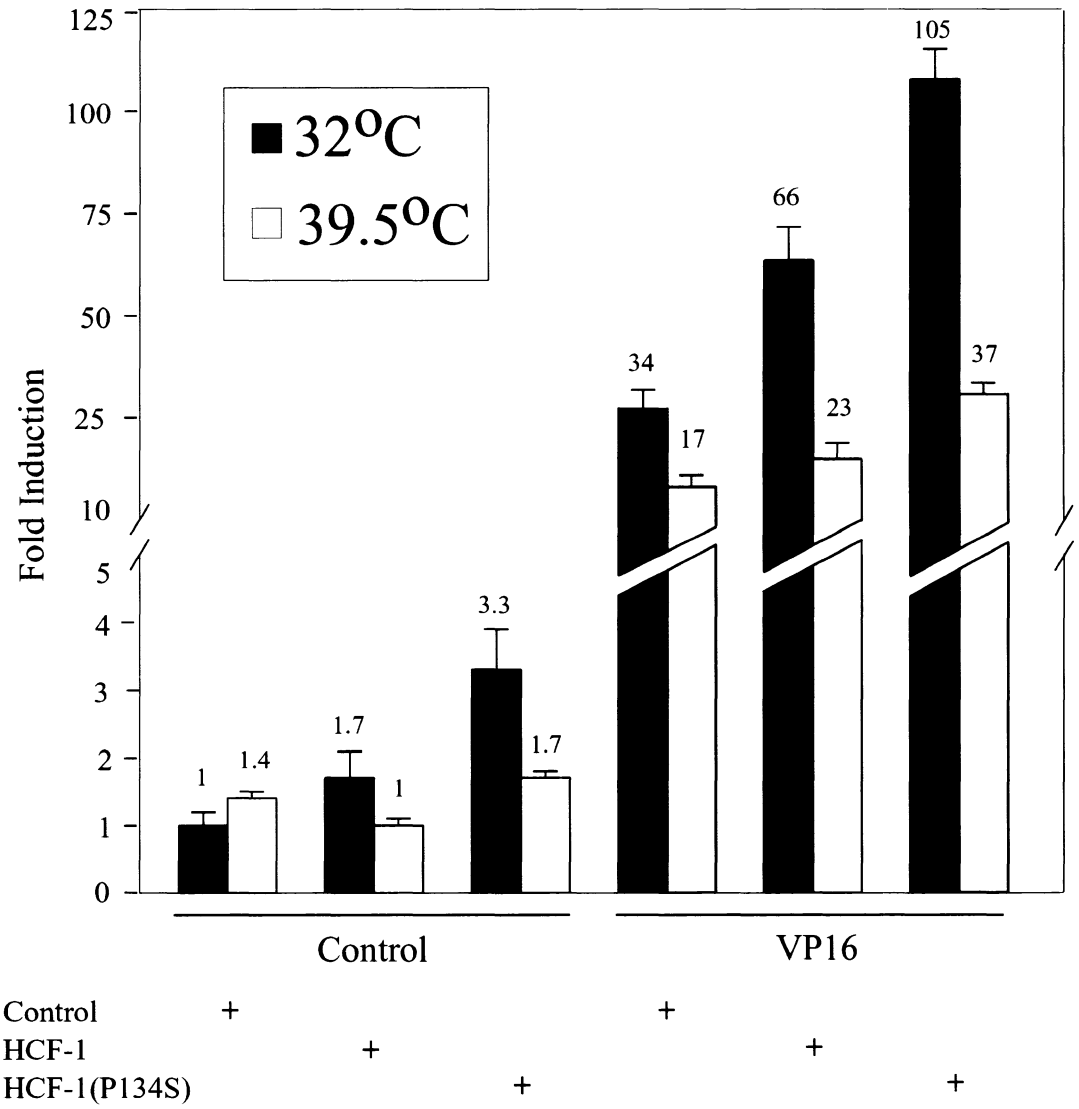

Figure 4.4.3.1. HCF-1 mediated synergy of VP16 activation is diminished in tsBN67 cells. tsBN67 cells contain a temperature sensitive proliferation defect which was localized to a P134 to S missense mutant in HCF-1 (Goto *et al.*, 1997). Duplicate sets of tsBN67 cells were transiently transfected with VP16 (10 ng of pEVRF65), HCF (1.8 μg), and 0.1 μg of the α4-*luc* reporter plasmid. One set of transfectants was placed at the permissive temperature (32°C), and the other at the non-permissive temperature (39.5°C). The activities were obtained from duplicates of three independent experiments. Error was calculated by standard deviation. The control plasmids at the permissive temperature were given a relative unit value of one.

originally transfected at the permissive temperature, and there does not appear to be a generalized effect on gene expression following a temperature shift to the non-permissive temperature (LaBoissierre *et al.*, 2000), thus the decreased activity attributed to HCF-1 may be due to a specific effect. Nonetheless, as with the other cell lines, the amount of potentiation of VP16 activation appears to be directly proportional to HCF-1 mediated activation in the absence of VP16 (compare 1.8 and 3.2-fold by HCF-1 and HCF-1$_{P134S}$ in the absence of VP16, and 60 and 115-fold in the presence of VP16). The lack of activity in the absence of VP16 also correlates with a lack of synergy when VP16 is present (Figure 4.4.3.1). In summary, these results appear to indicate that the amount of VP16-independent HCF-1 activation is a critical factor in determining the amount of synergy that occurs with VP16. Furthermore, these results are consistent with the findings from the other cell lines tested, which point toward a mechanism whereby HCF-1 is able to synergize with VP16; including those that are independent of VIC.

4.4.4 Discussion

Transcriptional co-regulatory proteins provide both specificity and diversity through combinatorial networks of protein interactions, while providing an opportunity for increased complexity in gene expression. Transcriptional co-activators modulate gene expression essentially through two mechanisms: First, through an association with sequence specific DNA, bound proteins provide a link to the transcription machinery resulting in the activation of target genes (Lemon and Tjian, 2000). Second, complexes can modify chromatin structures within the nucleus (Naar *et al.*, 2001). Although, the

precise biological function of HCF-1 is not yet clear, it is becoming increasingly apparent that it can function as a nuclear co-regulatory protein involved in transcriptional (Lu *et al.*, 1998, Vogel and Kristie, 2000a, Lin *et al.*, 2002, Luciano and Wilson, 2002, Scarr and Sharp, 2002) and post-transcriptional (Ajuh *et al.*, 2000, Ajuh *et al.*, 2002) gene regulation. It does possess an activation domain within its carboxyl- (Luciano and Wilson, 2002, Wong, 2002), and potentially within its amino- polypeptide fragment. Moreover, HCF-1 appears to function as both a co-activator and a co-repressor, depending upon its context (Scarr and Sharp, 2002, Piluso *et al.*, 2002, Wysocka *et al.*, 2003). In addition, it can form larger co-regulatory complexes with factors such as p300/CBP (Bannert *et al.*, 1999, Luciano and Wilson, 2000) and the nuclear receptor co-activator PGC-1 (Lin *et al.*, 2002). A recent finding also indicates that HCF-1 is indirectly involved in modifying chromatin structures through association with a methyltransferase complex (Wysocka *et al.*, 2003), and its binding to chromatin structures; which is correlated to its role in promoting cellular proliferation (Wysocka *et al.*, 2001b). It is clear, though, that aspects of HCF-1 function are emerging at a stunning pace both in terms of its role within the cell, and with respect to VP16 gene expression.

4.4.4.1 HCF-1: A(nother) link between the cell cycle and HSV?

HSV is able to replicate in both actively dividing and non-dividing cells, since it possesses a complement of genes that are capable of sustaining viral replication (Boehmer and Lehman, 1997). A viral infection has been shown to halt progression of infected cells from G1 to S, in part, through the regulation of the retinoblastoma protein

(Rb) and cdk2 (Song *et al.*, 2000, Ehmann *et al.*, 2000). In contrast, a specific cdk inhibitor (roscovitine) demonstrates the dependency of an HSV infection on transcription and replication factors that promote advancement of the cell cycle (Schang *et al.*, 1998, Schang *et al.*, 1999, Schang *et al.*, 2000). Thus, it appears that a delicate and complicated balance is involved in modulating the cell cycle status of the cell takes place in order for HSV to optimize virus replication and propagation (Flemington, 2001). Several viral proteins including ICP0 (Lomonte and Everett, 1999), ICP22 (Bruni and Roizman, 1998) and VP16 (Daksis and Preston, 1992) are specifically affected by the cell cycle. For example, a VP16 mutant defective in transcriptional activation was used to uncover a greater amount of IE gene accumulation in G1/S compared to G2. Moreover, in the absence of VP16 transactivation, without having an effect on complex formation, immediate-early gene expression and replication appears to vary during the cell cycle (Daksis and Preston, 1992, Jordan *et al.*, 1999).

A specific link between HSV gene expression and the cell cycle may exist with HCF-1, since it is a critical regulator of cellular proliferation and cytokinesis (Julien and Herr, 2003, Wysocka and Herr, 2003). In addition, proteins interacting with HCF-1 have largely consisted of cellular transcription factors, suggesting that HCF-1 functions at least in part, as a transcriptional regulatory protein that modulates cell cycle activity. HCF-1 was linked to the cell cycle through a temperature-sensitive mutant causing cell cycle arrest within tsBN67 cells, as a result of a nonsense mutant at residue 134 of HCF-1 (Goto *et al.*, 1997). The same mutation within HCF-1 also fails to support an interaction with VP16 and other transcriptional activators, possessing the HCF-1 binding motif (Lu

et al., 1997, Freiman and Herr, 1997, Lu and Misra, 2000). Although additional mutants within HCF-1 do not correlate rescue of the cell cycle defect with its ability to bind VP16 (Mahajan and Wilson, 2000), a functional link between the cell cycle and VP16 or other transcription factor(s) can not be ruled out. Furthermore, a loss in chromatin binding by HCF-1 precedes the induction of cell cycle arrest in tsBN67 cells, suggesting that HCF-1 modulates cellular gene expression in response to the cell cycle (Wysocka *et al.*, 2001b). The various transcription factors, most notably SP1 (Gunther *et al.*, 2000), GABP (Vogel and Kristie, 2000) and Miz-1 (Piluso *et al.*, 2002), that interact with HCF-1 through its basic domain, may be important for its activity. The basic domain, in conjunction with the Kelch domain, is required in order to overcome the proliferation block in tsBN67 cells (Wilson *et al.*, 1997). The basic domain of HCF-1 may provide an anchor for proteins within the nucleus, as evidenced through its ability to bind chromatin (Wysocka *et al.*, 2001b). The recruitment of HCF-1 into VIC might therefore serve as an indicator, or trigger, as a response to the corresponding infection by HSV. The cell cycle and transcriptional regulation are known to be linked (Dynlacht, 1997), and HCF-1 may provide important clues as to how HSV is able to exploit or subvert cells for optimizing the expression of its genes (Flemington, 2001, Wysocka and Herr, 2003).

Alternatively, a modification of HCF-1 may occur in response to signals within the cell cycle. Phosphorylation of ceHCF (an ortholog of HCF-1) by cdk2, takes place in worms and mammalian cells through a similar pattern (Wysocka *et al.*, 2001a). Interestingly, stimuli promoting reactivation of HSV from its latent state results in HCF-1 localization to the nucleus, as well as activation of cdk2. Roscovitine, which is a specific

cdk inhibitor (including cdk2), does not to affect VIC assembly, though it may affect its function or may be differentially regulated in neurons (Jordan *et al.*, 1999). Uncovering the precise role for HCF-1 in VP16 activation of IE genes will also require determining whether the basic region contains a functional activation domain(s) or whether additional co-activator interactions involved, that act in conjunction with the carboxyl-terminal acidic domain of HCF-1. The active participation of the HCF-1 in viral immediate-early gene regulation may therefore provide a basis to investigate the role of the cell cycle apparatus in modulating this activity, and provide useful clues with respect to future investigations. The co-dependent relationship between transcription factors and the cell cycle apparatus requires further exploration (Dynlacht, 1997).

4.4.4.2 The role of HCF-1 in VP16 transcriptional activation

Numerous questions surrounding the role of HCF-1 in VP16 transactivation of IE genes have arisen since it has been implicated with the cell cycle and as a transcriptional co-regulatory protein. Accordingly, Luciano and Wilson (2002) have demonstrated that HCF-1 contains an autonomous transcriptional activation domain within its carboxyl-terminus, which is important for transactivation by VP16 during a productive viral infection. In the studies presented here, we also demonstrate that VP16 transactivation of a reporter gene within transiently transfected cells is potentiated by the addition of HCF-1. In addition, we show that HCF-1 activates the IE reporter construct in the absence of VP16, implicating additional promoter-bound factors in this activity. Indeed, the ability of an HCF-1 mutant (P134S) to synergize with VP16 indicates that the potentiation of

VP16 activity takes place through a mechanism that is independent of VIC. Thus, HCF-1 regulation of HSV IE gene expression may occur through one or more factors present within the IE promoter, thereby uncovering a novel aspect of HCF-1 involvement in VP16 transactivation of viral IE genes.

HCF-1 is a critical component of the VP16-induced complex, and as such is required for promoting transcriptional activation by VP16 (Wu *et al.*, 1994, Lai and Herr, 1997, Simmen *et al.*, 1997, LaBoissiere *et al.*, 1999, Wysocka and Herr, 2003). However, the question regarding an additional role for HCF-1 within the VIC complex, apart from stabilizing it, has only recently been addressed. The amino-terminal 380 residues (Kelch domain) are sufficient for VIC assembly (Wilson *et al.*, 1997), although it is not as efficient as the wild-type protein (LaBoissiere *et al.*, 1997). One outcome of providing additional HCF-1 would then be to promote an increase in the number, or stability of VIC. Our results argue against that possibility, based on findings that synergy is promoted by the P134S mutant, and not by HCF_{1011}. HCF_{1011} promotes VIC, in addition to interacting with other transcription factors such as GABP (Vogel and Kristie, 2000) and SP1 (Gunther *et al.*, 2000), and co-activating GAL4-VP_{369} (section 4.3). Moreover, when compared to HCF-2 in transactivation assays using tsBN67 cells (Lee and Herr, 2001), HCF_{1011} also demonstrates a relative ability to promote transactivation within VIC. Lee and Herr (2001), through a number of HCF derivatives, show that formation of complex does not correlate with increased activity by VP16. The lack of activity with HCF_{1011} might therefore result from the absence of the carboxyl-terminal activation domain or its inability to co-activate via other factors. Consistent with the former, the

creation of an internal deletion within HCF-1 to remove its carboxyl-terminal AD, specifically affects transcriptional activation and not complex formation (Luciano and Wilson, 2002). The HCF-1$_{P134S}$ also argues against HCF-1 promoting additional VIC formation, since it is abrogated for VIC assembly (Wilson *et al.*, 1997, Goto *et al.*, 1997, LaBoissiere and O'Hare, 2000, Lee and Herr, 2001). Therefore, the robust synergy observed with HCF-1$_{P134S}$ within the various cell lines (see Figures 4.4.1 through 4.4.3.1) indicates that the potentiation of VP16 activity likely does not occur through additional complex assembly, but rather through some other mechanism, such as binding to a factor within the IE promoter. The role of other factors with respect to HCF-1 activity in HSV expression is further supported by biochemical studies indicating that HCF-1 co-localizes with HSV DNA, possibly in the absence of VP16, at early times post-infection (LaBoissiere and O'Hare, 2000).

4.4.4.3 The 'VP16-enhanceosome'

How does HCF-1 contribute to the transcriptional activation of VP16? A role for HCF-1 as a co-activator of VP16 within the context of VIC has recently been shown (Lee and Herr, 2002, Luciano and Wilson, 2002), and is consistent with the results presented here. Moreover, as postulated by others (Vogel and Kristie, 2000a, Wysocka *et al.*, 2003), and demonstrated here, HCF-1 can synergize with VP16 in the activation of HSV IE genes, possibly through interactions that occur simultaneously or independently with additional promoter associated factors upstream of HSV IE genes. Consequently, the synergy that is observed involving VP16 and HCF-1, and other promoter-bound factors,

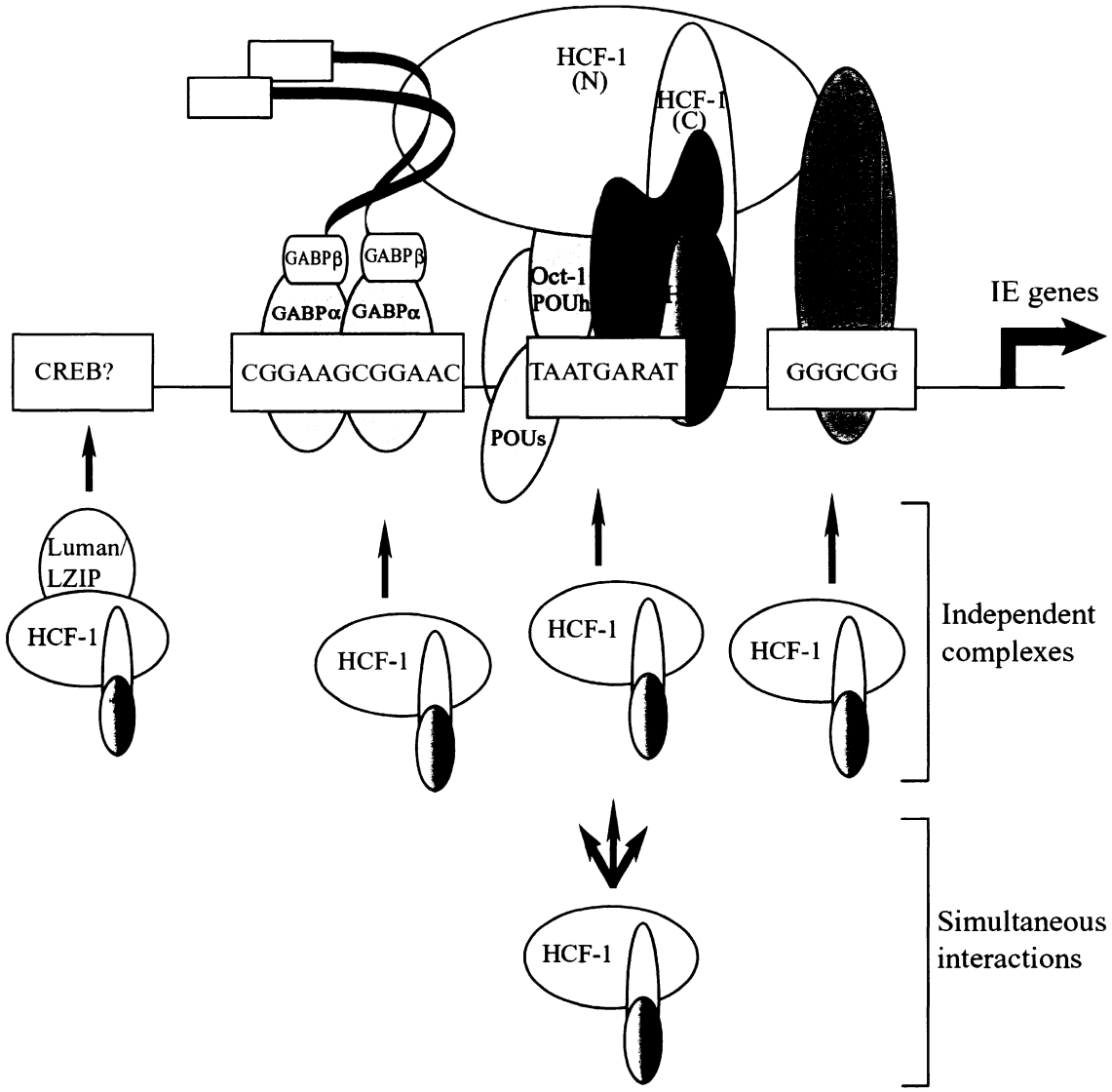

Figure 4.4.4.3. The 'VP16 enhanceosome': model for the role of HCF-1 in VP16 transactivation of HSV IE genes. Schematic model that illustrates the possible mechanism of HCF-1, in conjunction with the VP16-induced complex (VIC) and other promoter bound factors such as Sp1 and the GA-binding protein GABP, in modulating HSV IE gene expression. Luman (LZIP) may also play a role in the expression of HSV1 genes (Lu and Misra, 2000). The model envisions a multi-component complex involving multiple transcription factors, that is assembled or stabilized by HCF-1 on the HSV IE upstream promoter element; termed the 'VP16-enhanceosome'. HCF-1 can bind each transcription factor within the complex separately, or in conjunction with the other factors present, thus potentially affecting the stoichiometry of HCF-1 molecules that are present on the promoter.

can be manifested or modeled within functional structures termed 'enhanceosomes' (Carey, 1998, Merika and Thanos, 2001, Struhl, 2001). These assemblies consist of a specific complementation of transcriptional activators on a promoter (or enhancer), that are formed in order to regulate transcriptional activation of target genes through combinatorial interactions between participating transcription factors. VP16 responsive TAATGARAT-elements are usually found alongside binding sites for cellular transcriptional activators, such as Sp1 (Jones and Tjian, 1985, Triezenberg *et al.*, 1988b), GABP (Triezenberg *et al.*, 1988b, Douville *et al.*, 1995) and even Oct-1 (Wu *et al.*, 1994), which can independently contribute to modulating HSV promoter activity. Importantly, in addition to binding VP16, HCF-1 also interacts with Sp1 (Gunther *et al.*, 2000), GABP (Vogel and Kristie, 2000a) and Oct-1 (Mahajan and Wilson, 2000). Consequently, HCF-1 may serve as the common factor that co-ordinates the assembly of an enhanceosome complex on the IE gene promoter, thus regulating its expression. Accordingly, a model is proposed here to account for the specific co-ordination of a higher order complex based around VIC formation, and transcription factors GABP and SP1 (and perhaps other unidentified factors), which is termed the 'VP16 enhanceosome' (see Figure 4.4.4.3). The 'VP16 enhanceosome' constitutes a multi-order and component binding complex that is specifically generated on a HSV IE promoter, thus expanding upon the complexity of VP16 transcriptional activation and IE gene expression: in order to account for the expanded role of HCF-1 in viral gene expression. For instance, HCF-1 may function to nucleate various transcription factors to the IE promoter site, such that, as Vogel and Kristie (2000a) suggest in the case of GABP, VIC assembly serves as a

prerequisite to GABP activation. Assuming a similar scenario for Sp1, HCF-1 may be significantly enhancing VP16 mediated gene expression of its target genes through the localization of GABP and Sp1 to their respective promoter binding sites. In fact, both Sp1 and GABP do synergize with VP16 in the activation of the ICP4 promoter or other elements (Triezenberg *et al.*, 1988b, Wu *et al.*, 1994, Douville *et al.*, 1995, Hagmann *et al.*, 1997, Luciano and Wilson, 2002), and on other promoter elements in various contexts (Rosmarin *et al.*, 1998, Nuchparyoon *et al.*, 1999, Galvagni *et al.*, 2001). The arrangement of cis-acting elements and utilization of distinct protein interaction domains within HCF-1 to mediate binding to various factors can support the notion of a simultaneous interaction (Triezenberg *et al.*, 1988b). As a result, in addition to its role as a transcriptional co-activator in the context of each of the individual transcriptional activators on the ICP4 immediate-early promoter, HCF-1 may simultaneously coordinate or stabilize the assembly of the 'VP16 enhanceosome' on the HSV1 immediate-early promoter in order to promote synergy between these factors (Luciano and Wilson, 2000, Vogel and Kristie, 2000a). The co-ordinated assembly of the various activators on the promoter can also be envisioned, such that by increasing the effective activator concentration or stability of the 'enhanceosome' complex on the IE promoter, relatively few transcriptional activators would be required for generating robust activity (Carey, 1998). Furthermore, the presence of multiple activation domains within a one activator (Emani and Carey, 1992, Ghosh *et al.*, 1996), or the presence of multiple transactivator binding sites within a single promoter (Chang and Gralla, 1994), are known to promote

synergistic activity, particularly as a result of the convergence of diverse transcriptional activation domains (Chi *et al.*, 1995).

VP16 transcriptional activation of HSV1 immediate-early genes is continuously evolving into an increasingly complex mechanism. It is apparent through the work presented here, and by others (Luciano and Wilson, 2002, Wysocka and Herr, 2003), that HCF-1, in conjunction with VP16, has an active role in the activation of IE gene expression. This is particularly evident at low viral MOI, where VP16 relies increasingly upon the HCF-1 AD in activating its target genes (Luciano and Wilson, 2002). The synergistic activity that is promoted by HCF-1, however, must also be considered in light of the results of Hagmann *et al.* (1997), who point out that VP16 transcriptional activation is a 'paradox'. By this the authors point out that VP16 activation is limited to promoter-proximal, but not distal regions of the immediate-early promoter. It is termed a 'paradox' since VP16 contains a highly potent transcriptional activation domain, though it is muted within the context of VIC in order to provide localized, robust activity, and presumably to prevent activation of adjacent genes. The apparent 'under-utilization' of the VP16 AAD within VIC is not necessarily contradictory with the notion that HCF-1 serves to potentiate VP16 activity. VP16 activity is likely to be further enhanced by the involvement of multiple transcription factors, through the co-ordinated assembly of higher order protein-protein and protein-DNA interactions, thus providing functional diversity and increased specificity, as in the enhanceosome complexes (Struhl, 2001). In that sense, HCF-1 mimics co-activators (or co-regulatory factors) that are becoming part

of an increasingly important paradigm in gene regulation, through the formation of highly specific regulatory complexes on all genes (Cosma, 2002).

4.5 Future Directions

The synergistic activity promoted by transcriptional activators increases the flexibility and specificity of gene expression, which provides enhanced regulation of gene expression through the assembly of an array of combinatorial interactions (Chi *et al.*, 1995, Ptashne and Gann, 1997, Chen, 1999). The 'VP16 enhanceosome' is a model that accounts for the ordered assembly of VP16 into VIC, which is part of a larger complex of transcription factors; providing an increasingly complex regulatory scheme in the activation of HSV IE genes. It will be important to determine if the observed synergy with VP16 is occurring in conjunction with GABP, Sp1, or through another factor. In this regard, we currently have mutants that selectively disrupt binding to GABP (D. Piluso, pers. comm.). Likewise, nucleotide spacing or the context of transcription factor binding-sites may also be important in order to synergize with VP16. The mechanism of VP16 activation of IE genes, in relation to the role of HCF-1 and other transactivators, will likely yield important clues as to how HSV has developed its gene expression program.

Chapter 5. Synopsis

Viral proteins are necessarily, and perhaps invariably, multi-functional proteins that serve to maximize virus production and persistence. VP16 is a multi-functional HSV1 protein involved in the transcriptional activation of viral immediate-early (IE) genes, in addition to having distinct role in virus assembly. VP16 transcriptional activation has been studied extensively, and involves the formation of the VP16-induced complex (termed VIC), which includes at least two cellular transcription factors; Oct-1 and HCF-1 (Herr, 1998). This complex targets the TAATGARAT cis-acting DNA element upstream of IE genes, thereby promoting the viral lytic cycle. Various aspects of complex assembly and the role of the VP16 acidic activation domain (AAD) in mediating transcriptional activation continues to be the focus of a large body of research (Wysocka and Herr, 2003). Less well understood is the contribution that VP16 makes to virus assembly (Ace *et al.*, 1989, Weinheimer *et al.*, 1992, Poon and Roizman, 1995), though it is found to function during viral egress (Mossman *et al.*, 2001b). In this thesis, we sought to better understand the role of VP16 within HSV through the characterization of the interaction between VP16 and vhs and VP16 activation, in the absence of its AAD, through mutagenesis and structure-function analyses.

The interaction and downregulation of vhs activity by VP16 (Lam *et al.*, 1996) provided a unique opportunity to examine the importance of this interaction with respect to gene regulation, virus assembly, and the viral lytic cycle. In order to characterize the structural and functional aspects of the interaction, a mutational analysis of VP16 was undertaken. To accomplish this, and given that a relatively small region within VP16 was

important for mediating interactions between both vhs and VIC assembly (Stern and Herr, 1991, Shaw *et al.*, 1995, Lai and Herr, 1997, Simmen *et al.*, 1997), we chose to expand upon previous mutagenesis experiments (Smibert *et al.*, 1994). As a result, we determined that the region between residues 340 and 350 of VP16 contains a determinant for vhs binding. Based on these findings, alanine-scanning mutagenesis was used to isolate a single mutant (L344A) that was able to abrogate binding to vhs both *in vitro* and *in vivo*. The L344A mutant effectively uncoupled vhs binding from transcriptional activation and an interaction with HCF-1, thus providing evidence for a structural and functional difference between binding to vhs and VIC formation. Furthermore, through the development of virus complementation assays, we were able to utilize the VP16 (L344A) vhs-binding mutant in order to analyze virus growth, both in the presence and absence of a functional vhs protein. While the mutant was unable to effectively complement the 8MA (lacking only VP16) lytic cycle, it could with 8MAΔSma (lacking both VP16 and vhs); indicating that blocking the interaction between VP16 and vhs is incompatible with virus growth. Therefore, a specific interaction between VP16 and vhs is critical in maintaining a productive viral infection. These findings should provide a basis in which to investigate inhibitors that disrupt the interaction, which might eventually be useful as an antiviral therapeutic.

VP16 transcriptional activation of viral IE genes is not essential for virus growth in culture (Ace *et al.*, 1989, Smiley and Duncan, 1997), though it is important in determining a productive viral infection *in vivo*. Thus, the findings of a truncated VP16 activating a reporter when appended to a heterologous DNA binding domain (Popova *et*

al., 1995) were also further explored within this thesis. Transcriptional activation by VP16 in the absence of its AAD has been demonstrated (Ace *et al.*, 1989, Cleary and Herr, 1993, Wu *et al.*, 1994), also occurring with VP16 orthologs (Greaves *et al.*, 1991, Carpenter *et al.*, 1992, Moriuchi *et al.*, 1993, Purewal *et al.*, 1994, Elliott *et al.*, 1995). We demonstrate here, through deletion mutagenesis, that a region between residues 345 and 350 of VP16 is important for the activation a reporter construct in yeast when appended to the GAL4 DNA binding domain. Subsequent analysis of this phenotype also resulted in the segregation of this activity between mammalian and yeast cells, in addition to its interaction with vhs. Interestingly, experiments in mammalian cells resulted in the discovery that HCF-1 functions as a co-activator to VP16, as a GAL4 fusion protein. These findings indicated a novel role for HCF-1 in VP16-mediated gene expression. Furthermore, HCF-1, or mutants thereof, were also found to enhance reporter gene expression linked to the HSV1 α4 promoter in the absence of VP16. These results demonstrate a direct and novel role for HCF-1 in VP16 mediated transcriptional activation of viral immediate early genes. Therefore, the VP16-induced complex which utilizes multiple-interactions among proteins and DNA may take part in a higher-order array of structural and functional networks to activate gene expression, which was further discussed and modeled upon transcriptional regulatory complexes termed 'enhanceosomes' (Carey, 1998, Struhl, 2001, Merika and Thanos, 2001). Together, the findings presented here promote new directions in which to explore HSV1 gene expression with respect to the role of HCF-1.

Essential aspects of VP16 function, in terms of its role as a transcriptional activator and its interaction with vhs, are presented within this thesis. Determination of the mode of regulation of interaction between VP16 and vhs will be required to provide added insight as to the role of this interaction at various stages a viral infection, and could have significant implications with regard to finding an antiviral therapeutic. The synergy between HCF-1 and VP16 also adds to the complexity of VP16 mediated transcriptional activation of viral IE genes, and may have important implications not only for a productive viral infection, but also in reactivation from the latent state. Higher-order complex assembly, mediated by HCF-1, affects viral immediate-early gene expression, and possibly the mammalian cell cycle (Julien and Herr, 2003, Wysocka and Herr, 2003). Therefore, the results presented here not only enhance our understanding of the role of VP16 within HSV and the expression of viral IE genes, but are likely to have broad implications toward our understanding of mammalian gene expression and viral pathogenesis.

Chapter 6. Bibliography

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