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STUDY OF THE HERPES SIMPLEX VIRUS VIRION HOST SHUTOFF PROTEIN'S ENDORIBONUCLEASE ACTIVITY

By

Patricia Lu

A thesis submitted to the Faculty of Gradute Studies and Research in Partial Fulfillment of the requirements for the degree Master of Science

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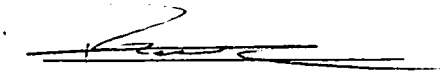

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Study of the Herpes Simplex Virus Virion Host Shutoff Protein's Endoribonuclease Activity" submitted by Patricia Lu in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

The virion host shutoff protein (vhs) of herpes simplex virus triggers accelerated mRNA turnover during virus infection, and induces endoribonucleolytic cleavage of exogenous RNA substrates in a rabbit reticulocyte (RRL) *in vitro* translation system. To address the question whether mammalian cellular factors are required for vhs activity, vhs was expressed in the budding yeast *Saccharomyces cerevisiae*. Despite the observation that expression of vhs inhibited colony formation, cell-free extracts of yeast expressing vhs displayed little or no vhs-dependent endoribonuclease activity. However, activity was detected when such extracts were mixed with blank RRL. These data suggest that the vhs-dependent endoribonuclease activity requires one or more mammalian macromolecular factors for its activity.

The vhs1 point mutation was previously considered to abolish vhs activity. Here I demonstrate that the vhs1 mutant protein induces cleavage of RNA substrates bearing the internal ribosome entry site (IRES) of encephalomyocarditis virus in the RRL system. These data raise the possibility that the vhs-dependant endoribonuclease employs more than one mode of substrate recognition.

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TABLE OF CONTENTS

CHAPTER 1 – GENERAL INTRODUCTION

1.1. Introduction to herpes simplex virus

Herpes simplex virus (HSV) belongs to the *alphavirinae* subfamily of the *Herpesviridae* family. The viruses in the *Herpesviridae* family consist of four characteristic complex structures: a core containing a linear, double-stranded DNA molecule; an icosadeltahedral capsid; an amorphous material surrounding the capsid designated as the tegument; and an envelope containing viral glycoprotein spikes on its surface (Roizman, 1996; Ward and Roizman, 1994).

HSV is the prototypic member of the alphavirinae subfamily, and includes HSV type 1 (HSV-1) and type 2 (HSV-2). The viruses in this subfamily have the following characteristics: variable host range; relatively short reproductive cycle; rapid spread in culture; efficient destruction of infected cells; and capacity to establish latent infections primarily, but not exclusively, in sensory ganglia (Roizman and Sears, 1996). Other viruses that belong to the *alphavirinae* subfamily include: herpes B virus, varicella-zoster virus (VZV), bovine mammillitis virus, pseudorabies virus and equine herpesvirus 1 (Roizman and Sears, 1996).

1.2. Overview of HSV pathogenesis

HSV is a human pathogen; the infection of the human host can be divided into four stages: the first stage is characterized by lytic infection in which primary mucocoutaneous infection in seronegative individuals leads to viral replication and tissue damage (ulceration) at the site of infection. The second stage is characterized by latent infection. Viruses released from the primary infection sites infect sensory nerve endings adjacent to the primary infection sites. The sensory nerve endings are long projections from a neuron, which is a large cell whose nucleus is found in sensory ganglia located at deeper anatomical sites. The HSV capsid travels along the neural projection to the cell body, and viral DNA is released into the nucleus and circularizes. After a round of replication, the virus can remain in a latent state for the life span of the host. During

latency, infectious virus and signs of replication can no longer be detected, but latent viral genomes persist in the nuclei of some of the sensory neurons. The third stage is characterized by reactivation of latent virus through an unknown mechanism in response to a range of stimului including damage to trigeminal ganglia, ultraviolet light, menstruation, immune suppression, and mental or physical stress. This reactivation leads to the transportation of virions from the neural cell body to sites at or near the site of primary infection. The fourth stage is characterized by recurrent infection at these peripheral sites.

Lytic HSV infection occurs at the mucosal surface or abrased skin of a seronegative susceptible individual following personal intimate contact with an infected individual actively excreting virus. Generally, HSV-1 is responsible for oral "cold sores" and is less severe than an HSV-2 primary infection of the genitals (Brugha *et al.*, 1997). On rare occasions, these viruses can cause reciprocal infections (Corey *et al.*, 1983). Primary HSV-1 infection, acquired during early childhood, is generally asymptomatic (Brugha *et al.*, 1997). When acquired in later life, primary HSV-1 infections are usually more severe and can cause pharyngitis and mononucleosis syndrome (Brugha *et al.*, 1997). Serological studies have repeatedly indicated that almost 90% of adults in North America are seropositive for HSV-1 (Nahmias, Lee, and Beckman-Nahmias, 1990).

HSV-2 is transmitted through intimate sexual contact and as such, primary infections do not usually occur until early adulthood (Brugha *et al.*, 1997). HSV infection is very prevalent in North America. HSV-2 prevalence varies widely, and depends on a number of factors, most significantly the number of sexual partners, but has been recently assessed at 23% in North America (Wald *et al.*, 1997).

1.3. HSV virion

The HSV genome is highly organized and packaged in the form of a toroid in the virion. The viral genome is about 152 kbp, and has a high GC content (68% for HSV-1 and 69% for HSV-2). It consists of two unique stretches of unequal length flanked by inverted repeats. The long unique sequence, U_L, contains 60 genes and is about 125 kbp, whereas the short unique sequence, U_S, contains 14 genes and is about 25 kbp (Roizman

and Sears, 1996). The genes are named according to their order within the U_L or U_S region. The repeated sequence flanking the U_L is designated ab and b'a', whereas the repeated sequence flanking the U_S is designated a'c' and ca (Becker, Dym, and Sarov, 1968; McGeoch *et al.*, 1988; McGeoch *et al.*, 1985). The presence of invert repeats allows the U_L and U_S regions to invert relative to each other. The significance of these inversion events is unknown. In addition to the genes located in the two unique regions, 4 genes are located in the repeated sequences. Of the 78 genes, more than 30 genes encode proteins that are contained in the infectious HSV virion as capsid structure proteins, tegument proteins and viral envelope glycoproteins (Heine *et al.*, 1974; Spear and Roizman, 1972).

The HSV capsid that encloses the viral DNA is an icosahedral structure, and consists of seven proteins (VP5, VP19C, VP21, VP22a, VP23, VP24, and VP26) which are products of six genes (UL18, UL19, UL26.5, UL35, and UL38) (Tatman *et al.*, 1994). The UL26 gene encodes a proteinase that cleaves its own gene product into VP21 and VP24, as well as cleaving preVP22 into VP22a (Tatman *et al.*, 1994). VP21, VP24, and pre-VP22a form the "scaffold" protein structure first, then, VP5, VP19C, VP23, and VP26 assemble outside of the "scaffold" (Tatman *et al.*, 1994).

The structure between the capsid and the viral envelope is the tegument. The tegument is a highly organized structure that is capable of self-assembling in the absence of the capsid (Elliott, Mouzakitis, and O'Hare, 1995; Elliott and O'Hare, 1999; Zhou *et al.*, 1999). It contains several molecules that are important for HSV infection such as: VP16, the virion host shutoff protein (vhs), UL13, VP1/2, VP11/12, VP13/14, and VP22 (Roizman and Sears, 1996). VP16 is a HSV transcriptional activator and is essential for formation of tegument. vhs is responsible for the early shutoff of host protein synthesis. UL13 is a protein kinase. VP1/2, VP11/12, VP13/14, VP22 are required for virion disassembly and release of viral DNA into the nucleus (Roizman and Sears, 1996). The function of VP16 and vhs will be discussed in detail in later sections.

The tegument is surround by a lipid bilayer called the envelope. The envelope of mature HSV particles contains at least 11 known glycoproteins (gB, gC, gD, gE, gG, gH,

gI, gJ, gL, gM) which play important roles in viral lytic infection such as virion attachment, viral entry, virion assembly, cell-to-cell spread, egress, and immune evasion (Ward and Roizman, 1994). The envelope is cellular in origin, but it is not clear which cellular membranes are used to form the final virion envelope (discussed in later section).

1.4. HSV lytic infection

HSV lytic infection occurs through the following steps: attachment of virions to the host cells, virion entry into cells, delivery of the viral genomes to the nucleus of the infected cells, expression of viral genes and viral genome replication, assembly and egress of the progeny virions (Roizman and Sears, 1996; Ward and Roizman, 1994). Details of each step are discussed in this section.

1.4.1. Attachment and entry

To infect a cell, herpes simplex virions must attach to the cell surface, and the viral envelope must fuse with the plasma membrane. The attachment of virions to cells is primarily mediated by gB and/or gC binding to cell surface heparan sulfate proteoglycans in a nonspecific way (Herold *et al.*, 1991; Sears, McGwire, and Roizman, 1991). gC appears to be the principal glycoprotein involved in this process as demonstrated by the following findings: mutants lacking gC adsorb to cells less efficiently than do wild type particles (Herold *et al.*, 1991); purified gC binds to cells through the cell surface heparan sulfate proteoglycans (Herold *et al.*, 1991; Svennerholm *et al.*, 1991; Tal-Singer *et al.*, 1995); antibodies directed against gC inhibit adsorption of HSV particles to cells (Fuller and Spear, 1985; Svennerholm *et al.*, 1991). The function of gB seems to provide low affinity binding because gB has been found to bind to cell proteoglycans in the absence of gC (Herold *et al.*, 1994; Herold *et al.*, 1991); this may explain why gC mutants can still bind to cells although less efficiently. Substantial evidence indicates that the heparan sulfate proteoglycan is the primary cellular factor required for attachment (Wittels and Spear, 1989). Loss or alteration of cell surface heparan sulfate results in significant reduction in viral attachment (Gruenheid *et al.*, 1993; Shieh *et al.*, 1992; WuDunn and Spear, 1989); as does the addition of molecules that are structurally similar to herparan sulfate. Also, HSV virions bind to immobilized heparan (WuDunn and Spear, 1989).

After the initial attachment, the viral envelope fuses with the cell membrane, then, virions enter the cell. At least five envelope glycoproteins, gB, gC, gD, gH, and gL are involved in this process as indicated by the following findings. HSV virions bearing mutations in any of these glycoproteins cannot enter the cell, despite their ability to bind to the cell (Cai, Gu, and Person, 1988; Forrester *et al.*, 1992; Ligas and Johnson, 1988; Roop, Hutchinson, and Johnson, 1993). Also, antibodies against these glycoproteins do not block viral attachment but prevent viral entry (Fuller, Santos, and Spear, 1989; Fuller and Spear, 1987; Highlander *et al.*, 1988). These glycoproteins (except gC) also represent the minimal set of viral glycoproteins essential for growth in tissue culture (Baines *et al.*, 1991; Cai, Gu, and Person, 1988; Desai *et al.*, 1993; Forrester *et al.*, 1992; Fuller and Spear, 1987; Johnson *et al.*, 1988; Ligas and Johnson, 1988; Longnecker and Roizman, 1986; Roop, Hutchinson, and Johnson, 1993; Weber, Levine, and Glorioso, 1987).

Viral entry into cells is believed to occur in two steps. Initially, gD binds to cell surface receptors; then, the viral envelope fuses with the cell membrane and the capsid enters the cell. Essentially all tissue culture cells have surface heparin sulfate proteoglycans, but not all tissue culture cells can support a lytic HSV infection. This observation leads to the hypothesis that binding of gD to specific cellular receptors is required for the entry of virions into the cells. Four cell surface proteins have been shown to mediate viral entry: HveA (herpesvirus entry mediator, also known as HVEM), HevB, HevC, and herpsvirus Ig-like receptor (HIgR). Expression of any of these four proteins in cells either enhances the HSV infection in a cell line, or renders an unsusceptible cell line susceptible to HSV infection (Cocchi *et al.*, 1988a; Cocchi *et al.*, 1988b; Geraghty *et al.*, 1998; Montgomery *et al.*, 1996; Sarrias *et al.*, 1999; Shukla *et al.*, 1999; Subramanian *et al.*, 1994; Warner *et al.*, 1998). gD was determined to be the principle molecule required for the entry process. HSV virions lacking gD bind to cells, but cannot enter (Johnson *et al.*, 1988; Ligas and Johnson, 1988). Cells transfected with gD are resistant to HSV infection (Campadelli-Fiume *et al.*, 1988; Johnson and Spear, 1989). Inactive wildtype HSV (gD intact) can block the entry of homologous virus (Johnson *et al.*, 1988), and soluble gD can block HSV infection (Johnson, Burke, and Gregory, 1990; Peng *et al.*, 1998).

The binding of gD to specific cell surface receptors brings the viral and cellular membranes into close proximity. HSV then enters cells through a pH-independent mechanism. This observation suggests that the mechanism of entry is though direct fusion of viral and cellular membranes (Koyana and Uchida, 1984). However, the mechanism involved in this membrane fusion process is not fully understood.

After entry into the cells, as shown by EM studies, most tegument proteins dissociate from the capsid and the viral capsid is transported to nuclear pores (Sodeik, Ebersold, and Helenius, 1997). Among the proteins present in the tegument, the following proteins are of particular interest and will be discussed in detail: vhs, which immediately induces termination of host protein synthesis; and the viral regulators ICP0, ICP4, and VP16, which migrate to the nucleus and induce HSV gene expression (Roizman and Sears, 1996; Spear and Roizman, 1972). The viral capsid is transported to the microtubule organizing center (MTOC), located adjacent to the nucleus, through association with microtubules (Whittaker and Helenius, 1998). A tegument protein VP22 is believed to be involved in this process because of its function in recognizing and stabilizing microtubules (Whittaker and Helenius, 1998). The viral capsid accumulates at the nuclear envelope and associates with nuclear pore complexes (Whittaker and Helenius, 1998). At this point, viral DNA is released into the nucleus, and immediately circularizes.

1.4.2. IE gene expression

Once viral DNA is in the nucleus of the infected cell, cellular RNA polymerase II is used to express HSV genes in three sequential classes: immediately early (IE), early (E), and late (L) (Roizman and Sears, 1996; Spear and Roizman, 1972). The IE gene products are primarily regulatory proteins, the E gene products are primarily involved in DNA replication and the L gene products are mainly structural proteins and proteins involved in virion assembly and egress (Roizman and Sears, 1996; Ward and Roizman, 1994).

IE genes are the first viral genes expressed and they are expressed in the absence of viral DNA synthesis and *de novo* viral protein synthesis. IE products are primarily

regulatory proteins that are essential for the expression of E and L genes, and include ICPs 0, 4, 22, 27, and 47. IE gene expression is transactivated by the tegument protein VP16 (also known as Vmw65, αTIF, ICP25), which is encoded by the late gene UL48 (Batterson and Roizman, 1983; Campbell, Palfreyman, and Preston, 1984; Pellett *et al.*, 1985; Post, Mackem, and Roizman, 1981). VP16 specifically transactivates the IE genes expression, and this specificity stems from the VP16 responsive elements within the IE gene promoters (ie. the TAATGARAT sequence) (Preston, Cordingley, and Stow, 1984). Although VP16 alone interacts with this element weakly, the interaction is greatly enhanced by binding this sequence in conjunction with the cellular proteins host factor HCF and the ubiquitous cellular transcription factor Oct-1 (Kristie, Lebowitz, and Sharp, 1989; Kristie and Roizman, 1987; Kristie and Sharp, 1990; McKnight, Kristie, and Roizman, 1987; O'Hare, Goding, and Haigh, 1988; O'Hare and Gooding, 1988; Preston, Frame, and Campbell, 1988). In addition to its function in enhancing the binding of VP16 to the TAATGARAT sequence, HCF has recently been suggested to be the nuclear transporter of VP16 since co-expression of HCF and VP16 leads to accumulation of VP16 in the nucleus and VP16 itself lacks a nuclear localization signal (La Boissiere, Hughes, and O'Hare, 1999).

As with other known transactivators, VP16 has two domains required for its activity. The C-terminal portion of VP16 is the activator domain which is dispensable for interaction with the cellular factors HCF and Oct-1, but is required for its transactivation activity. When the C-terminus of VP16 is fused to the DNA-binding domain of yeast GAL4 or *E. coli* TetR, the recombinant protein still activates transcription (Cousens *et al.*, 1989; Sadowski *et al.*, 1988; Triezenberg, Kingsbury, and McKnight, 1988; Triezenberg, LaMarco, and McKnight, 1988). The N-terminal portion of VP16 is the promoter binding domain which is required for interaction with the cellular factors HCF and Oct-1 (Ace *et al.*, 1988; Ace *et al.*, 1989; Freiman and Herr, 1997; Gerster and Roeder, 1988; Goto *et al.*, 1997; Greaves and O'Hare, 1989; Johnson, Mahajan, and Wilson, 1999; Katan *et al.*, 1990; Kristie, Lebowitz, and Sharp, 1989; Kristie and Sharp, 1990; La Boissiere, Hughes, and O'Hare, 1999; Liu *et al.*, 1999; Spector, Purves, and Roizman, 1991; Wilson *et al.*, 1997; Xiao and Capone, 1990). How VP16 transactivates gene expression is not yet clear. VP16 has been shown to interact with transcriptional

factors TBP, TFIIB, TFIIH, and RPA (Goodrich *et al.*, 1993; Gupta *et al.*, 1996; Ingles *et al.*, 1991; Lin *et al.*, 1991; Stringer, Ingles, and Greenblatt, 1990; Xiao *et al.*, 1994). These interactions lead to the hypothesis that VP16 stabilizes the interaction between the polymerase holoenzyme and DNA, thereby stimulating transcription initiation.

The transactivation function of VP16 is not essential for lytic infection of HSV. Lytic infection of HSV still occurs, although at a reduced rate, in cells infected with a VP16 C-terminal mutant and mutants that fails to interact with the HCF and Oct-1 (Ace *et al.*, 1988; Ace *et al.*, 1989; Smiley and Duncan, 1997). However, a VP16 null mutant is not viable, and the defect has been mapped to the assembly stage (Weinheimer *et al.*, 1992). This finding indicates that VP16 is required for virion assembly, in addition to its role as a transcriptional activator.

The products of IE genes that are transactivated by VP16 include ICPs 0, 4, 22, 27 and 47. ICP0 (also known as Vmw110, IE110, IE1, RL2) is a phosphoprotein that localizes to the nucleus. ICP0 alone or with ICP4, behaves as a promiscuous activator in transient-cotransfection assays, stimulating expression from a variety of HSV and heterologous promoters (Cai and Schaffer, 1992; Chen and Silverstein, 1992; Everett, 1984; O'Hare and Hayward, 1985b; Quinlan and Knipe, 1985). The regulatory function of ICP0 appears to lie "upstream" of the other IE genes since in noncomplementing cells, ICP0 mutants display a reduced levels of IE gene expression and a greatly increased particle-to-PFU ratio during infection (Cai *et al.*, 1993; Cai and Schaffer, 1992; Lium *et al.*, 1998; Lium and Silverstein, 1997; Stow and Stow, 1989; Stow and Stow, 1986; Yao and Schaffer, 1995).

The mechanism of ICP0 action has not yet been precisely defined. Nuclear runoff transcription assays indicate that ICP0 acts at the transcriptional or pretranscriptional level (Jordan and Schaffer, 1997; Samaniego, Wu, and DeLuca, 1997). An emerging hypothesis proposes that ICP0 acts by altering the stability of specific cellular proteins and nuclear structures, which circumvents a cellular, interferon-induced antiviral response pathway, thereby enhancing the environment for HSV-1 replication. This hypothesis is supported by the following observations: (1) In the absence of ICP0, HSV

genomes are silenced or repressed at sites at the periphery of ND10 sites (also known as nuclear dots, PML nuclear bodies, or promyelocytic oncogenic domains: PODs) (Everett, Orr, and Preston, 1998). ND10 sites have been suggested to play a role in intracellular defense against DNA virus infection based on the observation that interferons induce many of the identified ND10 proteins, including PML and Sp100 (Guldner *et al.*, 1992; Maul *et al.*, 1995). (2) ICP0 was found to localize to nuclear ND10 domains and disperse their constituent proteins such as PML and Sp100, an activity that correlates with activation function in mutational studies (Everett and Maul, 1994; Maul and Everett, 1994; Maul, Guldner, and Spivack, 1993). (3) ICP0 mutants were found to be hypersensitive to interferon (Mossman and Smiley, 1999). All these results suggest that ICP0 may act to circumvent a cellular, interferon-induced antiviral response pathway.

ICP0 is proposed to change the stability of cellular proteins and nuclear structures in infected cells through a ubiquitin-mediated pathway. Consistent with this hypothesis, ICP0 was found to directly interact with a cellular ubiquitin-specific protease, HAUSP (herpesvirus associated ubiquitin-specific protease), which is a protein found in a subset of ND10 (Everett *et al.*, 1997). HAUSP normally functions to remove the ubiquitin adducts from proteins to protect them from proteasome mediated degradation. The association between ICP0 and HAUSP may eliminate its protective ability, leading to changes in protein stability through a ubiquitin-mediated protein turnover pathway. Moreover, ICP0 has been shown to induce degradation of PML and Sp100 in a ubiquitin-specific proteasome-dependent process that is directly linked to the disruption of ND10 sites (Everett, Orr, andPreston, 1998). Both proteins are modified by covalent linkage to the small ubiquitin-like modifier peptide, SUMO-1 (Chelbi-Alix and de The, 1999; Muller and Dejean, 1999; Sternsdorf, Jensen, and Will, 1997). ICP0 specifically abrogates SUMO-1 modification of PML and Sp100, and this activity is directly related to its ability to disrupt ND10 sites (Muller and Dejean, 1999). ICP0 also induces proteasome-dependent degradation of the catalytic subunit of the DNA-dependent protein kinase, some isoforms of PML and the kinetochochore binding protein CENP-C (Everett *et al.*, 1999; Everett *et al.*, 1998). These data further indicate that the ability of ICP0 to induce the viral lytic cycle is directly linked to its ability to alter protein stability and nuclear architecture.

ICP4 (also known as Vmw175, α4, IE3) is a large phosphoprotein that exists as a homodimer and localizes to the nucleus of infected cells. ICP4 is essential for viral replication, and is required for activation of E and L viral gene expression and for the downregulation of HSV IE gene expression. ICP4 deletion or temperature-sensitive mutants have been shown to be inviable and only express IE proteins (DeLuca, McCarthy, and Schaffer, 1985; DeLuca and Schaffer, 1985; Dixon and Schaffer, 1980; Preston, 1979; Watson and Clements, 1978).

ICP4 contains discrete functional domains for DNA-binding, dimerization, nuclear localization, and transcriptional activation (DeLuca and Schaffer, 1985; Paterson and Everett, 1988a; Shepard, Imbalzano, and DeLuca, 1989). The DNA-binding activity is essential for transcriptional activation by ICP4 and its function as a repressor of activated transcription (Paterson and Everett, 1988a; Shepard and DeLuca, 1991; Shepard, Imbalzano, and DeLuca, 1989). Two additional regions of ICP4 contribute to the transcriptional activation: a large domain defined by the carboxy-terminal region of the protein (DeLuca, McCarthy, andSchaffer, 1985; DeLuca and Schaffer, 1985; Shepard, Imbalzano, and DeLuca, 1989), and a small serine-rich region near the amino terminus of the protein (Shepard, Imbalzano, and DeLuca, 1989). The carboxy-terminal region is required for high level activation by ICP4. Removal of the amino acids in the serine region completely abrogates the activation function (Samaniego, Webb, and DeLuca, 1995; Shepard, Imbalzano, and DeLuca, 1989; Smith *et al.*, 1993).

The mechanisms by which ICP4 mediates E and L gene expression and IE repression are not yet fully understood, but both functions seem to be related to its DNA binding ability and its association with the holo-TFIID complex. ICP4 has been shown to interact with TATA-binding protein (TBP), TFIID, TFIIB, and TAF250 (Carrozza and DeLuca, 1996; Gu and DeLuca, 1994; Gu, Kuddus, and DeLuca, 1995; Smith *et al.*, 1993). Mutations in the DNA binding domain of ICP4 result in loss of transactivation function (Paterson and Everett, 1988b; Shepard and DeLuca, 1991; Shepard, Imbalzano, and DeLuca, 1989). The ICP4-TFIID complex is required for enhancing transcription (Carrozza and DeLuca, 1996; Gu and DeLuca, 1994). Oddly, the TATA boxes which display the lowest affinity for TFIID are transactivated to the greatest extent by ICP4

(Cook *et al.*, 1995). These observations lead to the hypothesis that ICP4 acts to normalize TATA-box dependent gene expression. The repressor activity of ICP4 seems to also stem from its interaction with TBP and TFIIB and from its DNA binding ability. The formation of ICP4-TBP-TFIIB complex has been shown to be required for repression (Kuddus, Gu, and DeLuca, 1995). In addition, the ICP4 promoter contains a consensus ICP4 binding site that overlaps with the transcription start site; this site has been shown to be required for ICP4 mediated repression of its own gene through mutational analysis, and the orientation and position of this binding site influences the efficiency of repression (Gu, Kuddus, and DeLuca, 1995; Leopardi, Michael, and Roizman, 1995; Michael and Roizman, 1989; O'Hare and Hayward, 1987; Paterson and Everett, 1988b; Roberts *et al.*, 1988).

ICP22 (also known as α22) is the least understood of the five immediate early gene products of HSV. It is a phosphoprotein that localizes to the nucleus of the infected cells, and appears to play a role in defining the host range of HSV. ICP22 null mutants replicate well in Vero and HEp-2 cell, but grow poorly in human embryonic lung (HEL), Rat-1, and BHK cells (Poffenberger, Raichlen, and Herman, 1993; Post and Roizman, 1981; Purves, Ogle, and Roizman, 1993; Sears *et al.*, 1985). This phenotype has been correlated with a decrease in ICP0 and late gene expression (Purves, Ogle, and Roizman, 1993). This observation has lead to the suggestion that ICP22 is involved in regulation of gene expression. ICP22 has been suggested to promote HSV gene expression by altering RNA polymerase II (RNAP II) which is utilized by HSV for its own gene expression (Rice *et al.*, 1995; Rice *et al.*, 1994). Two different forms of RNAP II exist in normal cells: IIa, which is hypophosphorylated at the C-terminus domain (CTD) of the largest subunit and is involved in transcription initiation; and IIo, which is hyperphosphorylated at the CTD of the largest subunit and is involved in transcription elongation. Upon HSV infection, IIa and IIo are depleted, and a novel phosphorylated form of RNAP II, IIi, is observed (Long *et al.*, 1999; Rice *et al.*, 1995). Both ICP22 and the protein kinase encoded by gene UL13 are required for this change of RNAP II. In cells infected with a ICP22 mutant, 22/n199, which is an HSV mutant with a nonsense condon insertion in the ICP22 gene, IIi formation and IIo depletion do not occur efficiently (Long *et al.*, 1999; Rice *et al.*, 1995). In cells infected with a UL13-deficient mutant, IIi induction and IIa

depletion do not occur efficiently (Long *et al.*, 1999; Rice *et al.*, 1995). The significance of ICP22 dependent IIi formation is still unknown, but depletion of the IIo form is correlated with repression of host cell transcription (Spencer, Dahmus, and Rice, 1997). Moreover, ICP22 has been shown to localize to small dense nuclear structures early in infection, and at the onset of DNA replication, ICP22 colocalizes with ICP4, nascent DNA, RNAP II (Leopardi *et al.*, 1997). All these data suggest that ICP22 is involved in regulation of gene expression at the transcriptional level; modification of RNAP II may favor the expression of viral genes.

ICP27 (also known as Vmw63, IE63, α27, IE2, UL54) is a phosphoprotein, and is essential for viral replication. Genetic studies of ICP27 mutants demonstrate that it is required for the down-regulation of IE genes, the enhancement of viral DNA replication, the expression of some late genes, and the efficient shutoff of host cell protein synthesis at later times of infection (Everett, 1986; McCarthy, McMahan, and Schaffer, 1989; McMahan and Schaffer, 1990; Rice and Knipe, 1988; Rice and Knipe, 1990; Sacks *et al.*, 1985; Sekulovich, Leary, and Sandri-Goldin, 1988; Uprichard and Knipe, 1996).

The mechanism by which ICP27 controls gene regulation is unknown. The current thinking is that ICP27 regulates gene expression by altering the cellular mRNA processing machinery (Sandri-Goldin, 1998). This alteration presumably increases the level of viral mRNAs in the cytoplasm, and at the same time, reduces the level of competing cellular mRNAs, therefore enhancing viral gene expression. ICP27 has been shown to alter the specificity of the host polyadenylation machinery, therefore allowing more efficient use of a subset of viral polyadenylation signals (McGregor *et al.*, 1996; McLauchlan *et al.*, 1992; McLauchlan, Simpson, and Clements, 1989). This activity of ICP27 is important because the late genes of HSV contain weak polyadenylation processing signals. In addition, substantial evidence suggests that ICP27 impairs RNA splicing, thereby causing the intron-bearing pre-RNAs to accumulate in the nuclei of infected cells. ICP27 has been suggested to inhibit expression of reporter genes bearing certain introns in transient-cotransfection assays (Sandri-Goldin and Mendoza, 1992). In addition, ICP27 co-immunoprecipitates with splicing factors and alters the phosphorylation status of some of these proteins (Sandri-Goldin and Hibbard, 1996).

ICP27 also causes the redistribution of cellular snRNP and splicing factors during infection (Phelan *et al.*, 1993). Moreover, nuclear extracts prepared from cells infected with wildtype virus carry out *in vitro* splicing reactions less efficiently than those prepared from uninfected cells, and ICP27 is required for this reduction (Hardy and Sandri-Goldin, 1994). Since most of the viral RNAs do not contain introns and most cellular pre-RNAs do contain introns, this leads to the increased levels of viral mRNAs in the cytoplasm. This inhibition of splicing is also thought to be the reason why ICP27 is required for late shutoff of host protein synthesis (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994).

Recently, ICP27 has been shown to bind RNA directly, and shuttle between the nucleus and cytoplasm of infected cells (Ingram *et al.*, 1996; Mears and Rice, 1996; Mears and Rice, 1998; Phelan and Clements, 1997; Sandri-Goldin, 1998; Soliman, Sandri-Goldin, and Silverstein, 1997). This observation leads to the suggestion that ICP27 stimulates the cytoplasmic accumulation of intron-less viral mRNAs by binding to these transcripts and mediating their nuclear export. Very recent data shows that ICP27 induces cytoplasmic accumulation of unspliced polyadenylated α-globin pre-mRNA in infected HeLa cells (Cheung *et al.*, 1999). Since the expression of the endogenous chromosomal human α-globin is activated by HSV infection (Cheung, Panning, and Smiley, 1997), this finding seems to suggest that ICP27 also stimulates the cytoplasmic accumulation of intron-bearing viral mRNAs, possibly by binding to these transcripts and mediating their nuclear export. How the nuclear export activity of ICP27 relates to its effect on intranuclear processing is yet unknown.

In addition to their individual effects, ICPs 0, 4, and 27 cooperatively regulate viral gene expression at the transcriptional and post-transcriptional levels. Substantial evidence shows that ICP0 interacts with ICP4 directly, and these proteins act together to activate gene expression (Everett, 1984; Everett, 1988; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985a; Yao and Schaffer, 1994). ICP27 also modulates ICP4 transactivation activity through physical interaction with ICP4 (O'Hare and Hayward, 1985a; Panagiotidis, Lium, and Silverstein, 1997; Samaniego, Webb, andDeLuca, 1995; Sekulovich, Leary, andSandri-Goldin, 1988; Su and Knipe, 1989). In transfected and

infected cells, these interactions lead to the redistribution of these IE proteins (Gelman and Silverstein, 1985; Knipe and Smith, 1986; Mullen *et al.*, 1995; Zhu, Cai, and Schaffer, 1994; Zhu, DeLuca, and Schaffer, 1996; Zhu and Schaffer, 1995). All these data indicate that a complex process is required to regulate the activities of IE proteins.

ICP47 is the only IE gene product which does not have a role in gene regulation. It is also nonessential for HSV growth in tissue culture, but it blocks peptide supply to MHC class I molecules by binding to the transporter associated with antigen processing (TAP), thereby preventing transport of viral peptide to the endoplasmic reticulum. This function of ICP47 enables the virus to escape anti-HSV $CD8^+$ CTL (cytotoxic T-lymphocyte) mediated death (Hill *et al.*, 1995; York *et al.*, 1994).

1.4.3. E gene expression and DNA replication

The expression of IE genes leads to the expression of HSV E and L genes. E gene expression reaches peak rates at about 5 to 7 hours postinfection, and includes several viral proteins that are essential in viral DNA replication such as: UL5 (which forms a complex with UL8 and UL52 in a 1:1:1 ratio, and functions as a helicase-primase), UL8, UL9 (a sequence-specific origin-binding protein), UL29 (a ss-DNA binding protein), UL30 (the catalytic subunit of viral DNA polymerase), UL42 (part of the viral DNA polymerase), and UL52 (Boehmer, 1997).

The presence of E gene products signals the onset of viral DNA replication. Viral DNA replication is carried out in an origin-dependent manner through a rolling-circle mechanism (Roizman and Sears, 1996). Three origins of replication have been identified; Ori_L which is a lytic replication origin located in the U_L region of the HSV DNA and two copies of Ori_S which located in the inverted repeat region flanking the U_S region of the HSV DNA (Roizman and Sears, 1996). The mechanism of replication has not yet been precisely defined. Analysis of the structure of the replicative intermediates of HSV-1 DNA using field inversion or pulsed-field gel electrophoresis shows that intra-cellular HSV-1 DNA is held together in a large complex by frequent branches (Severini *et al.*, 1994; Severini, Scraba, and Tyrrell, 1996; Zhang, Efstathiou, and Simmons, 1994).

This finding suggests that, in addition to a rolling-circle mechanism, recombination events occur during replication, thereby forming links between DNA molecules.

1.4.4. L gene expression, virion assembly and egression

The onset of viral DNA replication signals L gene expression. The L genes encode proteins that are involved in virion assembly and egression of progeny virion. Accumulation of L gene products leads to the assembly of HSV procapsids in distinct nuclear components referred to as assemblons (Desai and Person, 1998; Ward, Ogle, and Roizman, 1996). Seven L gene products: VP5, VP19C, VP21, VP22a, VP23, VP24, and VP26 are required for the assembly of the capsid (Tatman *et al.*, 1994). The requirement of these gene products has recently been confirmed by *in vitro* assembly systems using purified proteins (Newcomb *et al.*, 1996; Newcomb *et al.*, 1994; Thomsen *et al.*, 1995). VP21, VP24, and pre-VP22a form the "scaffold" protein structure first, then VP5, VP19C, VP23, and VP26 assemble outside of the "scaffold". After this process, the UL26 gene that encodes a proteinase cleaves its own gene product into VP21 and VP24, as well as cleaving preVP22 into VP22a, in the maturation step (Preston *et al.*, 1994).

After the assembly and maturation of the capsid, newly synthesized, highly branched viral DNA is resolved into unit-length genomes and is packaged into the pre-formed empty capsids. A full-length genome is packaged into the capsid by cleavage of concatameric replication intermediates at cis-acting sequences (pac sites) within the *a* sequence of the viral DNA, and the cleavage is probably mediated by proteins bound to the pac sites (Deiss, Chou, and Frenkel, 1986; Deiss and Frenkel, 1986; Stow, McMonagle, and Davison, 1983; Varmuza, 1985). The viral proteins that are required for this process include UL6, UL12, UL15, UL17, UL25, UL28, UL32, UL33 (Martinez *et al.*, 1996; Roizman and Sears, 1996). The precise functions of these proteins are not yet known.

After the incorporation of the viral genome into the capsid, the next step in the virion maturation process is the acquisition of the tegument layer and the envelope. The site of tegument assembly and acquisition remains to be defined, as does the egression route. Regardless of the mechanism, viral proteins UL34, UL20, UL11, gD, gH and gK

have been shown to be required for capsid maturation and egression (Baines and Roizman, 1991; Baines *et al.*, 1991; Browne *et al.*, 1996; Jayachandra, Baghian, and Kousoulas, 1997; Roller *et al.*, 2000).

Two competing theories have been proposed for the acquisition of the tegument; one is that tegument acquisition occurs near the inner nuclear membrane. Consistent with this theory, tegument proteins US11 and VP16 have been found to localize to sites near the inner nuclear membrane at late times of infection of Vero cells (Ward, Ogle, and Roizman, 1996). In addition, at these sites, thick patches which are presumably due to the presence of the tegument proteins and glycoproteins have been observed (Roizman and Sears, 1996). Another theory is that tegument acquisition occurs at a stage downstream of capsid translocation through the nuclear membrane. Consistent with this theory, tegument proteins VP22 and VP16 have been found to colocalize to the cytoplasmic compartment of infected and transfected cells (Elliott, Mouzakitis, and O'Hare, 1995; Elliott and O'Hare, 1999).

Mature capsids are believed to exit the nucleus by binding to the inner nuclear membrane (a process designated as budding). Two theories have been proposed to explain how HSV virions exit the cell after budding (Whittaker and Helenius, 1998). One is that the capsids bud into the perinuclear space, acquiring a membrane containing immature glycoproteins. These structures then move into the endoplasmic reticulum (ER) where glycoprotein processing occurs, and finally pass through the Golgi apparatus to reach cell surface (Whittaker and Helenius, 1998). Another theory proposes that capsids pass through the nuclear membrane, travel into the cytoplasm as unenveloped capsids, then enter transport vesicles containing mature glycoproteins to reach cell surface (Whittaker and Helenius, 1998). Consistent with this hypothesis, recombinant viruses which express Golgi-targeted forms of gD were recently found to grow to wild-type levels on noncomplementing cells with unaltered particle/infectivity ratio and wildtype levels of gD, but recombinant viruses expressing ER-retained forms of gD produced virions of low specific infectivity with greatly reduced levels of gD when grown on noncomplementing cells (Whiteley *et al.*, 1999).

1.5. Host protein synthesis shutoff

HSV infection leads to the shutoff of host protein synthesis. This strategy, in combination with other strategies (such as altering the RNAP II, cellular structures and mRNA processing) employed by HSV, allows the efficient expression of viral genes. The shutoff of host protein synthesis occurs in two phases: one occurs at early times in infection and does not require *de novo* viral protein synthesis; one occurs at late times in infection and requires *de novo* viral protein synthesis. The conclusion that shutoff happens at two different phases comes mainly from the studies of viable HSV mutants and their revertants. The HSV temperature-sensitive mutant, HFEM tsB47 cannot grow in permissive cells at the non-permissive temperature, and cannot induce host shutoff at this non-permissive temperature (Fenwick and Clark, 1982; Knipe *et al.*, 1981). However, at the non-permissive temperature, its revertant can grow and induces late host shutoff, but can not induce early host shutoff. These results indicate that the late host shutoff requires *de novo* viral protein synthesis, while the early host shutoff does not require *de novo* viral protein synthesis (Fenwick and Clark, 1982; Knipe *et al.*, 1981). This observation is further confirmed by the isolation of a series of viable host shutoff defective HSV mutants which can not induce early shutoff, but are able to induce late shutoff of host protein synthesis (Read and Frenkel, 1983).

How the late shutoff of host protein synthesis occurs is unknown. The HSV IE gene product ICP27 has been suggested to be responsible for the late shutoff because of its ability to alter the cellular mRNA processing and transport pathway. Presumably, alteration of these pathways leads to retention of intron-bearing cellular mRNAs in the nucleus of infected cells, thereby reducing the level of cellular mRNAs in the cytoplasm.

The early shutoff of cellular protein synthesis is accompanied by disruption of pre-existing polysomes and the rapid degradation of pre-existing mRNAs (Fenwick, 1984; Fenwick and Everett, 1990a; Fenwick and McMenamin, 1984; Fenwick and Owen, 1988; Fenwick and Walker, 1978; Jones, Smibert, and Smiley, 1995; Nishioka and Silverstein, 1977; Nishioka and Silverstein, 1978; Oroskar and Read, 1987; Oroskar and Read, 1989; Read and Frenkel, 1983; Roizman, Borman, and Rousta, 1965; Schek and

Bachenheimer, 1985; Smibert, Johnson, and Smiley, 1992; Sydiskis and Roizman, 1966; Sydiskis and Roizman, 1967; Sydiskis and Roizman, 1968). There is evidence suggesting that these processes are linked, but it is unknown whether the degradation of mRNAs is the cause or the result of shutoff of protein synthesis. So far, the very little evidence that has been generated suggests that the degradation of mRNAs is the consequence of shutoff of protein synthesis. Greater inhibition of protein synthesis than can be accounted for by the degradation of mRNAs has been observed (Schek and Bachenheimer, 1985). In addition, polysome disaggregation before mRNA degradation has been detected in infected cells (Nishioka and Silverstein, 1978).

The early shutoff of host protein synthesis is believed to be caused by a virion protein since it is independent of *de novo* protein synthesis as demonstrated by the following experiments: (1) UV inactivated HSV viruses still cause the early shutoff of host protein synthesis in infected cells, and this early shutoff is accompanied by the disaggregation of polysomes (Fenwick, 1984; Fenwick and Clark, 1982; Fenwick and Owen, 1988; Fenwick and Walker, 1978; Schek and Bachenheimer, 1985; Strom and Frenkel, 1987). (2) The same phenotype is observed when infection is carried out in the presence of drugs that block transcription or translation (Fenwick and McMenamin, 1984; Fenwick and Walker, 1978; Hill *et al.*, 1995; Schek and Bachenheimer, 1985; Strom and Frenkel, 1987). (3) Rapid shutoff of protein synthesis is observed in enucleated cytoplasts (which lack nuclei, and therefore, cannot support HSV gene expression) (Fenwick and McMenamin, 1984; Fenwick and Walker, 1978). (4) Early shutoff of host protein synthesis still occurs when cells are infected with HSV viruses that lack capsid and DNA, and therefore, cannot produce viral products (McLauchlan *et al.*, 1992).

A virion tegument protein, the virion host shutoff protein (vhs), encoded by the late gene UL41, is responsible for inducing the early shutoff of host protein synthesis. Three lines of data indicate that vhs is responsible for the early shutoff of host protein synthesis. First, mutations in viable mutants that are deficient in early shutoff have been mapped to the UL41 locus by marker rescue analysis (Fenwick and Owen, 1988; Kwong, 1988; McGeoch *et al.*, 1988). Second, targeted disruption of the UL41 gene produces an

early shutoff-deficient phenotype, and these mutants cannot cause polysome disaggregation or degradation of mRNAs (Fenwick and Everett, 1990a; Smibert, Johnson, and Smiley, 1992; Smibert and Smiley, 1990). Third, HSV-2 displays a stronger shutoff phenotype than HSV-1 (Powell and Courtney, 1975); viral recombinants in which the UL41 gene of HSV-1 has been replaced by the corresponding gene from HSV-2 display the more robust shutoff phenotype characteristic of HSV-2 (Fenwick and Everett, 1990b).

vhs is believed to be a protein of 58 kDa. *In vitro* translation of an mRNA mapping to the UL41 locus produces a ca. 58 kDa protein (Anderson *et al.*, 1981). A 58 kDa protein was identified in HSV-1 infected cells using antiserum generated against a synthetic peptide corresponding to a region of the predicted vhs sequence or against a bacterially expressed lacZ fusion UL41 protein (Read, Karr, and Knight, 1993; Smibert, Johnson, and Smiley, 1992). In addition, no 58 kDa protein was detected in cells infected with mutant HSV bearing a truncation in this region (Read, Karr, andKnight, 1993; Smibert, Johnson, and Smiley, 1992). The presence of the vhs protein in the tegument of HSV virions was also demonstrated by using the previously described antiserum; vhs protein was detected in a HSV virion that has been chemically depleted of the capsid and DNA (McLauchlan *et al.*, 1992).

Recent data suggest that vhs is sufficient to induce the early shutoff of host protein synthesis, although data suggesting that other viral proteins are also involved have been obtained. HSV mutants that carry defects in the UL13 protein kinase have been shown to induce the early shutoff inefficiently, and this inefficiency cannot be attributed to the level of vhs protein in the tegument of the virion (Overton *et al.*, 1994). This result suggests that the UL13 protein kinase is required for the efficient early shutoff of host protein synthesis. However, a different observation was obtained regarding level of vhs in the tegument in UL13 (or ICP22) mutants by Ng et al. (Ng, Chang, and Roizman, 1997). These mutants display reduced early shutoff, but the level of vhs in the tegument of the virion is also reduced, suggesting that the reduced host shutoff is due to the reduced level of vhs in the tegument (Ng, Chang, and Roizman, 1997). Furthermore, in our laboratory, UL13 mutants either fail to reduce the shutoff, or reduced shutoff only

very marginally (Shivak, 1998). Recently, definitive data has been obtained to show that vhs is sufficient to induce the early shutoff of host protein synthesis. When expressed as the only viral protein in transiently transfected mammalian cells, vhs is sufficient to block the expression of the reporter genes β-galactosidase and chloramphenicol acetyl transferase (CAT) (Jones, Smibert, and Smiley, 1995; Pak *et al.*, 1995).

Although vhs is not essential for HSV infection, vhs mutants display a ca. 10-fold reduction in virus yield in tissue culture (Read, Karr, and Knight, 1993; Smibert, Johnson, and Smiley, 1992) and cause severe defects in the nervous system of the mouse in terms of establishing/reactivating from latency (Leib *et al.*, 1999; Strelow, 1995). This observation indicates that vhs plays a important role in the HSV infection cycle. In addition to degrading cellular mRNAs in the cytoplasm, vhs also significantly destabilizes HSV mRNAs belonging to all three temporal classes (Oroskar and Read, 1987; Oroskar and Read, 1989; Strom and Frenkel, 1987). This effect is believed to sharpen the transitions between the successive phases of viral protein synthesis by tightly coupling changes in the rate of mRNA synthesis to altered mRNA levels. Consistent with this hypothesis, in vhs mutants infected cells, not only is there continued host protein synthesis, but also continued accumulation of viral IE and E transcripts and delayed onset of L gene expression (Kwong and Frenkel, 1987; Oroskar and Read, 1987; Read and Frenkel, 1983; Strom and Frenkel, 1987).

Although vhs destabilizes all viral mRNAs, its activity is presumably downregulated by the HSV late protein VP16, and this downregulation allows viral mRNAs to accumulate after host mRNAs have been degraded. Several lines of evidence support this hypothesis. First, the vhs activity delivered by the infecting virion is partially downregulated by a newly synthesized viral protein, allowing viral mRNAs to accumulate after host mRNAs have been degraded (Fenwick and Clark, 1983; Fenwick and Owen, 1988). Second, vhs binds directly to VP16 *in vitro* and *in vivo* (Smibert *et al.*, 1994). Third, VP16 null mutants undergo vhs induced termination of viral protein synthesis at intermediate times post infection, and this termination is inhibited by VP16 supplied in *trans* and inactivation of the UL41 gene (Lam *et al.*, 1996).

The mechanism of vhs action remains to be precisely defined. There is strong evidence indicating that vhs acts as a ribonuclease, but these data have not shown conclusively whether cellular factors are required for this ribonuclease activity. vhs displays weak but significant amino acid sequence similarity to the fen-1 family of nucleases. These nucleases are involved in DNA replication and repair in eukaryotes and archaebacteria (Doherty, Serpell, and Ponting, 1996), and have recently been shown to cleave both RNA and DNA substrates (Stevens, 1998). Cytoplasmic extracts prepared from HSV infected HeLa cells contain a vhs-dependent ribonuclease activity; both host and viral mRNAs are rapidly degraded in extracts from wildtype virus but not a vhs1 mutant (Krikorian and Read, 1991). The same phenotype was observed with HSV infected MEL cell extracts; a post polysome fraction of extracts from wildtype virus but not from vhs1 mutant infected cells extract triggered the degradation of exogenous mRNAs (Sorenson, Hart, and Ross, 1991). Extracts from partially purified HSV virions contain a vhs-dependent ribonuclease activity, and this activity is inhibited by anti-vhs antiserum (Zelus, Stewart, and Ross, 1996). More importantly, vhs expressed as the only HSV protein in a rabbit reticulocyte lysate (RRL) expression system induces the endoribonucleolytic cleavage of a variety of reporter mRNAs (Elgadi, Hayes, and Smiley, 1999; Elgadi and Smiley, 1999).

The mechanism of vhs action has recently been studied in more detail using the RRL system. Because vhs has the intriguing property of inducing degradation of mRNAs but sparing rRNAs and tRNAs *in vivo* (Krikorian and Read, 1991; Kwong and Frenkel, 1987; Oroskar and Read, 1989), experiments have been done to determine how vhs differentiates between these RNAs. It is of special interest whether features common to most mRNAs such as the 5' cap structure or 3' poly(A) tail, may select mRNAs from the RNA pool for vhs induced degradation. Using the RRL system, Elgadi and colleagues (Elgadi, Hayes, and Smiley, 1999) have shown that these specific features of mRNAs do not target them for vhs induced degradation. In addition, they also showed that ribosomes are not required for vhs induced degradation (Elgadi, Hayes, and Smiley, 1999).

Although vhs activity is not influenced by the presence of a 5' cap or 3' poly(A) in the RNA substrate, and does not require ribosomes in order to target RNAs, Elgadi and colleagues have recently obtained evidence which may link vhs to the translation initiation process. These investigators showed that vhs activity is selectively targeted to RNA sequences located 3' to picronavirus IRES elements (Elgadi and Smiley, 1999). The IRES elements provide an alternative, cap-independent mode for translation in eukaryotic cells. In the standard cap-dependent mechanism, the cap-binding translation initiation factor eIF4F binds to the cap structure at the 5' end of mRNAs. eIF4F consists of 3 proteins: eIF4E which is the cap-binding protein, eIF4A which is an RNA helicase, and eIF4G which serves as a scaffold for assembly of the complex. The N-terminal of eIF4G contains the eIF4E binding site, the C-terminal of eIF4G contains the binding sites for eIF4A and eIF3. Once bound to RNA, eIF4F recruits eIF3, which then serves as bridge between eIF4F and the 40S ribosomal subunit. In IRES-dependent of translation, the C-terminal portion of eIF4G binds to the IRES, which in turn serves as a scaffold for assembly of the initiation complex. This IRES-dependent translation mechanism is in fact how some of the viruses such as poliovirus and encephalomyocarditis virus preferentially translate their mRNAs and inhibit the translation of cellular mRNAs. The majority of cellular mRNAs are normally translated through the cap-dependent mechanism and viruses like polio encode a protein which cleaves the eIF4G into two fragments, uncoupling the cap recognition and ribosome recruitment of functions of eIF4F, thereby inhibiting cap-dependent translation.

Elgadi and colleagues showed that transcripts bearing the IRES of encephalomyocarditis virus or poliovirus are preferentially endoribonucleolytically cleaved by vhs (Elgadi, Hayes, and Smiley, 1999; Elgadi and Smiley, 1999). Using primer extension and RNA probing, these researchers further showed that cleavage occurs at sits clustered immediately downstream of the IRES element. In addition, targeting of mRNAs was found to be independent of the IRES position or sequence context. The authors hypothesized that this cleavage of IRES containing transcripts by vhs is possibly due to a secondary structure or a component of the translational machinery considering the translational function of IRES.

1.6. Experimental rationale and summary

Although there is evidence suggesting that vhs acts as a ribonuclease, these data do not exclude the possibility that this ribonuclease activity requires cellular factors. Several years ago, to address the question whether or not vhs is a ribonuclease itself or requires cellular factors for its activity, a Ph.D student in our lab, Frank Jones, expressed vhs in the yeast *Saccharomyce cerevisiae* using the GAL10 inducible promoter. He found that expression of vhs in yeast inhibits colony formation. Importantly, this inhibition of colony formation required the same regions of vhs as the shutoff of host protein synthesis in mammalian cells (Jones, 1995). These observations argue for the biological relevance of the effect. However, when the rate of protein synthesis was evaluated in yeast induced to express vhs, no change was observed (Frank Jones unpublished observation). This observation was not consistent with the prediction that vhs acts as a ribonuclease in yeast. One possible explanation for this finding was that, to induce vhs expression from the GAL10 promoter, galactose has to be added. Addition of galactose is known to globally elevate the cell growth rate, and greatly enhances protein synthesis. Because of this elevation, effects of vhs expression on yeast protein synthesis rate could have been masked. In addition, the plasmid used in these experiments to express vhs in yeast is a single-copy plasmid, and the level of expression was relatively low. Therefore, I started my project trying to repeat these results in yeast using a multiply-copy plasmid containing a inducible CUP1 promoter. The CUP1 promoter can be induced by copper sulfate, a treatment which has no major effect on cell growth or protein synthesis (Fürst *et al.*, 1988). As detailed below, I found that expression of vhs using the CUP1 promoter in *S. cerevisiae* inhibits colony formation, and the severity of inhibition is dependent on the carbon source used. Moreover, the level of vhs expressed using the CUP1 promoter was significant higher than level of vhs expressed from the GAL10 promoter. In the interum, Mabrouk Elgadi in our lab had developed a sensitive assay to test vhs ribonuclease activity *in vitro*. Therefore, I decided to test whether or not whole cell extracts of yeast containing vhs show any activity *in vitro*, instead of pursuing the *in vivo* study of the effects of vhs expression in yeast. These experiments revealed that yeast extract containing vhs show no detectable endoribonuclease activity *in vitro*,

and that this inability of vhs to induce cleavage of substrate RNAs is not due to the presence of an inhibitor but rather is due to the absence of a required mammalian cellular factor. This result demonstrates that mammalian cellular factors are required for the activity of vhs *in vitro*.

While I was testing vhs activity *in vitro*, I unexpectedly found that the vhs point mutant, vhs1, retains significant *in vitro* activity. This observation was a surprise, as previous data had suggested that the vhs1 mutation completely abolishes vhs activity. vhs1 is one of the best-studied vhs mutants, and it contains a single base change resulting in a threonine to isoleucine substitution at vhs residue 214 (Jones, Smibert, and Smiley, 1995). Much of our current knowledge about vhs has been gained through the study of this vhs1 mutant. Marker rescue of the vhs1 mutant led to the mapping of the vhs function to the UL41 gene (Fenwick and Owen, 1988; Kwong, 1988). Study of the vhs1 mutant resulted in the identification of vhs as the protein responsible for the early shutoff of host protein synthesis (Daksis *et al.*, 1987; Fenwick, 1984; Fenwick and Everett, 1990a; Fenwick and Owen, 1988; Jones, Smibert, and Smiley, 1995; Kwong, 1988; Kwong, 1989; Kwong and Frenkel, 1987; Oroskar and Read, 1989; Read and Frenkel, 1983; Read, Karr, and Knight, 1993; Strelow, 1995). Several pieces of evidence have led researchers to believe that the point mutation in vhs1 completely inactives vhs function. First, this mutation is located in one of the regions of strongest homology to the fen-1 family of nucleases. Second, the vhs1 mutant shows a vhs-deficient phenotype during infection (Daksis *et al.*, 1987; Fenwick, 1984; Fenwick and Everett, 1990a; Fenwick and Owen, 1988; Jones, Smibert, and Smiley, 1995; Kwong, 1988; Kwong, 1989; Kwong and Frenkel, 1987; Oroskar and Read, 1989; Read and Frenkel, 1983; Read, Karr, and Knight, 1993; Strelow, 1995). Third, in transfection assays, the vhs1 protein does not inhibit the expression of a reporter gene, lacZ (Jones, Smibert, and Smiley, 1995). Fourth, in the RRL expression system, when tested on an exogenous mRNA lacking the picornavirus internal ribosome entry site (IRES) element, the activity of vhs1 is significantly reduced compared to vhs (Elgadi, Hayes, and Smiley, 1999). Intriguingly, I found that the vhs1 protein expressed in RRL system shows significant endoribonuclease activity on a RNA bearing the EMCV IRES element. These data raise the possibility that

the vhs-dependant endoribonuclease employs more than one mode of substrate recognition.

1.7. References

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CHAPTER 2 – THE HERPES SIMPLEX VIRUS VIRION HOST SHUTOFF PROTEIN REQUIRES A MAMMALIAN FACTOR FOR EFFICIENT *IN VITRO* ENDORIBONUCLEASE ACTIVITY

Submitted to the Journal of Virology.

Preface.

The data presented in this chapter, submitted to the Journal of Virology, have been formatted to comply with the paper thesis format. The data presented in Figure 1 and 2 were generated by Frank Jones. I carried out all the rest of the experiments presented in this chapter (from Figure 3 to 11). I also prepared the data for publication and wrote the first draft of the manuscript. A major editorial contribution from my supervisor, Dr. James R. Smiley, led to the final version of the paper.

The Herpes Simplex Virus Virion Host Shutoff Protein Requires A Mammalian Factor for Efficient *In Vitro* Endoribonuclease Activity

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Running title: cellular factor required for vhs activity

2.1. Abstract

The virion host shutoff protein (vhs) of herpes simplex virus triggers global shutoff of host protein synthesis and accelerated mRNA turnover during virus infection, and induces endoribonucleolytic cleavage of exogenous RNA substrates when it is produced in a rabbit reticulocyte (RRL) *in vitro* translation system. Although vhs induces RNA turnover in the absence of other HSV gene products, it is not yet known whether cellular factors are required for its activity. As one approach to addressing this question, we expressed vhs in the budding yeast *Saccharomyces cerevisiae*. Expression of vhs inhibited colony formation, and the severity of this effect varied with the carbon source. The biological relevance of this effect was assessed by examining the activity of five mutant forms of vhs bearing previously characterized in-frame linker insertions. The results indicated a complete concordance between the growth inhibition phenotype in yeast and mammalian host cell shutoff. Despite these results, cell-free extracts of yeast expressing vhs displayed little if any vhs-dependent endoribonuclease activity. However, activity was readily detected when such extracts were mixed with RRL. These data suggest that the vhs-dependent endoribonuclease requires one or more mammalian macromolecular factors for efficient activity.

2.2. Introduction

Herpes simplex virus (HSV) is a large enveloped DNA virus that replicates in the nuclei of infected mammalian cells. During lytic infection, more than 80 genes are expressed in the order of immediately early (IE), early (E) and late (L) through execution of a complex genetic regulatory program. Several of the viral regulatory proteins are contained in the tegument of the HSV virion. One of the best characterized of these is the virion host shutoff protein (vhs) encoded by HSV gene UL41. Vhs triggers early shutoff of cellular protein synthesis, disruption of polysomes and rapid degradation of pre-existing mRNAs (Fenwick and Clark, 1982; Fenwick and McMenamin, 1984; Fenwick and Owen, 1988; Fenwick and Walker, 1978; Kwong and Frenkel, 1987; Kwong, Kruper, and Frenkel, 1988; Oroskar and Read, 1987; Oroskar and Read, 1989; Read and Frenkel, 1983; Strom and Frenkel, 1987). Three lines of evidence indicate that the vhs protein is both necessary and sufficient for early host shutoff. First, several mutations that lead to a vhs-deficient phenotype have been mapped to the UL41 locus, and targeted disruption of the UL41 gene eliminates early shutoff (Fenwick and Evrett, 1990; Read, Karr, and Knight, 1993; Smibert, Johnson, and Smiley, 1992; Smibert and Smiley, 1990). Second, viral recombinants in which the UL41 gene of HSV-1 has been replaced by the corresponding gene from HSV-2 display the more robust shutoff phenotype characteristic of HSV-2 (Fenwick and Everett, 1990). Third, vhs blocks reporter gene expression when it is produced as the only viral protein in transiently transfected mammalian cells (Jones, Smibert, and Smiley, 1995; Pak *et al.*, 1995).

In addition to triggering degradation of cellular mRNAs, vhs also significantly destabilizes HSV mRNAs belonging to all three temporal classes. This effect is believed to sharpen the transitions between the successive phases of viral protein synthesis, by tightly coupling changes in the rate of mRNA synthesis to altered mRNA levels (Fenwick and Owen, 1988; Kwong and Frenkel, 1987; Kwong, Kruper, and Frenkel, 1988; Oroskar and Read, 1987; Oroskar and Read, 1989; Read and Frenkel, 1983; Strom and Frenkel, 1987). Although vhs significantly destabilizes viral mRNAs, the vhs activity delivered by the infecting virion is partially dampened by a newly synthesized viral protein,

allowing viral mRNAs to accumulate after host mRNAs have been degraded (Fenwick and Owen, 1988). Two lines of evidence suggest that the virion transactivator VP16 serves this negative regulatory role. First, vhs binds directly to VP16 (Smibert *et al.*, 1994). Second, VP16 null mutants undergo vhs-induced termination of viral protein synthesis at intermediate times post infection, and this effect is inhibited by VP16 supplied in *trans* (Lam *et al.*, 1996).

Although the mechanism of action of vhs has yet to be precisely defined, the currently available evidence strongly suggests that vhs is either itself a ribonuclease, or a subunit of a ribonuclease that also includes one or more cellular subunits. Extracts of HSV infected cells and partially purified virions contain a vhs-dependent ribonuclease activity (Karr and Read, 1999; Krikorian and Read, 1991; Sorenson, Hart, and Ross, 1991; Zelus, Stewart, and Ross, 1996) that is inhibited by anti-vhs antibodies (Zelus, Stewart, and Ross, 1996). In addition, vhs induces endoribonucleolytic cleavage of a variety of reporter mRNAs when it is expressed as the only HSV protein in a rabbit reticulocyte lysate (RRL) expression system (Elgadi, Hayes, and Smiley, 1999; Elgadi and Smiley, 1999; Zelus, Stewart, and Ross, 1996). Moreover, vhs displays weak but significant amino acid sequence similarity to the FEN-1 family of nucleases that are involved in DNA replication and repair in eukaryotes and archaebacteria (Doherty, Serpell, and Ponting, 1996), and recent studies have shown that human FEN-1 cleaves both RNA and DNA substrates (Stevens, 1998). Although the foregoing data indicate that the vhs protein is a required component of the vhs-dependent endoribonuclease, they leave open the possibility that the enzyme also contains one or more cellular subunits, or requires cellular factors for its activity.

As one approach to testing a possible requirement for cellular factors, we studied the effects of expressing vhs in a heterologous eukaryotic system, the budding yeast *Saccharomyces cerevisiae*. *S. cerevisiae* has been successfully utilized for the expression of other HSV proteins including glycoprotein B (Nozaki *et al.*, 1985), DNA polymerase (Haffey *et al.*, 1988) and thymidine kinase (McNeil and Friesen, 1981; Zealey *et al.*, 1988; Zhu, Ward, and Weissbach, 1984). We report here that expression of vhs inhibits colony formation, and that this effect displays the same mutational sensitivity spectrum

as host shutoff in mammalian cells. However, cell-free extracts of yeast expressing vhs displayed little if any vhs-dependent endoribonuclease activity. Activity was restored by adding RRL to the extracts, indicating that the vhs-dependent ribonuclease requires one or more mammalian factors.

2.3. Materials and Methods

Plasmids. Two different yeast expression vectors were used to express wild-type and mutant forms of vhs in yeast: pYGAL and pYEX-BX. pYGAL bears the galactose-inducible GAL10 promoter, and pYEX-BX contains the copper-inducible CUP1 promoter. pYGAL was generated from pJAY99 by inserting a 375 bp BglII-HindIII fragment from pPGK (generously donated by Dr. John Glover, McMaster University) into the SphI-HindIII sites of pJAY99 (after making the BglII and SphI sites flush with T4 DNA polymerase). This fragment of pPGK contains the 3' untranslated region (UTR) of the yeast 3-phosphoglycerate kinase (PGK) gene which bears a yeast polyadenylation signal and transcription termination sequence (Hitzman *et al.*, 1982). pJAY99 was derived by Jacques Archambault in the Friesen laboratory (University of Toronto) by cloning the GAL10 promoter region (Guarente, Yocum, and Gifford, 1982) into the EcoRI-SmaI sites of pFL39. pFL39 is a pUC19-based low copy-number plasmid bearing the TRP1 gene, an autonomously replicating sequence (ARS), and the centromere of yeast chromosome VI (CEN6) (Bonneaud *et al.*, 1991).

A yeast expression vector bearing the vhs ORF under the control of the GAL10 promoter was constructed in two steps. First, a plasmid (pvhsRI) (Smibert *et al.*, 1994) containing the vhs ORF and 0.3 kb of 3' flanking sequences with an engineered NcoI site at the vhs initiation codon was modified by inserting a StuI linker immediately upstream of the NcoI site, generating pvhs Stu. Second, the StuI-HincII fragment of pvhs Stu bearing the vhs ORF and 3' flanking sequences was subcloned between the SmaI-HincII sites of pYGAL, generating pYGAL vhs. The vhs1 point mutation and several vhs linker insertion mutations were transferred into pYGAL vhs by replacing the 1.7 kb NcoI-PstI fragment of pYGAL vhs with the corresponding 1.7 kb NcoI-KpnI fragment from pCMV vhs1, R27, pN138-HA, pSc243, pS344-HA, and pM384 (after making the KpnI and PstI sites flush with T4 DNA polymerase) (Jones, Smibert, and Smiley, 1995), generating the

plasmids pYGAL vhs1, pYGAL R27, pYGAL N138-HA, pYGAL Sc243, pYGAL S344-HA, and pYGAL M384, respectively.

pYEX-BX (Clontech) contains the yeast CUP1 promoter (pCUP1), the leu2-d gene (a LEU2 gene with a truncated, but partially functional promoter), the 2μ origin of DNA replication, and the URA3 gene. The NcoI-HindIII fragment of pYGAL vhs bearing the vhs ORF and 3' flanking sequences was cloned between the BamHI-SalI sites of pYEX-BX (after making all four ends flush with the Klenow fragment of DNA polymerase I), generating pYEX-BX vhs. pYEX-BX vhs1 was generated in the same way, using the NcoI-HindIII fragment of pYGAL vhs1. A pYEX-BX vector bearing a doubly tagged vhs ORF (pYEX-BX 2.1vhs) was generated by inserting the NcoI-EcoRI fragment from pSP6 2.1vhs (Elgadi, Hayes, and Smiley, 1999) into the BamHI-EcoRI sites of pYEX-BX (after repairing the NcoI and BamHI ends with the Klenow fragment of DNA polymerase I). pYEX-BX 2.1vhs1 was generated in the same way, using the NcoI-EcoRI fragment of pSP6 2.1vhs1.

The vhs *in vitro* translation vector (pSP6vhs), and *in vitro* transcription vectors (pCITE-1 and pSPSR19N) encoding substrate RNAs (pCITE-1 and SRPα RNA, respectively) have been described previously (Elgadi, Hayes, andSmiley, 1999; Elgadi and Smiley, 1999).

Bacterial strains and growth media. Plasmids were maintained and amplified in two *Escherichia coli* strains. Plasmids derived from pYGAL were maintained and amplified in strain DH5α (*F⁻ endA1 hsdR17[rk-mk+] supE44 thi-1 λ recA gyrA96 relA1 Δ[argF-laczya] U169 θ80 lacZΔM15*) (Hanahan, 1983), while plasmids derived from pYEX-BX were maintained and amplified in strain HB101 (*F⁻, Δ[gpt-proA] 62 leuB6 supE44 ara-14 galK2 lacY1 Δ[mcrC-mrr] rpsL20 [Str^r] xyl-5 mtl-1 recA13*) (Maniatis, Fritsch, and Sambrook, 1989). Both strains were cultured at 37°C in Luria-Bertani medium (LB; 1.0% w/v bacto-tryptone, 0.5% w/v yeast extract, 1.0% w/v NaCl) in a shaker incubator set at 250 rpm. Derivatives transformed with recombinant plasmids were isolated on LB agar plates (LB with 1.5% w/v agar) containing 100 μg/ml of ampicillin.

Yeast strains. Two different yeast strains were used: YPH500 (*MATα ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 lec2-Δ1*) (Sikorski and Hieter, 1989), generously supplied by Dr. John Glover (McMaster University), and W303-1A (*MATa SUC3 ade2-1 can1-100 his3-11, 15 leu2-3, 112trp1-1, ura3-1*) (Livingstone-Zatchej *et al.*, 1997), generously supplied by Dr. Ivan Sadowski (University of British Columbia). YPH500 was used as the host for plasmids derived from the pYGAL vector, while W303-1A was used as the host for plasmids derived from pYEX-BX.

Yeast media and growth conditions. YPH500 and W303-1A were cultured and maintained in YEP/D (2% bacto-peptone, 1% yeast extract, 2% dextrose). Yeast strains transformed with plasmids were cultured in YNB/D or YNB/G (0.67% yeast nitrogen base without amino acids, containing 2% dextrose or 2% galactose respectively) supplemented with the appropriate nutrients for plasmid selection (uracil, L-lycine, adenine, L-histidine, and L-leucine in the case of pYGAL derivatives and strain YPH500; adenine, L-histidine, and tryptophan in the case of pYEX-BX derivatives and strain W303-1A). All yeast strains were cultured at 30°C in a shaker incubator set at 200 rpm.

Yeast transformation. YPH500 was transformed using either of the following methods. The first is a modification of a protocol described elsewhere (Becker and Guarente, 1991). Briefly, yeast cells cultured to log phase in YEP/D were washed three times with ddH_2O and once with ice-cold 1.0 M sorbitol (Sigma). The final cell pellet was resuspended into ice-cold 1.0 M sorbitol and placed on ice until use. 1 μg of transforming plasmid DNA was added to 20 μl of the yeast cell suspension. The cells were electroporated at 250 V with 4 KOhm resistance in a cell-Porator (Gibco-BRL). The electroporated cells were immediately removed, suspended in 100 μl of ice-cold 1.0 M sorbitol, and the entire cell suspension was spread onto selective YNB/D plates. Alternatively, cells were transformed using a modification of the lithium-acetate procedure (Elble, 1992). Briefly, 1.0 ml of log phase yeast cells in YEP/D were pelleted by centrifugation. 1 μg of transforming plasmid DNA was mixed with the cell pellet. 500 μl of PLATE (40% w/v PEG 4000, 100 mM Li-acetate, 10 mM Tris, pH 7.5, 1 mM EDTA) was added to the mixture and the cells were re-suspended by pipetting up and down several times. The cell suspension was incubated at room temperature without

mixing. After incubating for at least 1 day, 50 µl of the mixture was taken from the bottom of the tube, mixed with 50 µl of ddH_2O and the entire cell suspension was spread onto selective YNB/D plates.

W303-1A was transformed using a slightly different lithium-acetate method (Elble, 1992), with the following modification. Briefly, log phase cells were collected by centrifugation, washed with 1 ml of TE/LiAc, which was made fresh from 10X filter sterilized stocks (10X TE {0.1 M Tris-HCL, 0.01 M EDTA, pH7.5}; 10X LiAc {1M LiAc, pH7.5 adjusted with diluted acetic acid}) and resuspended at 2 X 10^9 cells/ml in 1X TE/LiAc. 50 µl of the cells were mixed with 1µg of transforming DNA, 50 µg of single stranded salmon sperm carrier DNA and 300 µl of 40% PEG 4000 solution (40% PEG 4000, 1X TE, 1X LiAc, which was made fresh from 50% PEG 4000, 10X TE and 10X LiAc). The mixture was mixed thoroughly, then incubated at 30°C for 30 minutes with agitation. After incubation, the mixture was heat shocked for 15 minutes at 42°C. Cells were collected and resuspended in 1 ml of 1X TE, then plated onto selective YNB/D plates.

Preparation of cell extracts for Western blot analysis. Yeast cultures were grown to an OD_{600} of 2-3, then split into two. One culture was induced with 0.5 mM CuSO4 for 5 hours, while the other was left untreated. Cells were collected, washed once with ice-cold ddH_2O, then lysed by boiling 10 minutes in 2 volumes of 2X sample buffer (125 mM Tris, pH 6.8, 600 mM 2-mercaptoethanol, 6% SDS, 20% glycerol, 0.005% bromophenyl blue). The lysate was clarified by centrifugation for 10 minutes and stored at –70°C until use.

Extracts of HSV-1 infected Vero cells were prepared as described elsewhere (Jones, Smibert, and Smiley, 1995), with the following modification. Briefly, Vero cells in 35 mm diameter dishes were infected with the mutant virus HSV-1 Pvhs N138-HA at 10 PFU per cell. After infection for 12 hours, the cells were lysed by boiling for 10 minutes in 2 volumes of 2X sample buffer. The lysate was stored at –70°C until use. HSV-1 Pvhs N138-HA encodes a mutant version of vhs bearing the HA tag following residue 138 (Jones, Smibert, and Smiley, 1995).

Western blot analysis. Western blot analysis was conducted as described elsewhere (Jones, Smibert, and Smiley, 1995). Briefly, samples were separated by electrophoresis through a 9% SDS-PAGE gel, then transferred to a 0.45 mm nitrocellulose filter. The protein was detected with a 1/500 dilution of rabbit antiserum against the HA epitope (Boehringer Mannheim) and a 1/500 dilution of sheep anti-rabbit Ig conjugated with horseradish peroxidase (Boehringer Mannheim). Bound secondary antibody was visualized by Renaissance chemiluminescence Reagent (NEN) according to the manufacturer's protocol.

Preparation of yeast extracts for vhs activity assay. Frozen cells were prepared as described elsewhere (Schultz, Choe, and Reeder, 1991). Briefly, cells grown in YNB/G to an OD_{600} of ~3 were induced with 0.15 mM $CuSO_4$ for 5 hours, then harvested by centrifugation at 4,000 rpm at 4°C for 4 minutes in a Beckman J-Lite™ rotor. The wet weight of the cells was measured, then the cells were washed sequentially in ice-cold ddH_2O, 1.3 volumes of extraction buffer (100 mM Hepes - KOH, pH 7.9, 245 mM KCl, 5 mM EGTA, 1 mM EDTA, and freshly made 2.5 mM dithiothreitol), and 1.3 volumes of extraction buffer supplemented with protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine hydrochloride, 3.5 µg/ml pepstatin A, 5 µg/ml leupeptin, and 10 µg/ml aprotinin). The cell pellet was loaded into a syringe, squeezed into liquid nitrogen, then stored at –70°C.

Extracts were prepared by the method of Schultz et al. (Schultz *et al.*, 1997). Briefly, ~3 g of frozen cells were used to prepare the extract in an unmodified home coffee mill. The mill was chilled by covering the blades with dry ice and running it until the dry ice was a powder. Frozen cells were added and processed in the cold room for 5 minutes. The powder was transferred to a cold beaker, and 1.3 volumes of extraction buffer with protease inhibitors was added. The powder was thawed, mixed, then centrifuged at 100,000 X g for 2 hours. The supernatant (minus lipid pellicle) was collected by tube puncture and dialyzed overnight against 50 volumes of vhs assay buffer (1.6 mM Tris-acetate, 80 mM potassium acetate, 2.0 mM Mg acetate, 0.1 mM DTT, 0.25 mM ATP, 20 U of RNase inhibitor {Sigma}, adjusted pH to 7.8 with acetic acid). The

extracts were stored at –70°C. Protein concentration in the yeast extract was determined by the method of Bradford (Bradford, 1976).

***In vitro* transcription and RNA labeling.** vhs and vhs1 mRNAs destined for *in vitro* translation were produced according to a procedure that has been described elsewhere (Elgadi, Hayes, and Smiley, 1999). Uncapped, internally labeled reporter RNAs were generated in a similar way, except that the cap primer was omitted and the reaction was shortened to 30 minutes. The pCITE-1 reporter RNA transcribed from pCITE-1 was generated using T7 RNA polymerase and Eco47III-linearized plasmid DNA as a template to yield a run-off transcript of ca. 2.3 kb (Elgadi and Smiley, 1999). The SRPα reporter mRNA was generated using SP6 RNA polymerase and EcoRV-linearized pSPSR19N as a template to yield a run-off transcript of 2.4 kb (Elgadi, Hayes, and Smiley, 1999).

***In vitro* translation.** *in vitro* translation of vhs using RRL has been described previously (Elgadi, Hayes, and Smiley, 1999; Elgadi and Smiley, 1999).

vhs activity assay. Reporter mRNAs were added to RRL controls (blank RRL and RRL containing the pretranslated vhs), yeast extracts or yeast extracts mixed with blank RRL. The amount of yeast extract used for each reaction was based on the total protein concentration and ~ 100 μg of total protein was used in each reaction. All reactions were adjusted to the same final volume using the vhs assay buffer. Where indicated, an equal volume of blank RRL was added to the yeast extracts. Reactions were incubated at 30°C. At various times post addition of the reporter RNA, aliquots were taken and RNA was recovered using the RNeasy mini kit (Qiagen) according to the manufacturer's procedure. Briefly, samples were added to a mixture of RNase-free H_2O and 100 mM EDTA to bring the final EDTA concentration to 10 mM, and the final volume of the mixture to 100 μl. To this mixture, 350 μl of buffer RLT (with 10 μl β-mercaptoethanol/ml RLT, Qiagen) and 250 μl ethanol (95%) were added. The mixture was loaded onto an RNeasy mini spin column and centrifuged for 15 seconds at ≥10,000 rpm. The column was washed twice with wash buffer RPE (Qiagen), then the RNA was eluted with RNase-free water. The eluted RNA was precipitated with 95% ethanol and

1/10th volume 3 M sodium acetate. The RNA pellet was washed with 70% ethanol, dried, then resuspended in RNase-free water.

Agarose gel electrophoresis and Northern blot analysis. The details of electrophoresis and Northern blot analysis have been described elsewhere (Elgadi, Hayes, and Smiley, 1999).

Markers. RNA markers were generated as previously described (Elgadi, Hayes, and Smiley, 1999). Briefly, pSPSR19N DNA was linearized with EcoRV, PvuII, SmaI and NruI. These linearized DNA templates were used in *in vitro* transcription to produce run-off transcripts of 2422, 1628, 800 and 429 nt, respectively.

2.4. Results

vhs inhibits colony formation in *S. cerevisiae*. As reviewed in the Introduction, previous data strongly suggest that vhs is an integral component of the vhs-dependent endoribonuclease, but leave open the possibility that one or more mammalian subunits or cofactors are required for its activity. As one approach to testing the possible involvement of cellular cofactors, we explored the consequences of expressing vhs in the budding yeast *S.cerevisiae*. Strain YPH500 was transformed with a plasmid bearing the vhs ORF under the control of the galactose-inducible GAL10 promoter (Yvhs); as controls, we also derived strains bearing the empty expression vector (YGAL) and a vector specifying the inactive vhs1 mutant form of vhs (Yvhs1). Strains were grown to saturation in selective glucose medium, then serial dilutions were spotted onto minimal plates containing either galactose or glucose as the carbon source (Figure 1). As expected, the control YGAL strain harboring the empty expression vector formed equivalent numbers of colonies on glucose and galactose plates. In contrast, the Yvhs strain displayed a large reduction in the number of visible colonies when cells were plated in the presence of galactose; only a few small colonies were observed at the 10^{-2} dilution, and no colonies were observed at dilutions greater than 10^{-2} (Fig. 1, see also Fig. 2). The vector encoding the vhs1 mutant form of vhs had little if any effect (Yvhs1). These results indicate that induction of vhs expression from the galactose-inducible

GAL10 promoter strongly inhibits colony formation, and that the vhs1 point mutation eliminates this phenotype.

The vhs induced phenotype displays the same mutational sensitivity spectrum as host shutoff in mammalian cells. We compared the mutational sensitivity spectrum of the growth inhibition phenotype to that of mammalian host shutoff, as an additional test of the biological relevance of the foregoing results. Previous mutational studies have indicated that two regions of the vhs polypeptide tolerate in-frame insertions, while a minimum of three regions of the protein are essential for its function (Jones, Smibert, and Smiley, 1995). These results are exemplified by the phenotypes of five representative mutants: R27, Sc243, and M384 bear in-frame insertions that disrupt highly conserved regions of the vhs polypeptide, and are inactive in mammalian cells; in contrast, N138-HA and S344-HA alter regions of vhs that are deleted from the vhs homologues of some alphaherpesviruses, and retain full activity (Jones, Smibert, and Smiley, 1995). We cloned these five mutations into the pYGAL vector, and determined their effects on yeast cell growth using the dilution patch test (Fig.2). The results revealed a complete concordance between the yeast and mammalian assay systems: expression of vhs, N138-HA and S344-HA strongly inhibited colony formation, while the empty vector, R27, Sc243, and M384 had no effect (Fig. 2). These data provide a strong indication that the growth inhibition phenotype in yeast requires the same regions of the vhs polypeptide as does the shutoff phenotype in mammalian cells, and are consistent with the hypothesis that this effect reflects a biological relevant activity of vhs.

Carbon source-dependent inhibition of growth. The pYGAL plasmid used in the proceeding experiments is a single copy vector that directs accumulation of relatively low levels of vhs protein (data not shown). In addition, the GAL10 promoter is not well suited for eventual studies of the *in vivo* effects of vhs on protein synthesis and RNA turnover in yeast, because addition of the inducer (galactose) causes a large increase in cellular growth rate, protein synthesis and mRNA levels. In order to increase the levels of vhs expression and avoid global effects of the inducer on cell growth rates, we decided to use a multicopy plasmid containing the copper-inducible promoter derived from the yeast metallothionine (CUP1) gene (induction of the CUP1 promoter has no effect on cell

growth or protein synthesis (Fürst *et al.*, 1988)). To this end, strain W303-1A was transformed with a plasmid bearing the vhs ORF under the control of the CUP1 promoter; the empty expression vector and a vhs1 mutant construct served as controls. Cells harboring each of these plasmids were grown to saturation in selective glucose medium, then equal numbers of cells (based on OD_{600}) were spotted and streaked out on selective glucose plates containing or lacking the inducer (copper sulfate). Surprisingly, the vector encoding wild-type vhs had little, if any, effect on colony formation on glucose plates in the presence of copper sulfate (Fig. 3A).

Although this observation at first glance appears to conflict with the results described above (Fig.1 and 2), the GAL10 constructs were assayed in the presence of galactose as the sole carbon source (in order to avoid catabolite repression of the GAL10 promoter), while glucose was used as the carbon source in the experiment shown in Fig. 3A. We therefore tested the effects of varying the carbon source (Fig 3). As shown in Fig. 3B and C, induction of vhs expression from the CUP1 promoter severely inhibited cell growth when galactose or raffinose was used as the carbon source. Moreover, the vhs vector strongly inhibited cell growth in both the presence and absence of copper sulfate when maltose was used as the carbon source (Fig. 3D). In all three cases, the vhs1 vector had no effect. Taken in combination, these data demonstrate that the severity of vhs-induced growth inhibition varies markedly with the carbon source. Presumably, the failure of cells harboring the vhs vector to grow on maltose plates in the absence of inducer stems from low constitutive levels of vhs expression obtained with the CUP1 promoter (see Fig. 5).

Although induction of vhs expression prevented the formation of visible colonies within 5 days on galactose plates, the expected number of colonies were observed when the plates were incubated for 10 days (data not shown). Thus, expression of vhs reduces the growth rate of yeast, but is not lethal.

The foregoing data demonstrate that vhs inhibits the growth of yeast, and that this effect is eliminated by mutations that inactivate shutoff function in mammalian cells. The failure of certain mutant forms of vhs to inhibit the growth of yeast might stem from

loss of one or more functions of the vhs protein. Alternatively, the mutations might reduce accumulation of the mutant protein. In order to distinguish between these possibilities in the case of the vhs1 mutant protein, we examined the levels of accumulation of epitope-tagged protein by Western blot analysis. The 2.1 version of vhs (Elgadi, Hayes, and Smiley, 1999) bears eight tandem histidine residues inserted after residue 138, an influenza virus hemagglutin (HA) epitope following residue 344, and retains full activity in the RRL *in vitro* system. We placed wild-type and vhs1 mutant versions of the 2.1vhs open reading frame under the control of the CUP1 promoter, then tested for effects on cell growth and accumulation of vhs protein. As shown in Figure 4, the 2.1 versions of wild-type and mutant vhs produced the same effects on cell growth as the corresponding untagged proteins, and were therefore suitable for the experiment. Cells harboring the 2.1vhs and 2.1vhs1 expression vectors were grown to saturation in selective galactose medium, induced with copper sulfate, then harvested 5 hours later. Extracts were then examined by Western blot analysis using an anti-HA monoclonal antibody (Fig. 5). As a control, we also examined an extract of Vero cells infected with HSV-1 Pvhs N138-HA (Jones, Smibert, and Smiley, 1995), which encodes a mutant version of vhs bearing the HA tag following residue 138. The 2.1vhs and 2.1vhs1 vectors gave rise to the expected band of ca. 58kDa, while cells containing empty vector showed no signal. Significant signals were observed for both wild-type and vhs1 mutant protein in the un-induced culture, but in both cases the level was markedly higher after induction. The vhs1 mutant protein accumulated to somewhat higher levels than wild-type vhs after induction, demonstrating that the vhs1 mutation does not prevent protein accumulation. This observation in turn suggests that the failure of the vhs1 mutant protein to inhibit the growth of yeast stems from loss of one or more functions of vhs.

Extracts of yeast expressing vhs do not display vhs-dependent endoribonuclease activity. We next asked if cell-free extracts prepared from yeast expressing vhs display endoribonuclease activity comparable to that previously observed in RRL containing pre-translated vhs (Elgadi, Hayes, and Smiley, 1999; Elgadi and Smiley, 1999). Strains harboring the 2.1vhs expression plasmid and empty vector were induced with copper sulfate, and whole cell extracts were prepared as described in Materials and Methods. Western blot analysis confirmed that the extract prepared from

2.1vhs-expressing cells contained readily detectable amounts of full-length vhs protein (Fig. 6).

Two RNA substrates were used for the *in vitro* assays for vhs-dependent ribonuclease activity: pCITE-1 RNA, which bears the internal ribosome entry site (IRES) of encephalomyocarditis virus (EMCV) at its 5' end, and signal recognition particle α mRNA (SRPα RNA, Fig. 7A&B). Internally labeled substrate RNA was added to the extracts, and samples withdrawn at various times were then analyzed by agarose-formaldehyde gel electrophoresis (Fig. 7C&D, respectively). As a control, the RNA substrates were also incubated in RRL containing pre-translated vhs (RRLvhs) and blank RRL (RRL). Previous studies using the RRL assay system have shown the vhs-dependent endoribonuclease preferentially cleaves pCITE-1 RNA immediately 3' of the IRES, generating 5' and 3' products of ca. 600 nt and 1800 nt respectively (Elgadi and Smiley, 1999). The 600 nt fragment is stable throughout the course of the reaction, while the 1800 nt product is subject to further decay. We observed a similar pattern when pCITE-1 RNA was added to RRLvhs, with the exception that additional products of 1500 and 1000 nt were also observed. In marked contrast, extracts of yeast expressing 2.1vhs were devoid of detectable vhs-dependent ribonuclease activity: the pCITE-1 RNA was as stable as in the extract of cells harboring empty expression vector. Moreover, no discrete vhs-induced degradation intermediates were observed. Similar results were obtained with SRPα RNA (Fig. 7D). In this case, the RRL system does not produce stable degradation products (Elgadi, Hayes, and Smiley, 1999); rather the RNA is initially cleaved at a cluster of sites located over the 5' quadrant of the RNA, and the 5' and 3' products of these initial cleavage are subjected to rapid further decay to low molecular weight species (Elgadi, Hayes, and Smiley, 1999). SRPα RNA was as stable in extracts of yeast expressing vhs as in control extracts.

Taken in combination, these data demonstrate that extracts of yeast expressing 2.1vhs display little if any vhs-dependent endoribonuclease activity. Similar results were also obtained with extracts of yeast cells expressing unmodified vhs (data not shown). Inasmuch as the yeast extracts analyzed contain far more vhs protein than the RRL

reactions (data not shown), these results suggested that vhs protein produced in yeast has little or no endoribonuclease activity.

Reconstitution of endoribonuclease activity by a mammalian factor(s). The failure to detect vhs-dependent ribonuclease activity in extracts of yeast containing the 2.1vhs protein could be due to the presence of an inhibitor of the enzyme in the yeast extract, or reflect the absence of a required mammalian cofactor. We evaluated these possibilities in a series of mixing experiments. To test for the presence of an inhibitor, we mixed 20 µl of extract prepared from yeast harboring empty expression vector (total protein concentration: ~1 mg/ml) with an equal volume of RRL containing pre-translated vhs (total protein concentration ~100-200 mg/ml), then assayed for activity on pCITE-1 and SRPα RNA (Fig. 8 A&B, respectively). The yeast extract had no significant effect on the vhs-dependent endoribonuclease produced in RRL, arguing that the inactivity of yeast extracts containing vhs does not stem from the presence of an inhibitor.

We next asked if RRL contains one or more factors capable of stimulating the activity of 2.1vhs present in yeast extracts. 20 µl of blank RRL (total protein concentration: ~10 mg/ml) was added to equal volume of extracts of control and 2.1vhs expressing yeast (total protein concentration: ~1 mg/ml), and the resulting mixtures were tested for activity on pCITE-1 and SRPα RNAs (Fig. 9 A&B respectively). The extracts used in this experiment are the same as those used in the experiment depicted in Figure 7, and these two experiments were conducted in parallel using the same reagents. Strikingly, addition of blank RRL clearly reconstituted activity on both substrates. Moreover, the pattern of degradation intermediates observed in the reconstituted reaction was similar to that in the RRL reaction; in particular, pCITE-1 RNA gave rise to the 5' and 3' products characteristic of cleavage immediately 3' to the EMCV IRES at early times, and the 5' product was stable throughout the course of the reaction. These results indicate that RRL contains one or more factors that greatly stimulate the *in vitro* activity of vhs produced in yeast.

As a first step in characterizing the nature of the required co-factor, we desalted blank RRL by passage over Sephadex G25, and assayed the excluded fraction for its

ability to reconstitute activity on extracts of yeast expressing 2.1vhs for multiple times (Fig. 10). The results clearly demonstrated that activity was recovered in the excluded fraction, indicating that the required factor is a macromolecule (ie, ≥5 kDa).

2.5. Discussion

The experiments described in this report have led to two seemingly contradictory sets of findings. First, expression of vhs in the budding yeast *S. cerevisiae* strongly inhibits cell growth. This effect displays the same mutational sensitivity spectrum as host shutoff in mammalian cells, arguing that growth inhibition stems from one or more biologically relevant functions of vhs. Inasmuch as vhs appears to trigger shutoff in mammalian cells through its associated ribonuclease activity, the simplest interpretation of these results is that vhs inhibits the growth of yeast by degrading one or more key cellular RNAs. Second, notwithstanding the foregoing, vhs protein produced in yeast does not display significant endoribonuclease activity in crude extracts. However, vhs-dependent endoribonuclease activity is restored by adding RRL to the extract. These data argue that the vhs-dependent endoribonuclease requires one or more mammalian factors for activity.

How can one reconcile these seemingly discrepant sets of observations? One possibility is that growth inhibition in yeast results from a fortuitous event that is entirely unrelated to mammalian host shutoff. For example, vhs might interact by chance with a yeast protein and interfere with its normal activity. According to this scenario, the concordance of growth inhibition in yeast with host shutoff in HSV-infected cells could simply be a consequence of impaired folding of the mutant proteins that lack activity in both systems. However, all of the mutant forms of vhs examined in the present study accumulate in infected cells to the same levels as the wild-type protein, and are packaged into the tegument of HSV virions (Jones, Smibert, and Smiley, 1995; Read, Karr, and Knight, 1993). Both of these observations seem incompatible with gross alterations in protein folding. In addition, the vhs1 point mutation (Thr 214 → Ile) maps to one of the regions of strongest homology to FEN-1 nucleases (Doherty, Serpell, and Ponting, 1996). These considerations suggest that the mutations abolish vhs function in yeast by

inactivating the enzymatic activity of the vhs protein, or by abolishing an interaction between vhs and a biologically relevant yeast factor (for example, the yeast homologue of a required mammalian cofactor).

If, as argued above, growth inhibition in yeast reflects the mammalian host shutoff function of vhs, then why do cell-free extracts of yeast expressing vhs lack detectable ribonuclease activity? The simplest explanation is that our failure to detect ribonuclease activity stems from technical limitations of the *in vitro* assay system. For example, the levels of ribonuclease activity may be too low to be readily detected in cell-free extracts but nonetheless sufficient for growth inhibition *in vivo*, or the putative yeast homologue of the required mammalian cofactor may be inactive or labile under our *in vitro* conditions. A second possibility is that the vhs-dependent endoribonuclease produced in yeast associates with a yeast cofactor that targets it in a sequence-specific fashion to a small subset of yeast RNAs, leading to growth inhibition. According to this hypothesis, the functional ribonuclease complex produced in yeast lacks activity on the pCITE-1 and SRPα RNA substrates used in the *in vitro* assay because it lacks an appropriate RNA targeting subunit. Another possibility is that vhs binds the yeast homologue of a mammalian cofactor and inhibits its normal function (leading to growth inhibition), without forming a functional ribonuclease. Distinguishing between these and other alternative explanations will likely require identification of the mammalian stimulatory factor detected in this study.

The observation that vhs inhibits the growth of yeast when cells are grown on galactose, raffinose, and (especially) maltose as the sole carbon source, but has little effect in glucose, is intriguing. However, understanding the molecular basis of this phenomenon will require determining precisely how vhs inhibits cell growth. Perhaps the effect stems from selective action of vhs on some of the mRNAs or proteins that required to metabolize these alternative carbon sources. Alternatively, it is conceivable that vhs activity is directly or indirectly altered by one of the signaling pathways that respond to changes in carbon source (reviewed in (Scheffler, de la Cruz, and Prieto, 1998)).

Our finding that desalted RRL reconstitutes vhs-dependent ribonuclease activity in yeast extracts containing vhs provides strong evidence that the ribonuclease requires one or more mammalian macromolecules for activity. We can think of at least three distinct possible mechanisms of action for the required mammalian factor(s): 1). It might be an enzyme needed for a required post-translational modification of vhs. It is interesting to note that vhs produced during HSV infection is phosphorylated (Smibert, Johnson, and Smiley, 1992), and the inactivating vhs1 mutation alters the pattern of phospho-isoforms that accumulate (Read, Karr, and Knight, 1993). These observations suggest that phosphorylation may be functionally important. 2). It might be a required regulatory or catalytic subunit of the ribonuclease. 3). It might serve as a targeting subunit that selectively delivers vhs to mRNAs as opposed to other cytoplasmic transcripts. In this latter context we note that vhs iritially degrades the 5' end of at least some mRNAs *in vivo* (Karr and Read, 1999) and *in vitro* (Elgadi, Hayes, andSmiley, 1999), and picornavirus IRES elements strongly target vhs-dependent cleavage events to adjacent RNA sequences (Elgadi and Smiley, 1999). IRES elements provide an alternative, cap-independent mode for translation initiation in eukaryotes, and function by recruiting translational initiation factors to the RNA (Jackson and Kaminski, 1995). We have previously argued that these observations are consistent with the possibility that vhs selectively targets mRNAs by interacting with one or more components of the translational initiation apparatus that act upstream of loading of the 40S ribosomal subunit (Elgadi and Smiley, 1999). Possibilities 2 and 3 are not mutually exclusive, and it is possible that RRL provides more than one required factor.

Our finding that the vhs-dependent endoribonuclease requires one or more mammalian factors for activity is inconsistent with Zelus et al.'s previous conclusion that the nuclease is active in the absence of cellular co-factors (Zelus, Stewart, and Ross, 1996). These authors based their conclusion on the observation that partially purified HSV virions contain a vhs-dependent ribonuclease, and the assumption that their virion preparations lack cellular proteins. However, virions purified by the protocol used in their study likely contain at least some contaminating cellular components, and it is in any case possible that the required cellular factor is packaged along with vhs into the virus particle.

The simple *in vitro* assay described in this report will allow rapid purification and identification of the mammalian factor(s) that are required for vhs action. We expect that this will greatly enhance our understanding of the mode and regulation of vhs activity.

2.6. Acknowledgements

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2.7. References

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2.8. Figure Legends

Figure 1. Expression of vhs inhibits yeast colony formation. Strains YGAL, Yvhs, and Yvhs1 were grown to saturation in YNB/D, diluted 10^{-1} to 10^{-4} in sterile ddH_2O, and 2 µl of each dilution was spotted onto YNB/D (glucose) and YNB/G (galactose) plates. The plates were incubated at 30°C for 3 and 5 days, respectively.

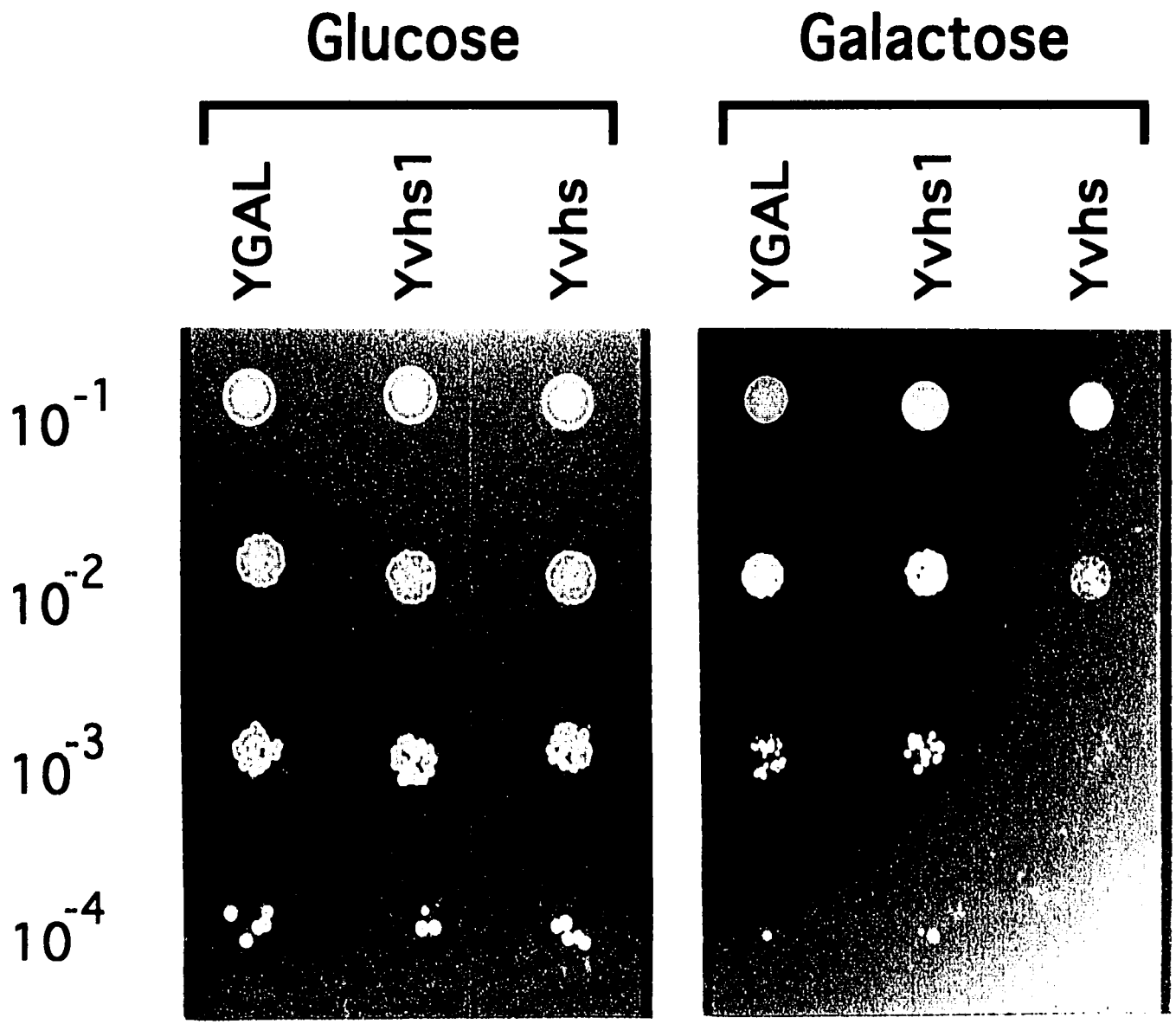

Glucose
Galactose
YGAL
Yvhs1
Yvhs
YGAL
Yvhs1
Yvhs
10^{-1}
10^{-2}
10^{-3}
10^{-4}

Figure 2. The growth inhibition phenotype requires the same regions of the vhs polypeptide as mammalian shutoff. Strains YGAL, Yvhs (vhs), YR27 (R27), YN138-HA (N138-HA), YSc243 (Sc243), YS344-HA (S344-HA), and YM384 (M384) were grown to saturation in YNB/D, diluted 10^{-1} to 10^{-4} in sterile ddH_2O, and 2 µl of each dilution was spotted onto YNB/D (glucose) and YNB/G (galactose) plates. The plates were incubated at 30°C for 3 and 5 days respectively. The upper portion of the figure displays a linear representation of the 489 residue vhs polypeptide with the positions of in-frame linker insertion mutation indicated. Regions conserved between the vhs proteins of alphaherpesvirus (conserved regions I, II, III, IV, and A, (Berthomme, Jacquemont, and Epstein, 1993; Jones, Smibert, andSmiley, 1995)) are presented as shaded and hatched boxes.

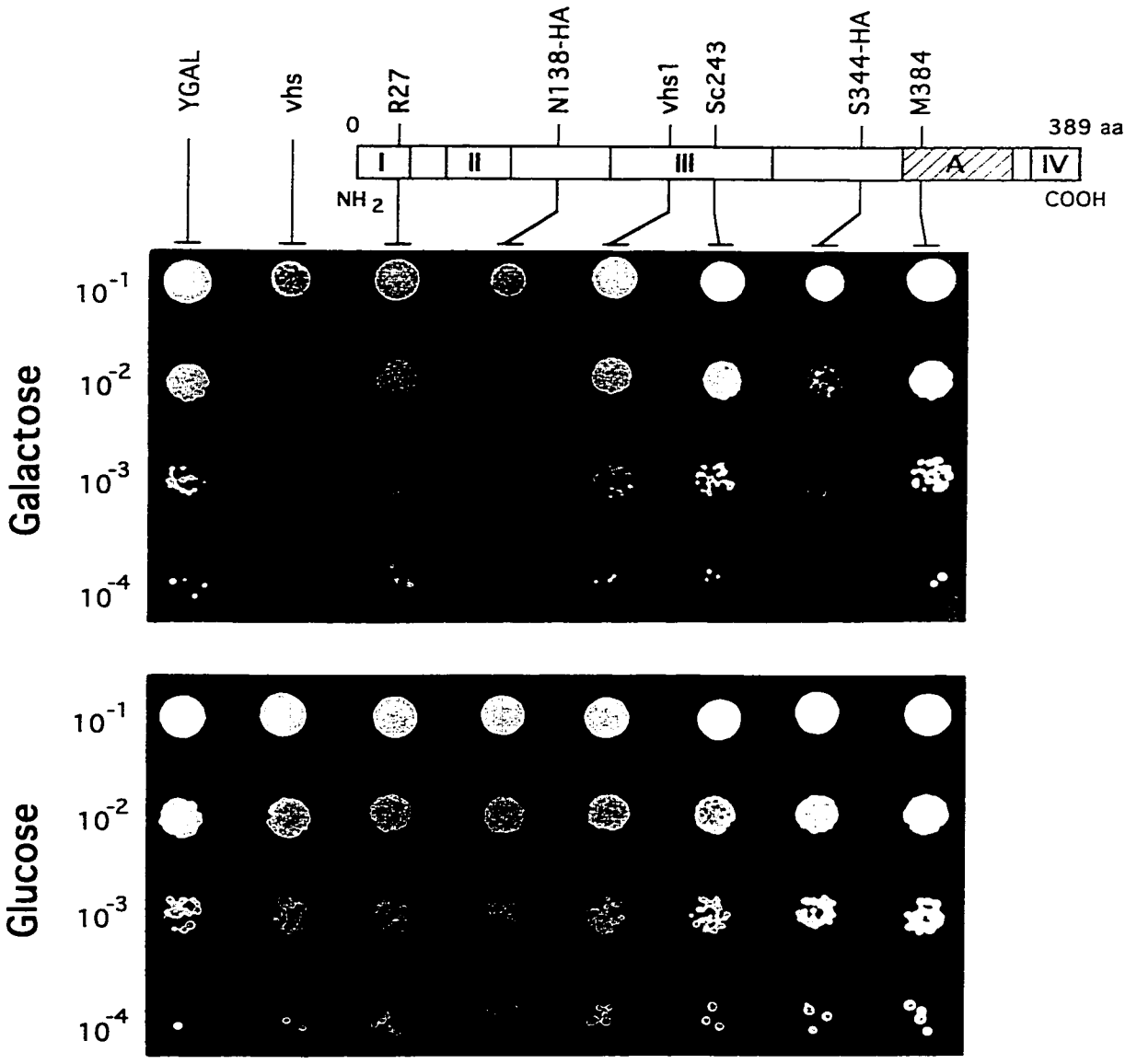

YGAL
vhs
R27
N138-HA
vhs1
Sc243
S344-HA
M384
0
389 aa
I
II
III
A
IV
NH 2
COOH
Galactose
Glucose
10-1
10-2
10-3
10-4

Figure 3. Expression of vhs from the inducible CUP1 promoter inhibits colony formation. Strains W303 pYEX-BX (vector), W303 pYEX-BX vhs (vhs), and W303 pYEX-BX vhs1 (vhs1) were grown to saturation in YNB/D, then equal amounts of cells from each strain were spotted and streaked on YNB/D (glucose), YNB/G (galactose), YNB/R (raffinose), and YNB/M (maltose) plates containing or lacking 0.5 mM copper sulfate (+ Cu or – Cu, respectively). The plates were incubated at 30°C for 5 days. This experiment has been repeated at least 5 times.

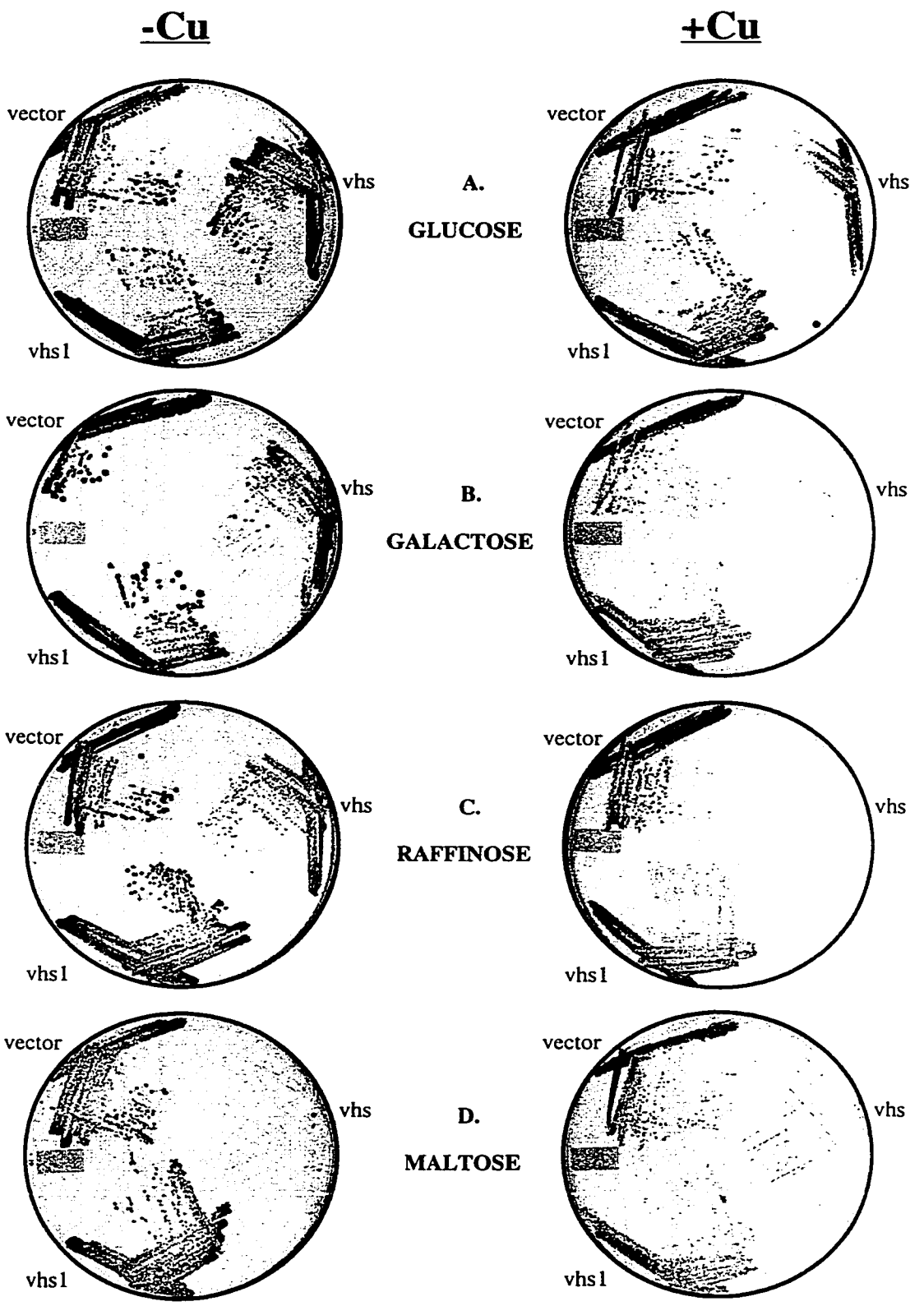

-Cu
+Cu
vector
vhs
vhs1
A.
GLUCOSE
B.
GALACTOSE
C.
RAFFINOSE
D.
MALTOSE

Figure 4. Expression of epitope-tagged vhs inhibits colony formation. Strains W303 pYEX-BX (vector), W303 pYEX-BX 2.1vhs (2.1vhs), and W303 pYEX-BX 2.1vhs1 (2.1vhs1) were grown to saturation in YNB/D, then equal amounts of cells from each strain were spotted and streaked on YNB/D (glucose), YNB/G (galactose), YNB/R (raffinose), and YNB/M (maltose) plates containing or lacking 0.5 mM copper sulfate (+ Cu or – Cu, respectively). The plates were incubated at 30°C for 5 dayis. This experiment has been repeated at least 5 times.

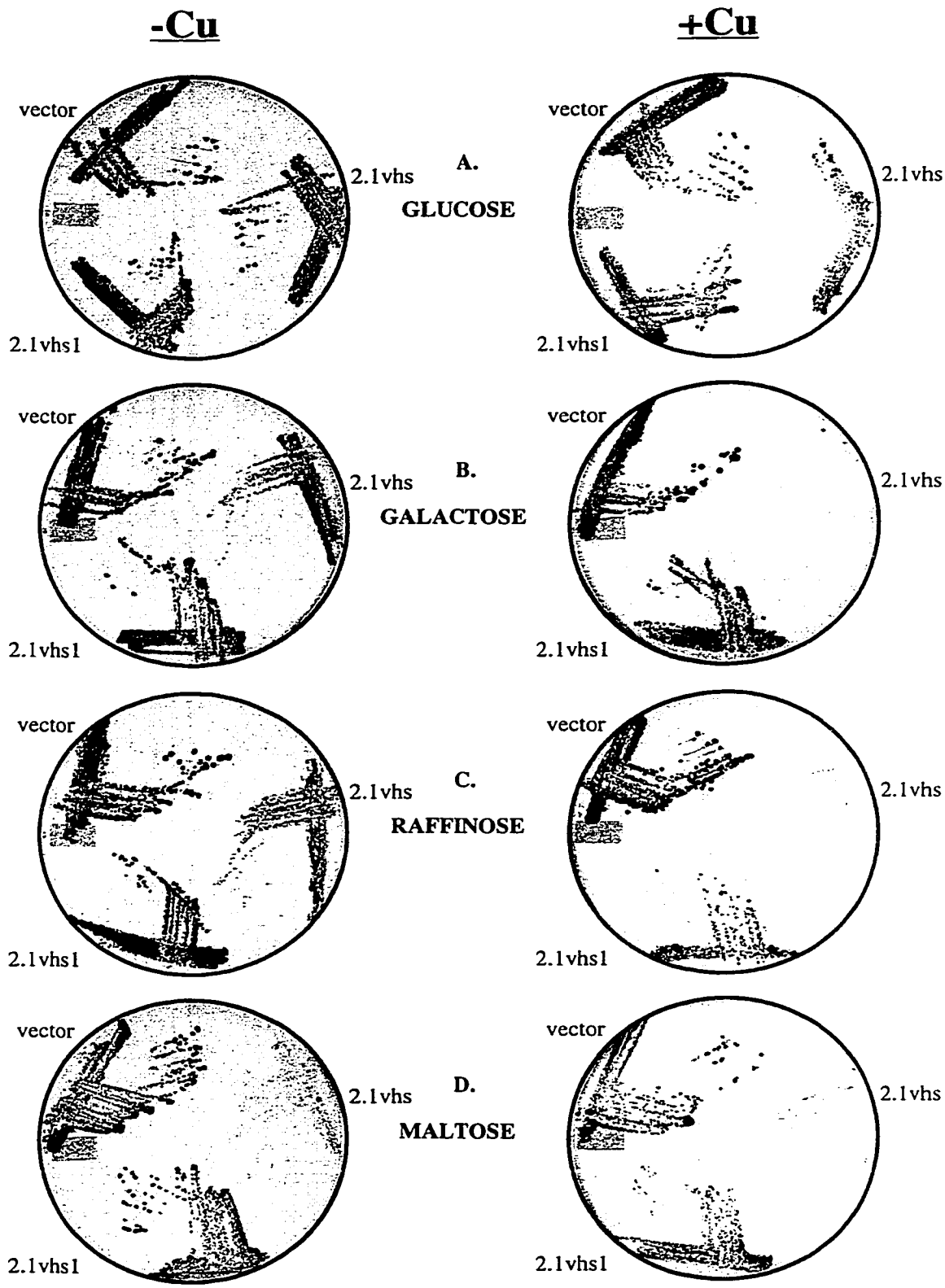

-Cu
+Cu
vector
2.1vhs
2.1vhs1
A.
GLUCOSE
B.
GALACTOSE
C.
RAFFINOSE
D.
MALTOSE

Figure 5. Levels of expression of 2.1vhs and 2.1vhs1 proteins. Strains W303 pYEX-BX (empty vector), W303 pYEX-BX 2.1vhs (2.1vhs), and W303 pYEX-BX 2.1vhs1 (2.1vhs1) were grown to saturation in YNB/G and the cultures were then split into two. One culture was induced with 0.5 mM $CuSO_4$ for five hours at 30°C (lanes I), while the other was left untreated (lanes U). Cell extracts were then analyzed for vhs expression by Western blot analysis using a monoclonal antibody directed against the HA epitope. A lysate of Vero cells infected with HSV-1 Pvhs N138-HA (lane Pvhs N138-HA) at a MOI of 10 for 12 hours was included as a positive control. This experiment has been repeated 3 times.

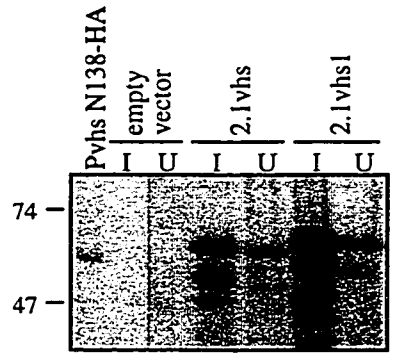

Pvhs N138-HA
empty vector
2.1vhs
2.1vhs1
I
U
I
U
I
U
74
47

Figure 6. Western blot analysis of yeast extracts. Strains W303 pYEX-BX (empty vector), and W303 pYEX-BX 2.1vhs (2.1vhs) were grown to an OD_{600} of 2-3 in YNB/G, then induced with 0.15 mM $CuSO_4$ for five hours at 30°C. Whole-cell extracts were prepared and the vhs protein in the extracts was detected by Western blot analysis using a monoclonal antibody directed against the HA epitope. A lysate of Vero cells infected with HSV-1 Pvhs N138-HA (lane Pvhs N138-HA) at MOI of 10 for 12 hours was included as a positive control. This experiment has been repeated at least 5 times.

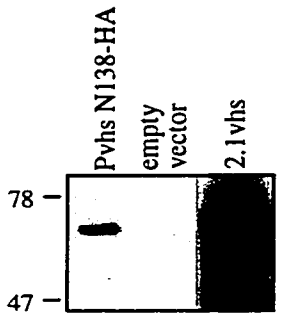

Pvhs N138-HA
empty vector
2.1vhs
78
47

Figure 7. Lack of endoribonuclease activity in extracts of yeast expressing 2.1vhs. Internally labeled pCITE-1 and SRPα RNAs were added to control RRL (RRL), RRL containing the pretranslated vhs (RRLvhs), and extracts of yeast containing (2.1vhs) and lacking (empty vector) 2.1vhs protein. RNA was extracted at indicated time points and resolved on a 1% agarose/1.8% formaldehyde gel, transferred to a Gene Screen Plus membrane, and the RNA signal was detected by autoradiography (panels C + D). (A) and (B). Diagrams of the pCITE-1 and SRPα RNA substrates, indicating the positions of the initial vhs-induced cleavage events in the RRL system. The EMCV IRES present on pCITE-1 is indicated. (C) and (D). Analysis of endoribonuclease activity on pCITE-1 and SRPα RNAs respectively. The filled square and diamond indicate the previously described 5' and 3' degradation products of pCITE-1, while the open square and diamond indicate the additional 1500 nt and 1000 nt products described in the text. Sizes of RNA marker (M) are indicated in nt at the left. This experiment has been repeated at least 5 times.

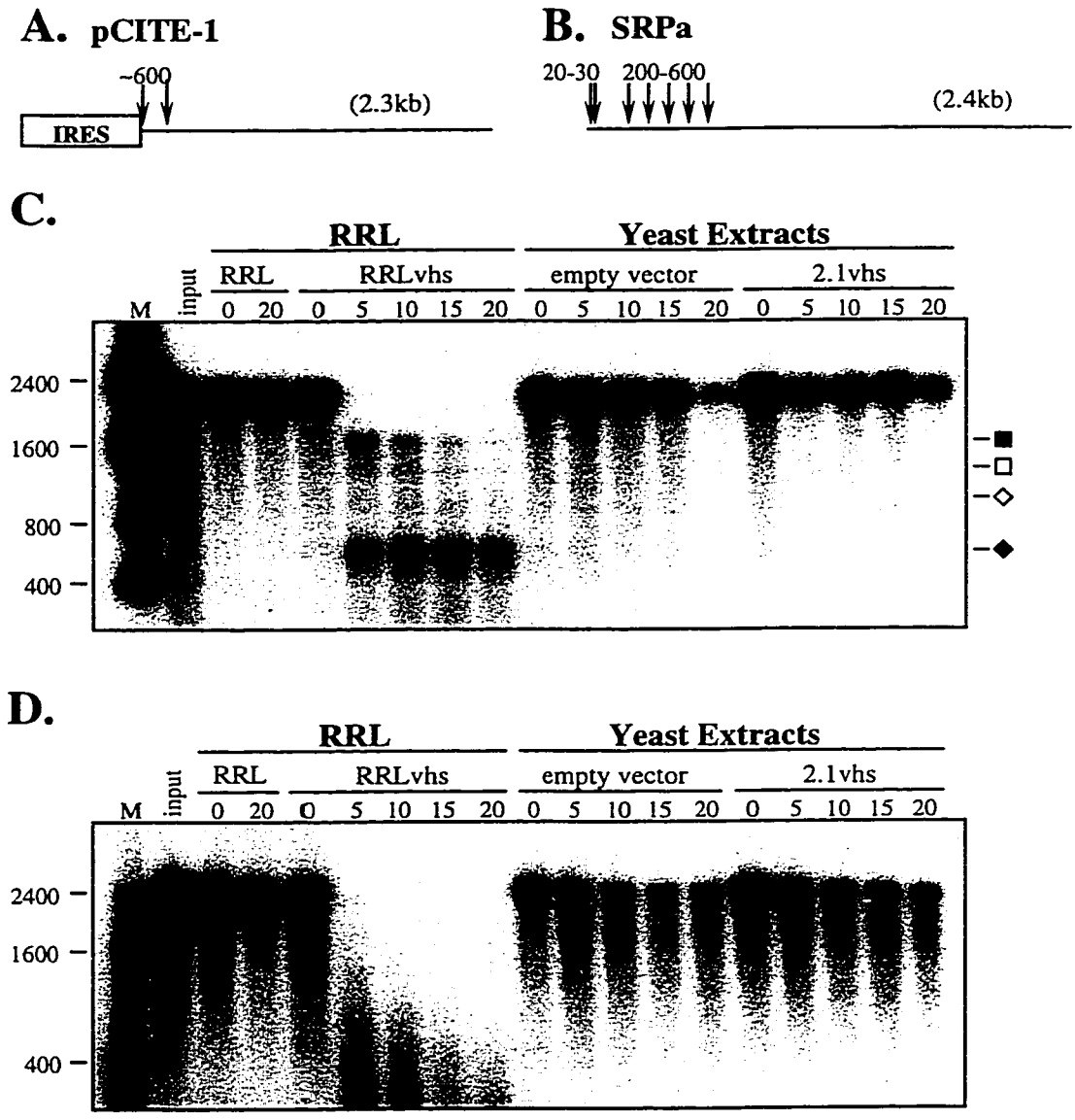

A. pCITE-1
~600
(2.3kb)
IRES
B. SRPa
20-30
200-600
(2.4kb)
C.
RRL
Yeast Extracts
RRL
RRLvhs
empty vector
2.1vhs
M
input
0 20 0 5 10 15 20 0 5 10 15 20 0 5 10 15 20
2400
1600
800
400
D.
RRL
Yeast Extracts
RRL
RRLvhs
empty vector
2.1vhs
M
input
0 20 0 5 10 15 20 0 5 10 15 20 0 5 10 15 20
2400
1600
400

Figure 8. Yeast extract does not contain an inhibitor of the vhs-dependent ribonuclease. Internally labeled pCITE-1 and SRPα RNAs were added to control RRL (RRL), RRL containing pre-translated vhs (RRLvhs), yeast extract from cells harboring empty vhs expression vector (empty vector), and yeast extract mixed with RRLvhs (empty vector + RRLvhs). Samples were incubated and processed as described in the legend to Figure 7. (A) and (B). Analysis of activity on pCITE-1 and SRPα RNAs respectively. Sizes of RNA markers (M) are indicated in nt at the left. This experiment has been repeated 2 times.

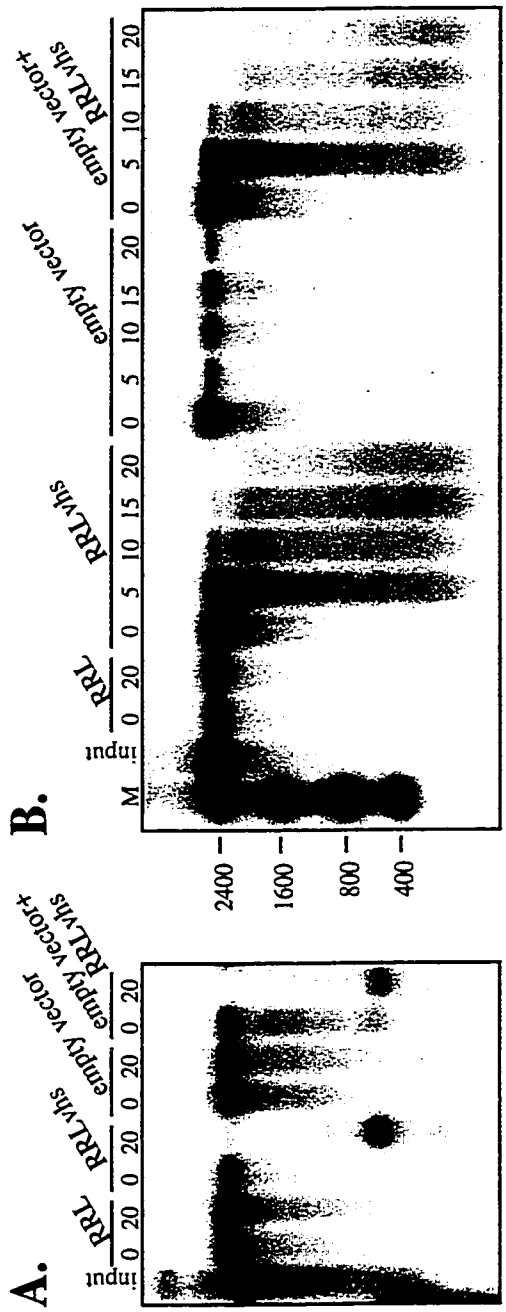

A.
input
RRL
0 20
RRL-vhs
0 20
empty vector
0 20
empty vector+
RRL-vhs
0 20
B.
M
input
RRL
0 20
RRL-vhs
0 5 10 15 20
empty vector
0 5 10 15 20
empty vector+
RRL-vhs
0 5 10 15 20
2400
1600
800
400

Figure 9. A mammalian cofactor(s) is required for reconstituting the endoribonuclease activity of the vhs protein produced in yeast. Internally labeled pCITE-1 and SRPα RNAs were added to control RRL (RRL), RRL containing pre-translated vhs (RRLvhs), yeast extract from cells harboring empty vhs expression vector mixed with blank RRL (empty vector + RRL), and yeast extract containing 2.1vhs mixed with blank RRL (2.1vhs + RRL). The yeast extract containing 2.1vhs used in this experiment is exactly the same as in Figure 7, and these two experiments were done at the same time on the same day. Samples were incubated and processed as described in the legend to Figure 7. (A) and (B). Analysis of activity of pCITE-1 and SRPα RNAs respectively. Sizes of RNA markers (M) are indicated in nt at the left. This experiment has been repeated 10 times.

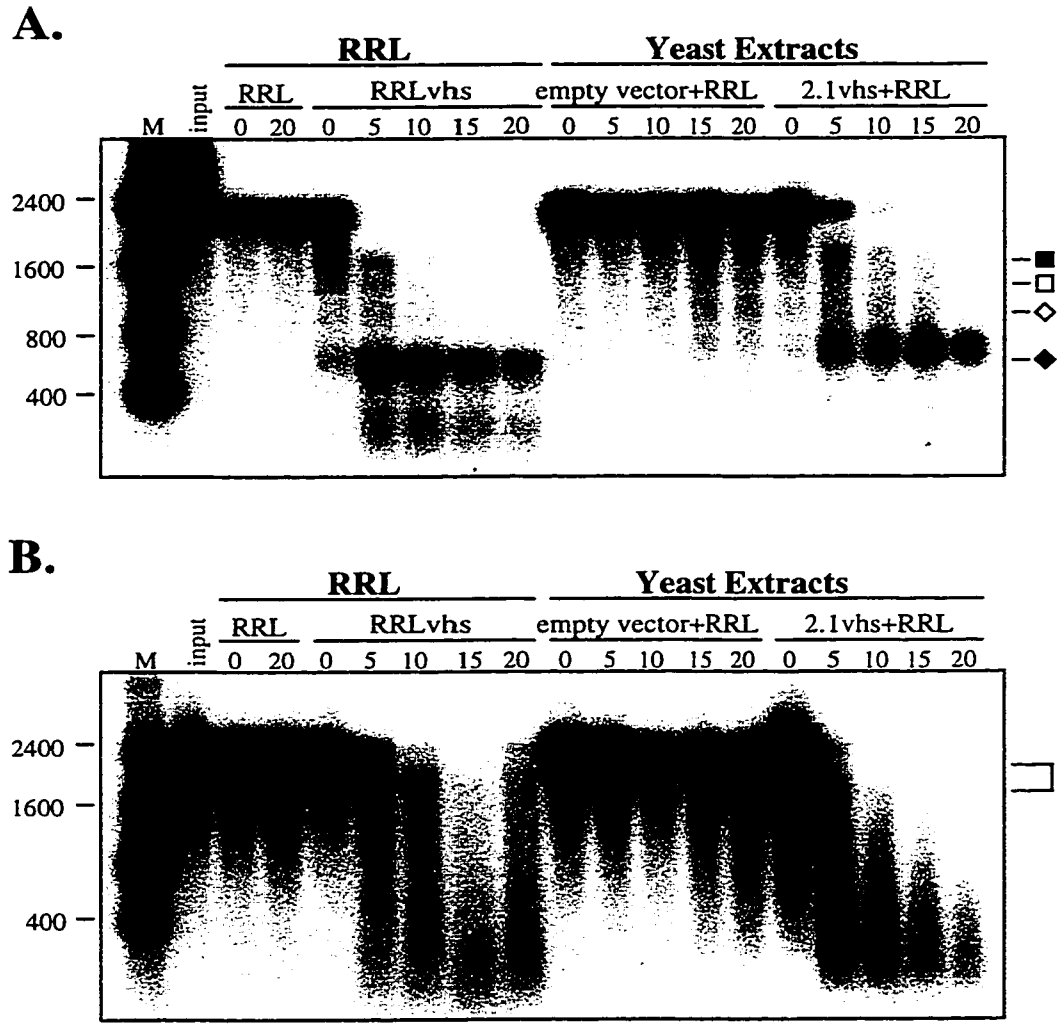
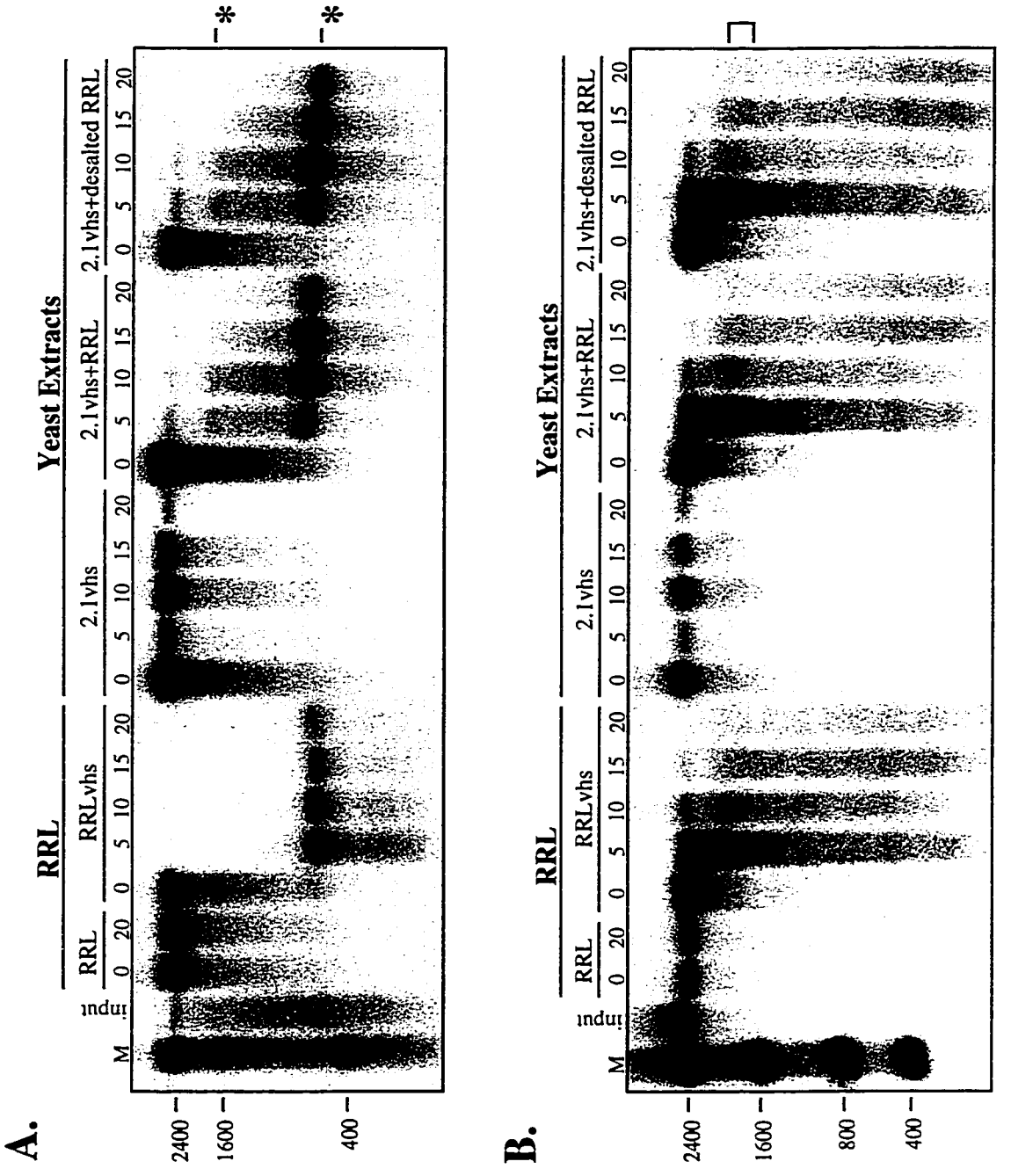

A.
RRL
Yeast Extracts
RRL
RRLvhs
empty vector+RRL
2.1vhs+RRL
M
input
0 20 0 5 10 15 20 0 5 10 15 20 0 5 10 15 20
2400
1600
800
400
B.
RRL
Yeast Extracts
RRL
RRLvhs
empty vector+RRL
2.1vhs+RRL
M
input
0 20 0 5 10 15 20 0 5 10 15 20 0 5 10 15 20
2400
1600
400

Figure 10. The mammalian cofactor is a macromolecule. Internally labeled pCITE-1 and SRPα RNAs were added to control RRL (RRL), RRL containing pre-translated vhs (RRLvhs), yeast extract containing 2.1vhs, yeast extract containing 2.1vhs mixed with blank RRL (2.1vhs + RRL), and yeast extract containing 2.1vhs mixed with desalted blank RRL (2.1vhs + desalted RRL). Samples were incubated and processed as described in the legend to Figure 7. (A) and (B). Analysis of activity of pCITE-1 and SRPα RNAs respectively. Sizes of RNA markers (M) are indicated in nt at the left. This experiment has been repeated at least 5 times.


A.
Yeast Extracts
RRL
RRLvhs
2.1vhs
2.1vhs+RRL
2.1vhs+desalted RRL
input
M
RRL
0 20 0 5 10 15 20 0 5 10 15 20 0 5 10 15 20 0 5 10 15 20
2400
1600
400
*
*
B.
Yeast Extracts
RRL
RRLvhs
2.1vhs
2.1vhs+RRL
2.1vhs+desalted RRL
input
M
RRL
0 20 0 5 10 15 20 0 5 10 15 20 0 5 10 15 20 0 5 10 15 20
2400
1600
800
400

CHAPTER 3 - THE VHS1 MUTANT FORM OF THE HERPES SIMPLEX VIRUS VIRION HOST SHUTOFF PROTEIN RETAINS SIGNIFICANT INTERNAL RIBOSOME ENTRY SITE-DIRECTED RNA CLEAVAGE ACTIVITY

Submitted to the Journal of Virology as a short note.

Preface.

The data presented in this chapter, submitted to the Journal of Virology, have been formatted to comply with the paper thesis format. The data presented in Figure 4 was generated by Holly A. Saffran. I carried out all the rest of the experiments presented in this chapter (from Figure 1-3, 5-6). I also prepared the data for publication and wrote the first draft of the manuscript. A major editorial contribution from my supervisor, Dr. James R. Smiley, led to the final version of the paper.

The vhs1 Mutant Form of the Herpes Simplex Virus Virion Host Shutoff Protein Retains Significant Internal Ribosome Entry Site-Directed RNA Cleavage Activity

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key words: herpes simplex virus, host shutoff, RNA turnover

running title: IRES-directed RNA cleavage by mutant vhs protein

3.1. Abstract

The virion host shutoff (vhs) protein of herpes simplex virus triggers global shutoff of host protein synthesis and accelerated turnover of host and viral mRNAs during HSV infection. As well, it induces endoribonucleolytic cleavage of RNA substrates when produced in a rabbit reticuloctye (RRL) *in vitro* translation system. The vhs1 point mutation (thr 214 → ile) eliminates vhs function during virus infection and in transiently transfected mammalian cells, and was therefore previously considered to abolish vhs activity. Here we demonstrate that the vhs1 mutant protein induces readily detectable endoribonuclease activity on RNA substrates bearing the internal ribosome entry site (IRES) of encephalomyocarditis virus in the RRL assay system. These data document that the vhs1 mutation does not eliminate catalytic activity, and raise the possibility that the vhs-dependant endoribonuclease employs more than one mode of substrate recognition.

3.2. Introduction, materials and methods, results, and discussion

Herpes simplex virus (HSV) is a large enveloped DNA virus that replicates in the nucleus of infected mammalian cells. Like many other viruses, HSV inhibits host cell protein synthesis as a key element of its strategy of reprogramming the cellular biosynthetic machinery. HSV-induced host shutoff can be divided into two phases which occur at early and intermediate times post-infection respectively. Early shutoff involves disruption of pre-existing polysomes and rapid degradation of host mRNAs (Fenwick and Clark, 1982; Fenwick and McMenamin, 1984; Fenwick and Owen, 1988; Fenwick and Walker, 1978; Kwong and Frenkel, 1987; Kwong, Kruper, and Frenkel, 1988; Oroskar and Read, 1987; Oroskar and Read, 1989; Read and Frenkel, 1983; Zelus, Stewart, and Ross, 1996). Substantial data indicate that the virion host shutoff (vhs) protein, a tegument protein encoded by HSV gene UL41, is both necessary and sufficient for the early shutoff effect (Fenwick, 1990; Fenwick and Evrett, 1990; Jones, Smibert, and Smiley, 1995; Pak *et al.*, 1995; Read and Frenkel, 1983; Read, Karr, and Knight, 1993; Smibert, Johnson, and Smiley, 1992).

Although the mechanism of vhs action remains to be precisely defined, several lines of evidence strongly indicate that it acts as a ribonuclease. First, cytoplasmic extracts prepared from HSV infected cells and extracts of partially purified HSV virions contain a vhs-dependent ribonuclease activity (Karr and Read, 1999; Krikorian and Read, 1991; Sorenson, Hart, and Ross, 1991; Zelus, Stewart, and Ross, 1996), and this activity is inhibited by anti-vhs antibodies (Zelus, Stewart, and Ross, 1996). Second, vhs induces endoribonucleolytic cleavage of a variety of reporter mRNAs when it is expressed as the only HSV protein in a rabbit reticulocyte lysate (RRL) *in vitro* translation system (Elgadi, Hayes, and Smiley, 1999; Elgadi and Smiley, 1999; Zelus, Stewart, and Ross, 1996). Third, vhs displays weak but significant amino acid sequence similarity to the fen-1 family of nucleases that are involved in DNA replication and repair in eukaryotes and archaebacteria (Doherty, Serpell, and Ponting, 1996), and human fen-1 has recently been shown to cleave both RNA and DNA substrates (Stevens, 1998). Although these data argue that vhs is an integral and required component of the vhs-dependent

endoribonuclease, they do not exclude the possibility that one or more cellular factors are also required for activity.

Much of our current knowledge about the role of the UL41 gene product in host shutoff has emerged from studies of the vhs1 mutant isolate of HSV-1 strain KOS (Read and Frenkel, 1983). This mutant harbors a single base change in the UL41 open reading frame that converts amino acid residue 214 from threonine to isoleucine (Jones, Smibert, and Smiley, 1995; Kwong, Kruper, and Frenkel, 1988). The mutation is located in one of the regions of strongest homology to the fen-1 family of nucleases (Doherty, Serpell, and Ponting, 1996), and previous studies have suggested that it completely inactivates vhs function. The vhs1 mutation abolishes early shutoff of host protein synthesis during HSV infection (Read and Frenkel, 1983) and eliminates vhs activity in transient co-transfection assays (Jones, Smibert, and Smiley, 1995; Pak *et al.*, 1995). In addition, the mutant protein fails to trigger accelerated RNA turnover in *in vitro* systems derived from extracts of HSV-infected cells and partially purified virions, and displays little if any activity on the mRNA encoding the α-subunit of the signal recognition particle (SRPα mRNA) in the RRL *in vitro* assay system (Elgadi, Hayes, and Smiley, 1999; Krikorian and Read, 1991; Sorenson, Hart, and Ross, 1991; Zelus, Stewart, and Ross, 1996).

Previous work from this laboratory has shown that internal ribosome entry sites (or IRES elements) derived from encephalomyocarditis virus (EMCV) or poliovirus act to strongly target vhs-dependent RNA cleavage events to a narrow zone located immediately 3'of the IRES (Elgadi and Smiley, 1999). During further studies of this effect, we obtained preliminary evidence that the vhs1 mutant form of vhs displays significant activity on substrates bearing the EMCV IRES (data not shown). This observation was both interesting and surprising, because it raised the possibility that the vhs1 mutation does not completely inactivate the vhs-dependent endoribonuclease. We therefore undertook studies designed to more carefully assess the activity of the vhs1 protein in the RRL assay system.

The vhs1 *in vitro* translation vector pSP6vhs1 (Elgadi, Hayes, and Smiley, 1999) was first subcloned by re-transforming *E. coli*, to eliminate possible contamination with

wild-type vhs sequences due to either mishandling or reversion. Following verification of the vhs1 mutation by DNA sequence analysis, DNA obtained from a single transformed colony was amplified and used for all of the experiments described in this report. Multiple RRL *in vitro* translation reactions were then programmed with vhs mRNA generated from pSP6vhs (Elgadi, Hayes, and Smiley, 1999) and pSP6vhs1 to generate wild-type and mutant vhs respectively. Template DNA was linearized with EcoRI, and transcriptions were done for 1 hour at 37^0C in a 20 µl reaction containing: 1 µg of template, 1 U of SP6 RNA polyermerase, 0.5 mM cap primer 7mG(5')ppp(5')G (Amersham Pharmaceutical), 2 U of RNase inhibitor (Gibco BRL) and 0.5 mM of GTP, CTP, ATP and UTP. The DNA template was then digested with 10 U of RNase-free DNase I (Boehringer-Mannheim) at 37^0C for 15 minutes, and the RNA product was recovered, precipitated with ethanol, and dissolved in RNase-free water. Approximately 2 µg of the vhs mRNA was then used to program each of several 40 µl aliquots of RRL (Promega) using the manufacturer's procedure. Reactions were incubated at 30^0C for 60 minutes in the presence of ^{35}S-methionine (NEN Life Science Products). Blank RRL controls were generated in the same way except that no mRNA was added to the translation reaction. A 2 µl aliquot of each reaction was then analyzed by SDS-polyacrylamide gel electrophoresis, and the remainder of each reaction mixture was stored at -70^0C for future use (Elgadi, Hayes, and Smiley, 1999). Following autoradiography, pairs of translation reactions containing comparable amounts of wild-type and mutant protein were identified (Figure 1), then thawed and used for the activity assays depicted in figures 2, 3, 5 and 6 below. This approach ensured that most of the activity comparisons presented in this report were performed with wild-type and mutant vhs preparations that were generated in parallel, and were closely matched for vhs protein content. Also each activity comparison presented in this report has been repeated at least 3 times.

We first compared the activity of wild-type and mutant vhs on a 2.3kb transcript which bears the EMCV IRES at its 5' end (pCITE-1 RNA, Figure 2A). Uncapped, internally labeled pCITE-1 RNA was generated by *in vitro* transcription as previously described (Elgadi and Smiley, 1999), then added to RRL containing pretranslated vhs.

Following incubation at 30^{0}C, RNA extracted at various time points was analyzed by electrophoresis on a 1% agarose-formaldehyde gel. Previous work has demonstrated that the initial sites of vhs-induced cleavage of pCITE-1 RNA are clustered immediately downstream of the IRES (diagrammed in Figure 2A). These IRES-directed cleavage events give rise to two relatively discrete early degradation products: a ca. 600 nt 5' fragment that contains the IRES, and a ca. 1800 nt 3' fragment. The 5' fragment is stable over the course of the reaction, while the 3' fragment is subject to further decay. We found that wild-type vhs generated the predicted 600 nt and 1800 nt products (Figure 2B). However, we also detected additional products of ca. 1500 and 1000 nt early during the reaction, and these declined in abundance as the reaction proceeded. The 1500 and 1000 nt fragments were not noted in our earlier study, and we have not yet determined their structure; it is possible that they represent degradation intermediates generated from the 1800 nt product. The vhs1 mutant protein also induced degradation of the pCITE-1 RNA (Figure 2B), leading to production of the same degradation intermediates as wild-type vhs. However, the reaction proceeded more slowly, and the 1800 nt 3' fragment was substantially more stable than in the presence of wild-type vhs. The data presented in Figure 2B were quantified by phosphoimager analysis, and are plotted in Figure 2C. These data demonstrate that the vhs1 mutant protein displays reduced but significant activity on pCITE-1 RNA.

We next re-examined the activity of wild-type and mutant vhs on SRPα RNA, a transcript that lacks an IRES (Figure 3). Elgadi *et al.* (Elgadi, Hayes, and Smiley, 1999) have previously shown that the most prominent sites of initial cleavage of this RNA are non-randomly clustered over the 5' quadrant of the transcript (diagrammed in Figure. 3A), leading to early production of relatively discrete sets of 5' and 3' degradation intermediates of ca. 200 - 600 nt. and 1800 - 2200 nt. respectively. Both sets of intermediates are then further degraded as the reaction proceeds. We observed a similar pattern in reactions containing wild-type vhs (Figure 3B). In contrast, reactions containing the vhs1 mutant protein did not display readily detectable quantities of these early 5' and 3' degradation intermediates. However, the RNA substrate was somewhat less stable than in control RRL, and gave rise to a heterogeneous set of degradation products that migrated as a broad smear during gel electrophoresis. These data suggest

that the vhs1 protein displays very weak activity on SRPα RNA in the RRL assay (see also Figure 4 below). However, this activity was not always detected in repeated trials ((Elgadi and Smiley, 1999) and additional data not shown), indicating that it is close to the lower detection limit of our assay system.

The reactions depicted in Figures 2 and 3 contained similar quantities of vhs protein, and the substrate concentrations were identical. pCITE-1 RNA decayed significantly more rapidly than SRPα RNA in the reactions containing the vhs1 protein (compare Figures 2 and 3), suggesting that pCITE-1 RNA serves as a preferred substrate for this mutant form of vhs. We were unable to determine whether pCITE-1 RNA also serves as a preferred substrate for wild-type vhs in these experiments, because both substrates were almost fully degraded at the earliest time point analyzed. As one approach to answering this question, we compared the activity of serially diluted samples containing wild-type and vhs1 mutant protein on both RNAs. ^{35}S-labeled wild-type and mutant vhs were generated by *in vitro* translation as described above. Following translation, reactions were serially diluted in naïve RRL. 2 µl aliquots of each dilution were analyzed by SDS-polyacrylamide gel electrophoresis as in figure 1, and the relative amount of vhs protein in each sample was quantified by phosphor-imager analysis. 5 µl aliquots of each dilution were then combined with internally labeled pCITE-1 or SRPα RNA, incubated for 4 minutes at 30^{0}C, and the RNA products extracted and analyzed by gel electrophoresis as described above. The proportion of undigested substrate was determined for each dilution by phosphor-imager analysis, and plotted against the relative amount of vhs protein present in the corresponding reaction (Fig. 4). The results of this experiment confirmed that the vhs1 protein displays substantially greater activity on pCITE-1 RNA than on SRPα RNA. For example, the concentration of vhs1 protein that led to 50% decay of pCITE-1 RNA had virtually no effect on the SRPα substrate over the 4 minute time course of the experiment (Fig 4B). This experiment additionally provided evidence that the wild-type vhs protein also displays greater activity on pCITE-1 RNA than on SRPα RNA, although in this case the difference was not as obviously apparent.

It seemed possible that the marked difference in activity of the vhs1 protein on pCITE-1 versus SRPα RNA reflected the presence of the EMCV IRES in the former

substrate. We tested this hypothesis, by examining the effect of deleting the IRES (construct pCITE Msc/RI RNA, ca. 1.7 kb, diagramed in Fig. 5A). Previous work has shown that vhs-induced decay of pCITE Msc/RI RNA proceeds in a fashion similar to that of SRPα RNA, in that the sites of initial cleavage are non-randomly clustered over the 5' quadrant of the transcript (diagramed in Fig. 5A) (Elgadi and Smiley, 1999). The vhs1 protein displayed little, if any, activity on this substrate; in contrast, wild-type vhs induced rapid decay (Fig 5B.). Thus, deletion of the IRES rendered the pCITE-1 transcript refractory to degradation by the vhs1 protein (compare Figures 2 and 5).

Taken in combination, the foregoing results suggested that the vhs1 protein retains significant IRES-directed cleavage activity. As an additional test of this hypothesis, we asked if the vhs1 protein was capable of inducing cleavage downstream of the EMCV IRES when the IRES was transplanted onto a heterologous RNA. pSRPα StuI IRES is a derivative of pSRPα which bears the EMCV IRES element inserted into the unique StuI site in SRPα RNA (Elgadi and Smiley, 1999). Using 5'-labeled RNA, Elgadi and Smiley (Elgadi and Smiley, 1999) have shown that the transplanted IRES efficiently targets vhs activity, resulting in a complex pattern of initial cleavage events. Specifically, some of the substrate RNA molecules are initially cleaved at sites clustered over the 5' quadrant of the transcript (in the same fashion as unmodified SRPα RNA), while others are initially cleaved immediately downstream of the IRES (diagrammed Fig. 6A). If, as suggested by data presented above, the vhs1 protein retains significant IRES-directed activity but is otherwise severely impaired, then one predicts that pSRPα StuI IRES RNA would be cleaved predominantly or exclusively downstream of the transplanted IRES. Such IRES-directed events would give rise to stable products of ca. 2300 and 700 nt., representing the 5' and 3' segments of the RNA respectively. Our results (Figure 6B) confirmed this prediction. In contrast, wild-type vhs rapidly degraded the substrate into low molecular weight products. As expected, no stable 2300 nt 5' fragment accumulated (previous work has shown that this early product is subject to rapid further decay in reactions containing wild-type vhs, (Elgadi and Smiley, 1999)). However, a stable ca. 700 nt product was observed, which may correspond to either the 3' end of the RNA (Elgadi and Smiley, 1999) or the excised IRES element (which is refractory to vhs-

induced attack, (Elgadi and Smiley, 1999)). Further experiments are required to clarify the nature of this product. The results of this experiment provided further evidence that the readily detectable endoribonuclease activity of the vhs protein is IRES-dependent.

Overall, the results presented in this report establish that the vhs1 point mutation does not abolish the activity of the vhs-dependent endoribonuclease. Thus, if the vhs polypeptide indeed forms an integral component of the endoribonuclease, then it follows that the mutation does not completely inactivate the active site of the enzyme. Our data also provide strong evidence that the vhs1 mutant form of vhs retains significant IRES-directed activity, but is essentially inactive on RNA substrates that lack an IRES. One interesting possibility is that wild-type vhs normally utilizes both IRES-dependent and IRES-independent modes of substrate recognition, and the vhs1 mutation selectively impairs the latter. If so, then screening other mutant forms of vhs may help to unveil which regions of vhs participate in each mode of substrate recognition, and clarify the mechanisms involved. Alternatively, it is possible that the apparently selective activity of the mutant protein simply mirrors an inherent preference of wild-type vhs for IRES-bearing substrates (Figure 4A). According to this hypothesis, the mutation coordinately reduces (but does not eliminate) activity on all substrates, and our inability to clearly detect activity on substrates lacking an IRES simply reflects limitations of our assay system. Distinguishing between these possibilities will require detailed kinetic analysis of the activity of wild-type and mutant vhs on a variety of substrates. However, such analyses are very difficult to perform using the present RRL assay system (data not shown).

It has been suggested previously that vhs may destabilize mRNAs by inducing a limited number of endoribonucleolytic cleavages, which predispose the transcript to further decay through cellular mRNA surveillance systems (Pak *et al.*, 1995; Sorenson, Hart, and Ross, 1991; Zelus, Stewart, and Ross, 1996) in a fashion analogous to the mechanisms that regulate the stability of some cellular mRNAs (for examples, human transferrin receptor mRNA and Xenopus X1hbox 2B and Xoo1 mRNAs (Binder *et al.*, 1994; Brown, Zipkin, and Harland, 1993)). However, our data provide strong evidence that vhs is required not only for initial endoribonucleolytic cleavage events, but also for

subsequent degradation to low molecular weight products, at least in the RRL system. Thus, although the vhs1 protein is able to perform IRES-directed cleavage of pCITE-1 RNA, the resulting 3' product is much more stable than in reactions containing wild-type vhs. The implication is that wild-type vhs actively induces further decay of the products of IRES-directed cleavage.

We suspect that further studies of the activity of IRES-directed activity of the vhs1 and other mutant forms of vhs will clarify the mechanism of vhs action.

3.3. Acknowledgments

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3.5. Figure Legends

FIG. 1. vhs protein used in activity assays. vhs and vhs1 mRNA were each used to program four RRL in vitro translation reactions in the presence of ^{35}S-methionine. RRL control (lane R) was generated as above except that no mRNA was added to the translation reaction. The products of each translation reaction were then analyzed by SDS-PAGE. Numbers to the left of the panel indicate the sizes of protein markers (lane M). The samples of wild-type and vhs1 mutant labeled 1, 2, 3, and 4 were used for the activity assays depicted in figures 2, 3, 5, and 6 respectively.

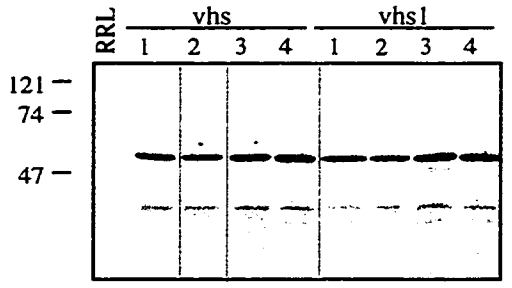

RRL
vhs
vhs1
1
2
3
4
1
2
3
4
121
74
47

FIG. 2. Activity of wild-type and mutant vhs on pCITE-1 RNA. (A) Diagram of the 2.3 kb pCITE-1 RNA, indicating the location of the IRES and the approximate location(s) of the sites of preferential initial cleavage by wild-type vhs . (B) Internally labeled pCITE-1 RNA was added to control RRL (lane R), and RRL containing pre-translated wild-type (lane vhs) or vhs1 mutant vhs (lane vhs1). RNA extracted at the indicated time (min) was resolved on a 1% agarose-1.8% formaldehyde gel, and transferred to a Gene Screen Plus membrane. Numbers to the left indicate the sizes of RNA markers (lanes M). Asterisks indicate the 3' and 5' products of IRES-directed cleavage (ca. 1800 nt and 600 nt. respectively). Open and closed squares indicate the ca. 1500 nt and 1000 nt. fragments. (C) The data displayed in panel B were quantified by phosphor-imager analysis.

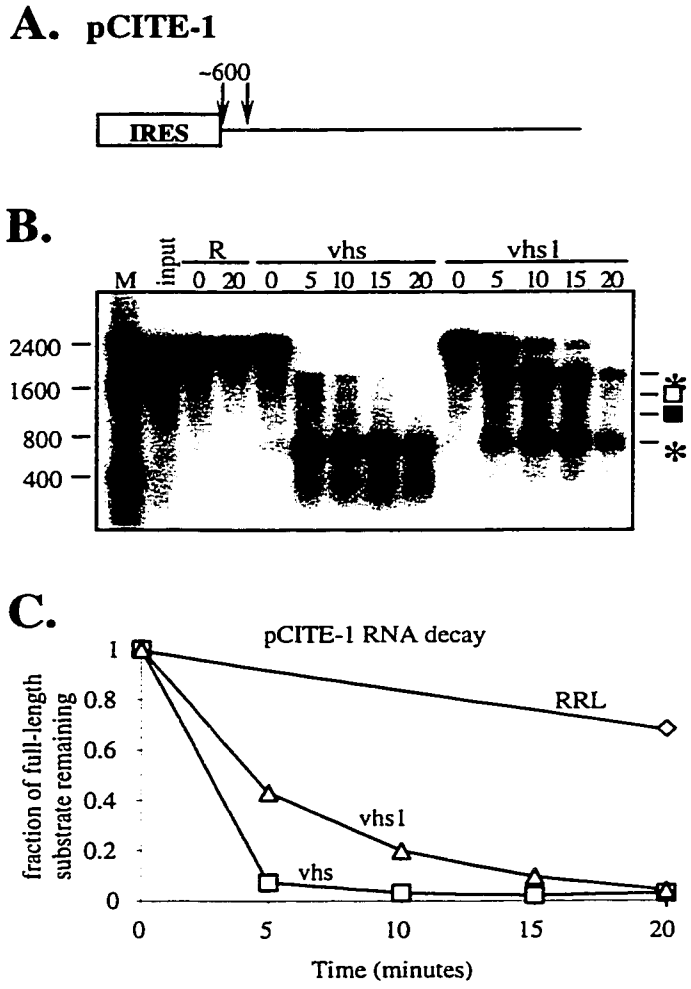

A. pCITE-1
~600
IRES
B.
input
R
vhs
vhs1
M
0 20 0 5 10 15 20 0 5 10 15 20
2400
1600
800
400
C.
pCITE-1 RNA decay
fraction of full-length substrate remaining
1
0.8
0.6
0.4
0.2
0
RRL
vhs1
vhs
0
5
10
15
20
Time (minutes)

FIG. 3. Activity of wild-type and mutant vhs on SRPα RNA. (A) Diagram of the 2.4 kb RNA substrate, indicating the approximate location(s) of the sites of preferential initial cleavage by wild-type vhs. (B) Internally labeled SRPα RNA was added to control RRL (lane R), and RRL containing pretranslated wild-type (lane vhs) or vhs1 mutant vhs (lane vhs1). The reaction products were analyzed as in Fig. 2. Numbers to the left indicate the sizes of RNA markers (lanes M). The bracket indicates the mobility of several discrete 3' products (1800 nt to 2200nt). (C) The data displayed in panel B were quantified by phosphor-imager analysis.

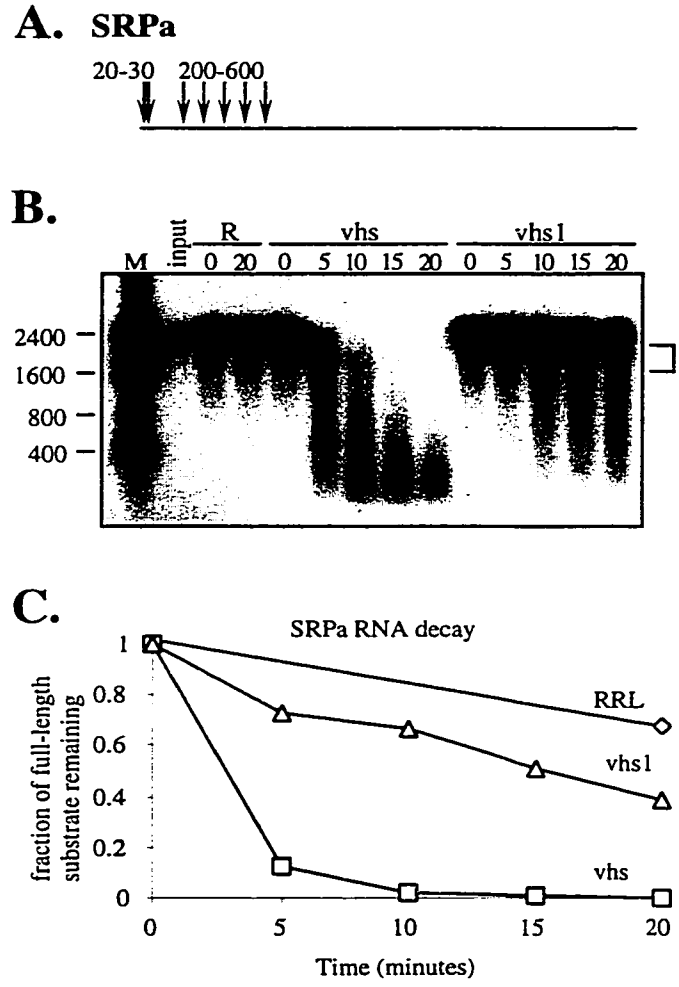
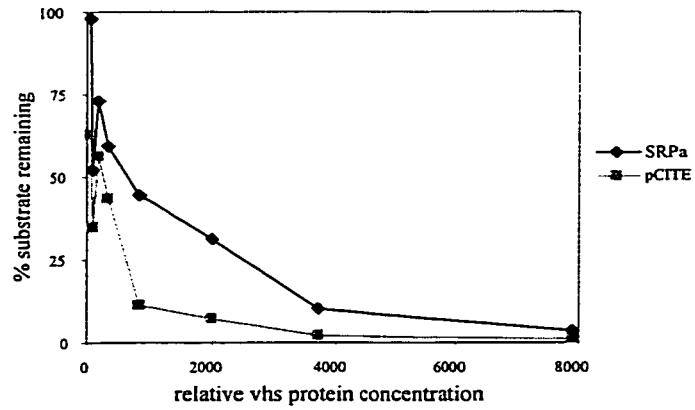

A.
SRPa
20-30
200-600
B.
M
input
R
vhs
vhs1
0 20 0 5 10 15 20 0 5 10 15 20
2400
1600
800
400
C.
SRPa RNA decay
fraction of full-length substrate remaining
1
0.8
0.6
0.4
0.2
0
RRL
vhs1
vhs
0
5
10
15
20
Time (minutes)

FIG. 4. Effect of dilution on activity of wild-type and mutant vhs. ^{35}S-Methionine labeled wild-type and vhs1 mutant vhs generated by *in vitro* translation were serially diluted in RRL. Aliquots of each dilution were assayed for vhs protein content by SDS-PAGE and phosphor-imager analysis, and tested for activity on internally labeled pCITE-1 and SRPα RNA as in figures 2 and 3. Reactions were incubated for four minutes. The activity data were quantified by phosphor-imager analysis, and plotted against the relative amount of vhs protein present in each reaction. (A). Activity of wild-type vhs; (B) activity of vhs1 mutant vhs.

A.


100
75
50
25
0
% substrate remaining
0
2000
4000
6000
8000
relative vhs protein concentration
SRPa
pCITE

B.

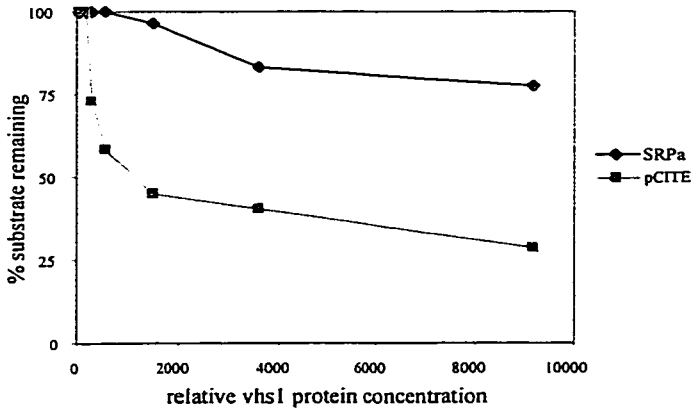

100
75
50
25
0
% substrate remaining
0
2000
4000
6000
8000
10000
relative vhs1 protein concentration
SRPa
pCITE

FIG. 5. Activity of wild-type and mutant vhs on pCITE Msc/RI RNA. (A) Diagram of the 1.7 kb transcript, indicating the IRES deletion and the approximate location(s) of the sites of preferential initial cleavage by wild-type vhs. (B) Internally labeled pCITE Msc/RI RNA was added to control RRL (lane R), and RRL containing pre-translated wild-type (lane vhs) or vhs1 mutant vhs (lane vhs1). The reaction products were analyzed as in Fig. 2. Numbers to the left indicate the sizes of RNA markers (lanes M).

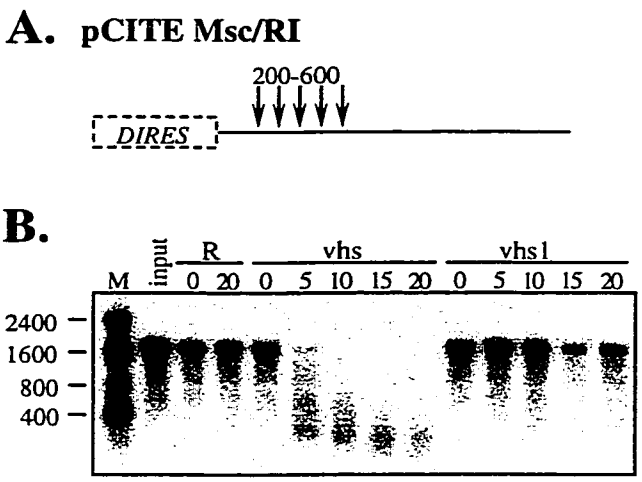
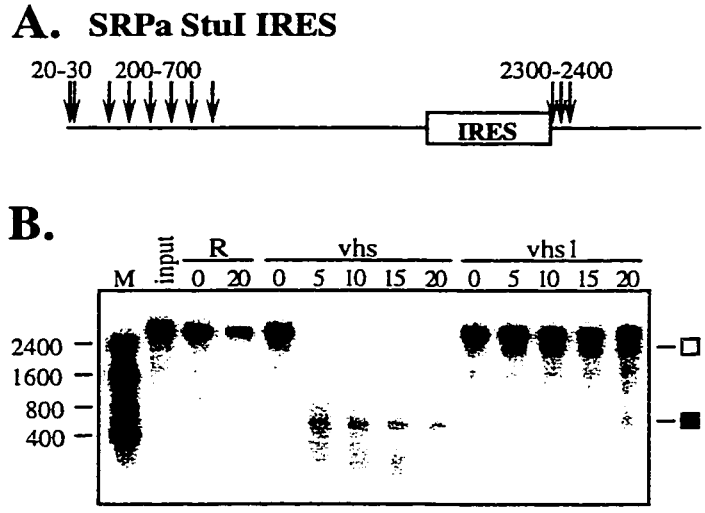

A.
pCITE Msc/RI
200-600
DIRES
B.
M
input
R
vhs
vhs1
0 20 0 5 10 15 20 0 5 10 15 20
2400
1600
800
400

FIG. 6. Activity of wild-type and mutant on SRPα StuI IRES RNA. (A) Diagram indicating the structure of the 3 kb RNA and the approximate location(s) of the sites of preferential initial cleavage by wild-type vhs. (B) Internally labeled SRPα StuI IRES RNA was added to control RRL (lane R), and RRL containing pre-translated wild-type (lane vhs) or vhs1 mutant vhs (lane vhs1). The reaction products were analyzed as in Fig. 2. Numbers to the left indicate the sizes of RNA markers (lanes M). The open and closed squares indicate the ca. 2300 and 700 nt fragments arising through IRES-directed cleavage.


A. SRPa StuI IRES
20-30
200-700
2300-2400
IRES
B.
M
input
R
vhs
vhs1
0 20 0 5 10 15 20 0 5 10 15 20
2400
1600
800
400

CHAPTER 4 – DISCUSSION AND FUTURE DIRECTIONS

To support efficient lytic infection, HSV employs several strategies to reprogram the host cell's replication machinery for its own replication and production of progeny. One of the strategies employed by HSV is to shut off host protein synthesis. Host shutoff is accompanied by disaggregation of pre-existing cellular polysomes, and degradation of pre-existing cellular mRNAs. The vhs protein of HSV is both necessary and sufficient for host shutoff.

The data described in this thesis show that expression of vhs in the yeast, *S. cerevisiae*, inhibits colony formation. The severity of this inhibition is dependent on the carbon sources used, and the vhs mutation, vhs1, eliminates this inhibition. Although the inhibition of colony formation displays the same mutational sensitivity spectrum as the shutoff in mammalian cells, I found that whole cell yeast extracts containing vhs show no endoribonuclease activity *in vitro*. One possible explanation for these observations is that the inhibition of colony formation is due to a previous unrecognized function of the vhs protein that is unrelated to host shutoff. Another possibility is that the inhibition of colony formation is due to the residual low level endoribonuclease activity of the vhs protein expressed in yeast. To distinguish between these two possibilities, mRNA collected from yeast cells expressing vhs could be tested on yeast DNA microarrays. mRNA from yeast cells expressing vhs and mRNA from yeast expressing vhs1 should be tested in parallel. These mRNAs can be used to generate fluorescently labeled cDNA. cDNA generated from yeast cells expressing vhs would be the test cDNA, while cDNA generated from yeast cells expressing vhs1 would be used as the control cDNA. Control and test cDNA would be mixed, then hybridized to DNA chips bearing PCR products representing the *S. cerevisiae* genome. Hybridization signals are detected with a high-resolution confocal DNA chip reader. If vhs expressed in yeast does cause accelerated mRNA turnover in yeast cells, at least a subset of mRNAs should be degraded if not the entire mRNAs. If vhs expressed in yeast inhibits colony formation through other mechanisms, vhs may affect only a specific protein or pathway, thereby affecting a specific mRNA or mRNAs involved in a specific pathway.

The mechanism of vhs function remains to be defined precisely. Recently, Elgadi and colleagues have started to study the details of vhs action, and have shown that vhs expressed as the only HSV protein in RRL induces endoribonucleolytic cleavage of substrate RNAs (Elgadi, Hayes, and Smiley, 1999; Elgadi and Smiley, 1999). These researchers observed that vhs induced cleavage of substrate RNAs produces initial cleavage products, which are then further degraded into low molecular weight products. Two hypotheses have been proposed for the mechanism of the further degradation of the initial cleavage products induced by vhs. One hypothesis is that the further degradation of these initial cleavage products is still a vhs dependent event; another hypothesis is that after the initial cleavage induced by vhs, cellular mRNA surveillance pathways in the RRL system take over to further degrade initial cleavage products. This hypothesis proposes that vhs is only needed for the initial cleavage. The stability of human transferrin receptor mRNA and Xenopus Xlhbox2B and Xoo1 mRNAs is regulated by a cellular pathway, in which these mRNAs undergo endonucleolytic cleavage in their 3' UTRs, generating unstable 5' and 3'products that are probably further degraded by cellular exonucleases (Binder *et al.*, 1994; Brown, Zipkin, and Harland, 1993). These initial cleavage events are independent of 5' cap structure, 3' poly(A) tail, or of ongoing translation, features which resemble the activity of vhs. The data described here show that in the presence of vhs, the initial cleavage products were further degraded into low molecular weight products, while in the vhs1 reaction, they were stable throughout the reaction (see result in Chapter 3). This result is consistent with the hypothesis that even after the initial cleavage induced by vhs, the further degradation of the initial cleavage products into low molecular weight fragments is still a vhs dependent event. To further investigate this possibility, one could study the degradation of initial cleavage products in blank RRL. After an initial reaction time (so the uniformly labeled substrate RNA has been cleaved to initial products, but the initial products have not been completely degraded), the RNAs are recovered and resolved on an agarose-formaldehyde gel. Initial cleavage products recovered from the gel are added to blank RRL, and observed for further degradation. This experiment will provide a definite answer as to whether or not further degradation of the initial vhs cleavage products is dependent on vhs.

In addition to the question of whether or not vhs is required for further degradation of initial cleavage products induced by vhs, another question regarding the mechanism of vhs action is whether any cellular factors are required for its activity. Although substantial data indicate that vhs is an integral part of a ribonuclease, this evidence does not exclude the possibility that this ribonuclease contains a mammalian cellular factor. The data presented in this thesis provide the first evidence that vhs expressed in yeast *S. cerevisiae* requires a mammalian macro- cellular factor(s) for its *in vitro* endoribonuclease activity. These data are consistent with our lab's previous observation that vhs translated in wheat germ extract does not trigger mRNA degradation, but the activity of vhs can be reconstituted by the addition of a post-ribosomal fraction from RRL (personal communication).

The mammalian cellular factor(s) required for the *in vitro* endoribonuclease activity of the vhs protein may serve four possible functions. One possibility is that the mammalian cellular factor(s) is a ribonuclease which has to be activated by vhs before being able to cleave target mRNAs. Alternatively, it is possible that the vhs protein is the ribonuclease, and the mammalian cellular factor(s) either acts as a co-factor(s) for the vhs endoribonuclease activity, is required for loading the vhs protein onto target mRNAs, or is required for post-translation modification of vhs (for example the cellular factor(s) acts as a kinase to phosphorylate vhs).

The idea that the cellular factor(s) is an enzyme required for post-translational modification of vhs is plausible because the vhs produced during HSV infection of mammalian cells is phosphorylated and the vhs1 mutation alters this phosphorylation pattern (Read, Karr, and Knight, 1993; Smibert, Johnson, and Smiley, 1992). Several experiments can be done to test this possibility. First, one can check the electrophoretic mobility of vhs produced in yeast with or without addition of RRL. Also, tests can be done to determine whether or not the addition of RRL stimulates labeling of vhs by γ-^{32}P-ATP in yeast extracts. Protein kinase inhibitors can be tested to see whether they reduce the stimulatory effect of RRL. Finally, commercially available mammalian protein kinases can be tested for their ability to stimulate the activity of vhs present in the yeast extract.

Alternatively, the cellular factor(s) could be required for loading vhs onto substrate RNAs. The data described in this thesis show that, although the vhs mutant, vhs1, was previously thought to be completely inactive, it is still active as a ribonuclease *in vitro* on substrates containing the EMCV IRES element. While wildtype vhs induces cleavage of RNAs independent of the EMCV IRES element, the vhs1 mutant form of vhs only retains significant IRES-directed activity. Given this result and my data indicating that vhs protein requires a cellular factor(s) for its activity *in vitro*, the simplest explanation for this vhs1 activity on IRES containing substrate is that vhs has two different mechanisms of loading onto substrates: one is IRES element dependent, and one is IRES element independent. According to this view, the vhs1 point mutation has no or little effect on ribonuclease activity, but it does have an effect on the mechanism of loading onto substrates. The vhs1 mutation may severely limit the ability of vhs to load onto substrates through the IRES element independent mechanism; therefore, vhs1 shows no activity on RNAs lacking the IRES element. The effect of the point mutation in vhs1 on its ability to load onto substrates through the IRES element dependent mechanism may be very moderate; therefore, vhs1 retains significant activity on RNAs bearing the IRES element.

IRES elements mediate cap-independent translation, by interacting with translation mediation factors (Goldstaub *et al.*, 2000). Therefore, an interesting and plausible possibility for IRES dependent cleavage is that the cellular factor(s) required for vhs activity could be a component of the translation apparatus. Recently, using a yeast two-hybrid system, vhs, but not the vhs1 protein, was shown to interact with a newly identified eukaryotic translation initiation factor eIF4H (GS Read, presented at the 24th International Herpesvirus Workshop). Although the function of eIF4H in translation is not clear, it has been shown to contain RNA binding domains and to stimulate the activity of the eIF4A/eIF4B helicase and eIF4F *in vitro* (Richter *et al.*, 1999; Richter-Cook *et al.*, 1998; Rogers, Richter, and Merrick, 1999). eIF4H could be one of the factors required for vhs endoribonuclease activity. This hypothesis has been supported by a very recent finding in our lab. A student in our lab, Rosalyn Doepker, found that when purified eIF4H protein was added to whole cell yeast extracts containing vhs, significant endoribonuclease activity on the substrate bearing the IRES element, pCITE-1, was

observed (personal communication). To further investigate whether other components of the translation apparatus are required for vhs activity, another approach would be to test vhs activity in the presence of the yeast small RNA inhibitor called IRNA. IRNA is a 60 nt small RNA which inhibits the translation of poliovirus and hepatitis mRNA in yeast *Saccharomyces cerevisiae* (Das *et al.*, 1998). The IRNA effectively competes with the poliovirus IRES for cellular factors required for cap-independent translation initiation including the La autoantigen (Das *et al.*, 1996). In fact, addition of exogenous La protein reverses the inhibitory effect of IRNA. If translation initiation and vhs activity are linked, the presence of the IRNA may inhibit the poliovirus IRES targeting vhs cleavage, and addition of La protein to the reaction will reverse the effect.

The previous sections discussed the possible experiments to identify the cellular factor(s) required for vhs activity based on the assumption that it may serve as a kinase or a component of the translation apparatus. If the required cellular factor(s) serves other functions, chromatographic fractions of naïve reticulocyte lysate could be tested with the vhs yeast extract to see which fraction leads to the complementation of its endoribonuclease activity. This approach could lead to purification of the cellular factor(s), and its identification by microsequencing. Alternatively, one might be able to use yeast genetics to identify the factor(s). The requirement of the same regions of vhs for inhibition of colony formation as the mammalian host shutoff suggests that a yeast homologue of the required mammalian factor(s) may be required for the inhibition of colony formation. This possibility could lead to the rapid selection of yeast chromosomal mutations that render cells resistant to vhs-induced growth inhibition. If such mutations produce a detectable phenotype in the absence of vhs expression, then identification of the gene involved will lead to the identification of the mammalian cellular factor(s).

Although there is evidence suggesting that vhs acts as a ribonuclease, my data strongly suggest that a mammalian macro- cellular factor is required for vhs ribonuclease activity. To definitely answer this question, purified vhs is needed. In the yeast expression system presented in this thesis, the levels of vhs protein were similar to these in HSV-infected mammalian cells. In addition to being very useful for identifying the mammalian cellular factor(s) required for the vhs endoribonuclease activity *in vitro*, this

yeast expression system could be very useful for eventually purifying the vhs protein. Although my attempt to purify the vhs protein using a nickel column (the 2.1vhs is also tagged with eight stretches of histidine) failed because the purification process seemed to inactivate the vhs protein (data not shown), other tags for protein purification such as FLAG and GST could be tried. In addition, the tag could be inserted at the C-terminus or N-terminus of the vhs protein if insertion at these locations does not affect the activity of vhs. Obtaining purified vhs will help lead to the definite answers as to whether or not vhs is a ribonuclease and whether any cellular factor is required for the activity of vhs.

As shown by Elgadi and colleagues, vhs preferentially induces cleavage of IRES substrates. The biological relevance of vhs preferentially inducing the cleavage of IRES containing substrates is still not clear. Recently, data have been generated to show that several cellular genes contain an IRES element, and under certain circumstances, their expression is controlled by the IRES element present in them. Under apoptosis conditions, in a variety of cell types, several components of the cap-dependent translation pathways such as the eIF4GI, eIF4E, eIF3, eIF2 and eIF4E-BP1 are cleaved by caspases (Bushell *et al.*, 2000). This leads to an inhibition of the cap-dependent translation pathway, which leads to the inhibition of the rate of overall protein synthesis. However, under conditions of apoptosis, several proteins were recently found to either maintain or upregulate their expression level through translation from an IRES segment in their mRNAs. These proteins include the proto-oncogene c-myc protein, death associate factor 5 (DAP5, which is highly homologous to eukaryotic translation initiation factor eIF4G), immunoglobin binding protein (BiP), the most potent member of mammalian inhibitor-of apoptosis (IAP) family, XIAP, and vascular endothelial growth factor (Henis-Korenblit *et al.*, 2000; Holcik *et al.*, 1999; Johannes and Sarnow, 1998; Levy-Strumpf *et al.*, 1997; Stein *et al.*, 1998; Stoneley *et al.*, 2000). Among these proteins, several are involved in the programmed cell death pathway. Overexpression of c-myc in the absence of survival factors has been shown to lead to apoptosis (Evan *et al.*, 1992; Stoneley *et al.*, 2000). During apoptosis, DAP-5 is cleaved by caspases, which yields a truncated protein that forms complex with eIF4A and eIF3, and this truncated form of DAP-5 protects the cells against apoptosis (Henis-Korenblit *et al.*, 2000; Levy-Strumpf *et al.*, 1997). IAP family members directly inhibit caspases 3 and 7 during apoptosis (Holcik *et al.*, 1999).

Recent data has shown that IRES-mediated XIAP translation was upregulated under different cellular stresses, and expression of XIAP protects cells from apoptosis under some of the stimulation conditions (Holcik *et al.*, 1999). These data seem to suggest that IRES mediated translation has a very important role in apoptosis.

Does the preferential cleavage of IRES containing substrates by vhs have any biological significance for the virus life cycle other than host shutoff? Viruses employ different methods of inhibiting apoptosis in order for them to survive and produce progeny. For example, baculoviral p53 protein and the crmA protein of the cowpox virus inhibit the activity of casapase 9, thereby inhibiting apoptosis in infected cells (Huang *et al.*, 2000). Could vhs be the HSV protein that has this apoptosis inhibition function through its endoribonuclease cleavage of pro-apoptosis protein such as c-myc? Also since some proteins that are translated through the IRES-mediated pathway under apoptosis condition are proteins that inhibit apoptosis, could vhs mediated degradation of these proteins fool the cells into thinking they are going through apoptosis? After the activity of vhs is downregulated by VP16, these proteins could then prevent cells from undergoing apoptosis. The observation that the vhs1 form of vhs is still active as a ribonuclease *in vitro* suggests that two regions on vhs are separately required for recognizing substrates. It would be interesting to explore other vhs mutants which preserve only the activity on substrates lacking the EMCV IRES element to help identify any involvement of vhs in apoptosis. The finding that vhs1 retains significant endoribonuclease activity has to be confirmed *in vivo* first. Vero cells can be transfected with a vhs1 expression plasmid and another plasmid expressing a reporter protein which is driven by the IRES element. If vhs1 retains its endoribonuclease activity *in vivo* on substrates containing the IRES element, other vhs mutants could be screened to find if any of these mutants only triggers the cleavage of substrates that lack the IRES element. If such a mutant is found, under apoptotic conditions, comparison between the mutant infected cells and wildtype vhs infected cells may provide an insight to the possible role of vhs in apoptosis.

The data presented in this thesis provide some insight into the mechanism of vhs action and begin to define some of the requirements for the vhs endoribonuclease

activity. Specifically, vhs expressed in yeast *S. cerevisiae* requires a mammalian macro-cellular factor(s) for its *in vitro* endoribonuclease activity. Also, the point mutant form of vhs, vhs1, retains significant endoribonuclease activity *in vitro* on substrates containing the IRES element.

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