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THE *IN VITRO* ACTIVITY OF THE HERPES SIMPLEX VIRUS VIRION HOST SHUTOFF (VHS) PROTEIN.

By

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A Thesis
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In Partial Fulfillment of the Requirements
for the Degree
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THE HERPES SIMPLEX VIRUS' VIRION HOST SHUTOFF (VHS) PROTEIN

DOCTOR OF PHILOSOPHY (1999) McMASTER UNIVERSITY
(BIOLOGY)

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DEDICATION.

This thesis is dedicated in loving memory to my brother

Khalid Mohammed Elgadi.

ABSTRACT.

The herpes simplex virus (HSV) virion contains the virion host shutoff protein (vhs, product of the UL41 gene) that induces inhibition of host cell protein synthesis, disaggregation of pre-existing polyribosomes, and degradation of mRNA during early times of infection. The data presented in this thesis describe the development of a rabbit reticulocyte lysate-based *in vitro* system for analysis of the vhs protein activity. I show that vhs protein produced, as the sole HSV protein, in rabbit reticulocyte lysates inhibits translation and induces degradation of reporter RNAs. Detailed analysis of the vhs-dependent RNA decay revealed that it proceeds through an endoribonucleolytic cleavage mechanism that is not affected by the presence of a 5' cap structure or a 3' poly(A) tail in the RNA substrate. The vhs-dependent RNA degradation activity requires Mg^{++} ions and occurs in the absence of ATP and ribosomes. Characterization of the decay profile of two unrelated substrates indicated that they were initially cleaved at preferential sites non-randomly distributed over their 5' quadrants. This activity is greatly altered by placing an internal ribosome entry site (IRES) from encephalomyocarditis virus (EMCV) or poliovirus in the transcript. Transcripts bearing the IRES were preferentially cleaved by the vhs-dependent endoribonuclease at multiple sites clustered in a narrow zone located immediately downstream of the element. "Targeting" was observed when the IRES was located at the 5' end or placed at internal sites in the substrate, indicating that it is independent of position or sequence context. As with cleavage over the 5' quadrant, cleavage downstream of the IRES elements requires Mg^{++} ion but not ATP and ribosomes. Transcripts containing internal IRES elements were also independently cleaved over their 5' quadrant. The vhs-induced RNA decay activity is not restricted to HSV-1 vhs, in that the vhs proteins from HSV-2 and pseudorabies virus also induce RNA degradation *in vitro*. I also show that the RNA degradation activity of the HSV-1 vhs protein is reproducible when vhs is produced in HeLa, but not wheat germ, cell-free extracts. These data indicate that the vhs-dependent nuclease can be selectively targeted by specific *cis*-acting elements in the RNA substrate, possibly through secondary structure or a component of the translational machinery.

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TABLE OF CONTENTS

CHAPTER 1: *Introduction.* **1**

1.1. Herpesviridae. 1

1.2. Diseases Associated With Human Herpesviruses. 3

1.3. The Herpes Simplex Virion. 8

1.4. Herpes Simplex Virus Lytic Cycle. 11

1.4.1. Entry into Host Cells. 11

1.4.2. Initiation of Viral Gene Expression 17

1.4.3. Immediate Early Genes. 21

1.4.4. Early Genes and Viral DNA Replication. 29

1.4.5. Late Genes, Virion Assembly, and Egress. 31

1.5. Virion Induced Host Shutoff. 36

CHAPTER 2: *Materials and Methods* **46**

2.1. Recombinant DNA manipulation protocols. 46

2.1.1. Propagation and maintenance of bacterial strains. 46

2.1.2. Restriction enzyme digestion and modifying enzyme treatment of DNA. 47

2.1.3. Agarose gel electrophoresis and gel purification of DNA fragments. 48

2.1.4. Ligation of DNA fragments. 48

2.1.5. Preparation of transformation competent *E. coli* 49

2.1.6. Transformation of competent *E. coli* with plasmid DNA. 49
2.1.7. Small scale preparation of plasmid DNA. 50
2.1.8. Large scale preparation and CsCl purification of plasmid DNA. 50

2.2. Radioactive labeling of DNA. 51
2.2.1. Random primer labeling of DNA. 51
2.2.2. 5' end labeling of DNA oligonucleotides. 52

2.3. Plasmids. 53
2.3.1. Vhs *in vitro* translation vectors. 53
2.3.2. *In vitro* transcription plasmids used to generate substrate RNAs. 55

2.4. *In vitro* transcription. 57
2.5. Preparation of HeLa cell-free translation extracts. 57
2.6. *In vitro* translation. 58
2.7. Ribosome depletion. 58
2.8. Depletion of the cap-binding protein eIF4E. 59
2.9. Western blot analysis. 60
2.10. Desalting of vhs containing and control RRL. 60
2.11. RNA labeling. 61
2.11.1. Internally labeled RNAs. 61
2.11.2. Cap labeled RNAs. 62

2.12. *In vitro* translational shutoff assay. 62
2.13. *In vitro* RNA degradation assay. 63
2.14. Agarose/formaldehyde gel electrophoresis. 64

2.15. Northern blot analysis........ 64

2.16. Primer extension analysis........ 65

2.17. Polyacrylamide sequencing gel electrophoresis........ 66

2.18. RNA and DNA markers........ 66

CHAPTER 3: ***The Herpes Simples Virus Vhs Protein Induces Endoribonucleolytic Cleavage of Target RNAs in Cell Extracts.*** **67**

Published in the Journal of Virology: 73(9):7153-7164. 1999........ 67

Preface........ 67

3.1. Abstract........ 70

3.2. Introduction........ 71

3.3. Materials and Methods........ 75

3.4. Results........ 83

3.5. Discussion........ 113

3.6. Acknowledgements........ 119

3.7. References........ 120

3.8. Addendum........ 125

3.8.1. Data not shown in chapter 3........ 125

3.8.2. Unpublished data relating to chapter 3........ 128

i) Vhs produced in wheat germ extracts does not induce translational arrest or 5' end RNA degradation........ 128

ii) Vhs proteins from HSV-1 and HSV-2 display the same activity *in vitro*.......................................129

CHAPTER 4: ***Picornavirus IRES elements target RNA cleavage events induced by the herpes simplex virus vhs protein*................................ 135**

Published in the Journal of Virology: 73(11):9222-9231. 135

Preface. 135

4.1. Abstract... 138

4.2. Introduction. 139

4.3. Materials and Methods. 144

4.4. Results.. 150

4.5. Discussion... 175

4.6. Acknowledgements. 181

4.7. References.. 182

4.8. Addendum. 191

4.8.1. Data not shown in chapter 4...191

4.8.2. Unpublished data relating to chapter 4.............................194

i) The EMCV IRES targets vhs-induced cleavage in extracts derived from HeLa cells..............................194

ii) Specificity of vhs-induced cleavage of SRPα RNA..197

CHAPTER 5: ***Discussion and Future Directions*.....................201**

CHAPTER 6: ***References*...214**

LIST OF FIGURES

Figure 3-1. HSV1 vhs induces translational arrest and mRNA 89

Figure 3-2. Analysis of vhs-induced degradation intermediates of SRPα RNA. 89

Figure 3-3. Primer extension analysis of the 5'-most degradation products of SRPα RNA. 92

Figure 3-4. Vhs induces degradation of a variety of RNA substrates. 95

Figure 3-5. Vhs synthesized in a HeLa cell translation extract induces degradation of SRPα RNA. 98

Figure 3-6. Vhs-induced RNA degradation is cap-independent. 101

Figure 3-7. Vhs-induced RNA degradation is not influenced by a 3' poly(A) tail. 104

Figure 3-8. Vhs-induced RNA degradation does not require ribosomes. 106

Figure 3-9. Vhs-induced RNA degradation requires magnesium. 109

Figure 3-10. Pseudorabies virus vhs displays RNA degradation activity *in vitro*. 112

Figure 3-11. The cap-binding protein, eIF4E, does not contribute to vhs-dependent RNA degradation *in vitro*. 127

Figure 3-12. Vhs produced in wheat germ extracts does not induce translational arrest and RNA degradation. 131

Figure 3-13.	HSV-1 and HSV-2 vhs proteins induce RNA degradation with similar kinetics *in vitro*.	134
Figure 4-1.	Vhs induces preferential endonucleolytic cleavage in the vicinity of the 3' boundary of the EMCV IRES.	152
Figure 4-2.	The pCITE transcript is cleaved immediately downstream of the IRES.	155
Figure 4-3.	The EMCV IRES serves as a movable targeting element for vhs-induced RNA cleavage.	159
Figure 4-4.	Vhs-induces cleavage downstream of an internally located EMCV IRES.	162
Figure 4-5.	The EMCV IRES targets vhs-induced cleavage of SRPα mRNA.	165
Figure 4-6.	The EMCV IRES targets cleavage to 3' flanking SRPα sequences.	167
Figure 4-7.	The poliovirus IRES serves as a moveable targeting element for vhs-induced RNA cleavage.	170
Figure 4-8.	IRES-targeted cleavage occurs in the absence of ribosomes.	174
Figure 4-9.	The presence of highly structured elements within the RNA is not sufficient to target vhs activity.	193
Figure 4-10.	The EMCV IRES functions as a vhs targeting element in extracts derived from HeLa cell.	196
Figure 4-11.	The cleavage sites clustered over the 5' end of SRPα are 5' end-independent.	199

CHAPTER 1: Introduction.

1.1. Herpesviridae.

The family *Herpesviridae* encompasses nearly 100 viruses that are widely disseminated in nature with most animal species revealing at least one example. Inclusion into this family is based on the architecture of the virion and the biological properties of the virus life cycle. Herpes virions are composed of an electron dense core containing the viral linear double stranded DNA genome, an icosahedral capsid surrounding the core, an "amorphous" proteinaceous material (termed tegument) encasing the capsid, and an outer most membrane bearing viral glycoprotein projections. Herpesvirus DNA replication, virion assembly, and envelopment occur in the nucleus, and release of progeny virions leads to the destruction of infected cells. A common feature of herpesviruses is their ability to remain latent, following acute primary infection, for the life span of the host with periodic reactivation episodes (reviewed by Roizman, 1996, Roizman & Sears, 1996).

The *Herpesviridae* family has been divided into three subfamilies (Alpha-, Beta-, and Gammaherpesvirinae) based on biological properties that include host range, length of replicative cycle, efficiency of host cell destruction, and target cell types in which latency is established (Roizman & Sears, 1996). Members of the *Alphaherpesvirinae* subfamily are characterized by wide host range, short replicative cycle resulting in a high

virus yield, efficient killing of host cells, and the ability to establish latency primarily in sensory ganglia. This subfamily is further divided into two genera: *Simplex-* and *Varicellovirus*. The *Simplexvirus* genus includes the herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2), circopithecine herpesvirus 1 (herpes B virus), and bovine mammillitis virus while the *Varicellovirus* genus includes varicella-zoster virus (VZV), pseudorabies virus, and equine herpesvirus 1 (reviewed by Murphy, 1996, Roizman, 1996).

Betaherpesviruses display a restricted host range, long replicative cycles, inefficient killing of host cells (infected cells often become enlarged, cytomegalia), and the ability to establish latent infection in a variety of tissues including sensory glands, lymphoreticular cells, and kidneys. This subfamily contains three genera: the *Cytomegalovirus* and *Muromegalovirus* genera are represented by the human cytomegalovirus (HCMV) and the murine cytomegalovirus (MCMV), respectively. The lymphotropic human herpesvirus 6 (HHV-6) and human herpesvirus 7 (HHV-7) have been assigned to the *Roseolovirus* genus of the *Betaherpesvirinae* subfamily (reviewed by Murphy, 1996, Roizman, 1996).

Herpesviruses belonging to the *Gammaherpesvirinae* subfamily are characterized by a narrow host range that is limited to the family or order of the natural host. Members of this subfamily are lymphotropic with specificity for either B- or T-lymphocytes. Latent infection is also primarily associated with lymphoid tissue. This family has been divided into two genera: *Lymphocryptovirus* (e.g., human Epstein-Barr virus (EBV)) and *Rhadinovirus* (e.g., herpesvirus ateles and herpesvirus saimiri) (reviewed by Murphy,

1996, Roizman, 1996). The recently identified human herpesvirus 8 (also known as Kaposi's sarcoma herpesvirus, KSHV) has also been assigned to the *Rhadinovirus* genus of this subfamily (reviewed by Neipel *et al.*, 1998).

1.2. Diseases Associated With Human Herpesviruses.

Of the nearly 100 herpesviruses identified thus far, 8 were isolated from humans. These are herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7), and human herpesvirus 8 (HHV-8 or KSHV). These viruses cause a variety of diseases ranging from a mild mucocutaneous infections to fatal systemic infection and infection of the central nervous system. Generally, herpes simplex virus infection can be divided into four stages: I) primary mucocutaneous infection in seronegative individuals leading to viral replication and tissue damage (ulceration) at the site of infection; II) neuronal retrograde transport of virions from the primary infection site to sensory ganglion where, after a round of replication, the viral DNA remains latent for the life span of the host; III) reactivation of latent virus in response to a given stimulus (including stress, injury, and exposure to ultraviolet light) leading to transport of virions to sites at or near the site of primary infection; IV) recurrent infection at these sites. Recurrent infections are usually less severe than primary infection and are often associated with asymptomatic shedding of the virus.

Infections with the prototype alphaherpesvirus, HSV, are most frequently associated with mucocutaneous infections of the oropharyngeal and genital regions. HSV-1 is most often associated with orelabial herpetic lesions and HSV-2 with genital herpetic lesions. Infrequently however, these viruses can cause the reciprocal infection (Corey *et al.*, 1983, Wolontis & Jeansson, 1977). Although HSV-1 can cause genital herpetic lesions, the infection is usually less severe and exhibits lower rate of recurrence than HSV-2 genital infection (reviewed by Brugha *et al.*, 1997). Primary HSV-1 infections, acquired during early childhood, are generally asymptomatic but can manifest clinical symptoms including labialis and gingivostomatitis. When acquired in later life, primary HSV-1 infections are usually more severe and can cause pharyngitis and mononucleosis syndrome. HSV-1 is transmitted by personal contact with an individual that has clinical (lesions) or subclinical (asymptomatic virus shedding) infection (reviewed by Whitley, 1996). HSV-2 is transmitted through intimate sexual contact and, as such, primary infections do not usually occur until early adulthood, with the commencement of sexual activity (reviewed by Brugha *et al.*, 1997). On rare occasions, primary HSV infection can lead to dissemination of the virus causing severe systemic (at times fatal) infection with multiorgan involvement (Whitley, 1996). An example of this is the multiorgan disease of pregnancy, which has higher than 50 % mortality rate and can lead to spontaneous abortion, neonatal HSV disease, or severe mental retardation in the newborn (Gutierrez *et al.*, 1999, Lahat *et al.*, 1999, Sullivan-Bolyai *et al.*, 1983, Whitley, 1996, Whitley, 1983, Whitley & Kimberlin, 1999). Primary HSV infection can also occur in the eye causing keratoconjunctivitis (most frequently caused by HSV-1), a disease that is difficult to treat and may lead to blindness (Binder, 1977, Whitley, 1996).

In addition to the diseases mentioned above, primary HSV infection can also lead to severe diseases including neonatal HSV infection and herpes simplex encephalitis (HSE) (Gutierrez *et al.*, 1999, Lahat *et al.*, 1999, Marton *et al.*, 1995, Whitley, 1983, Whitley, 1988, Whitley & Kimberlin, 1999). Approximately 11 to 20 per 100,000 live births are diagnosed with neonatal HSV infection with the virus being acquired from infected mothers during birth (Gutierrez *et al.*, 1999, Whitley, 1988). Neonatal HSV disease usually manifests skin, eye and mouth lesions and in approximately 50 % of neonatal HSV cases the virus spreads causing systemic multiorgan disease including encephalitis (Whitley, 1993, Whitley & Kimberlin, 1999). Mortality from neonatal HSV encephalitis is over 40 % despite anti-viral treatment. Furthermore, approximately two-thirds of babies that survive the disease develop neurological impairment (Whitley, 1993, Whitley & Kimberlin, 1999). HSE is an uncommon disease (2-4 cases per million per year in the USA, approximately 10% of encephalitis cases) that results from disseminated HSV infection. This potentially fatal disease (15 % mortality) is most often caused by HSV-1 and of those that survive the infection the rate of neurological problems (including retardation and paralysis) is high (Whitley & Kimberlin, 1999). Recurrent HSV infections are usually asymptomatic shedding of the virus and when symptoms appear, they are less severe and last for shorter duration than primary infections. In very rare instances, however, recurrent HSV infection can lead to systemic dissemination of the virus and the aforementioned diseases.

Varicella zoster virus (VZV) is another human pathogen belonging to the *Alphaherpesvirinae* subfamily. This virus is usually contracted in early childhood

through the respiratory tract or conjunctiva leading to the development of the highly contagious chickenpox (varicella) marked by widespread mucosal and cutaneous lesions. Reactivation of latent VZV (uncommon) results in Herpes zoster (shingles), a painful disease marked by a general skin rash. In neonates, immunocompromised individuals, and pregnant women, primary or recurrent VZV disease may be fatal due to complications that include varicella pneumonia, encephalitis, post-herpetic neuralgia, and meningoencephalitis (reviewed by Arvin, 1996).

Human cytomegalovirus (HCMV), human herpesvirus 6 (HHV-6), and human herpesvirus 7 (HHV-7) represent human pathogenic members of the *Betaherpesvirinae* subfamily. HCMV infection often leads to infectious mononucleosis in immunocompetent host (Peter & Ray, 1998). In neonates and immunocompromised individuals, however, the disease often leads to severe (at times fatal) complications including hepatitis, pneumonia, gastroenteritis, CNS infection (Guillain-Barr's disease), and aseptic meningitis. In addition, congenital CMV infection is marked by a high mortality rate (30 %) and 90 % of survivors develop significant neurological defects (Britt & Alford, 1996). Infection with the neurotropic HHV-6 is very common (nearly 100 % seroprevalence) and usually occurs in the first year of life. Although primary infection is asymptomatic in most cases, it may lead to the development of roseola infantum (also known as exanthem subitum) which is a common, mild febrile disease in infants (reviewed by Pellett & Black, 1996). Complications of primary HHV-6 infection including invasion of the CNS (focal encephalitis and meningoencephalitis), seizures, fulminant hepatitis, and disseminated infection are rare in immunocompetent hosts

(Campadelli-Fiume *et al.*, 1999, Kimberlin & Whitley, 1998, McCullers *et al.*, 1995, Torre *et al.*, 1998, Whitley & Kimberlin, 1999). HHV-7 infection usually leads to clinical manifestations similar to those caused by HHV-6 but primary infection occurs later in life (Frenkel & Roffman, 1996, Pellett & Black, 1996). Possible associations of HHV-6 with multiple sclerosis, Kaposi sarcoma, lymphoproliferative, and neoplastic disorders have been reported, however, the evidence is far from conclusive (Ablashi *et al.*, 1998, Campadelli-Fiume *et al.*, 1999, Kimberlin & Whitley, 1998, Pellett & Black, 1996).

Of the gamaherpesviruses, Epstein-Barr virus (EBV) and human herpesvirus 8 (HHV-8 or KSHV) cause disease in humans. EBV infection of epithelial cells in the oropharynx and B-lymphocytes is usually acquired in early childhood with 80 to 100 % seroprevalence worldwide. Early childhood EBV infection is usually asymptomatic, but when contracted in late childhood or later EBV infection frequently results in infectious mononucleosis (Peter & Ray, 1998, Rickinson & Kieff, 1996). EBV has been linked to the development of anaplastic nasopharyngeal carcinoma and circumstantially associated with Hodgkin's disease, Burkitt's lymphoma and T-cell lymphoma (EBV-associated). In addition, EBV has been linked to oral hairy leukoplakia in HIV/AIDS patients and posttransplant lymphoproliferative disease in solid organ transplant patients (Chapman & Rickinson, 1998, Flaitz & Hicks, 1998, Harris, 1998, Rickinson & Kieff, 1996, Yao *et al.*, 1998). The newly identified HHV-8 (or KSHV) has been causally linked to Kaposi's sarcoma (all forms), Castleman's disease and primary effusion lymphoma (Dupin *et al.*, 1999, Neipel *et al.*, 1998).

In conclusion, with the exception of HSV-2 and HHV-8, most infections caused by herpesviruses in immunocompetent individuals are asymptomatic or may cause mild disease that is self-resolving. These viruses may, however, cause life-threatening diseases in immunocompromised patients (such as HIV/AIDS and posttransplant patients) and in immunologically immature neonates.

1.3. The Herpes Simplex Virion.

Like all other herpesviruses, the HSV virion is composed of four elements: (i) an electron-dense core containing the viral DNA genome, (ii) an icosahedral capsid surrounding the core, (iii) a proteinaceous layer (tegument) surrounding the capsid, and (iii) an outer lipid membrane with viral glycoprotein projections.

The HSV genome is a linear double-stranded DNA molecule that is packaged in the form of a toroid in the virion. The size of the genome is approximately 152 kbp with a 68 % and 69 % GC content for HSV-1 and HSV-2, respectively. The genome is composed of two covalently linked elements, long (L) and short (S), that contain unique sequences termed unique long (UL) and unique short (US). These elements are flanked by inverted repeats, ab/b'a' bracketing the L component and ac/c'a' bracketing the S component (Becker *et al.*, 1968, Furlong *et al.*, 1972, Kieff *et al.*, 1971, McGeoch *et al.*, 1988, McGeoch *et al.*, 1985). The presence of these terminal repeat elements allows for the UL and US components to invert relative to each other giving rise to four equimolar linear isomers of the genome (Delius & Clements, 1976, Hayward *et al.*, 1975). The viral genome contains approximately 78 open reading frames: 60 contained within UL, 14

contained within US, and 4 contained within the repeats (3 in ab/b'a' and 1 in ac/c'a') (reviewed by Roizman & Sears, 1996). Of these more than 30 encode proteins that are contained in the infectious HSV virion (Heine *et al.*, 1974, Spear & Roizman, 1972). These include capsid structural proteins, tegument proteins, and viral envelope glycoproteins.

The HSV-1 capsid, approximately 125 nm in diameter, is composed of 162 capsomeres: 150 hexons and 12 pentons. The pentons are located at the vertices while the hexons are located at the edges and faces of the capsid shell (Roizman & Sears, 1996). VP5 (major capsid component; product of the UL19 gene) is the structural subunit of both hexon (composed of 6 VP5 molecules) and penton (composed of 5 VP5 molecules) capsomeres (Newcomb *et al.*, 1993, Schrag *et al.*, 1989). In addition, mature HSV capsids also contain three minor proteins: VP19C (UL38), VP23 (UL18), and VP26 (UL35). The VP26 protein binds to the outer tip of each of the hexon-associated VP5 protein subunit to stabilize the capsid structure (Newcomb *et al.*, 1999, Newcomb *et al.*, 1993, Trus *et al.*, 1996, Trus *et al.*, 1995, Zhou *et al.*, 1995). The VP19C and VP23 proteins assemble into hetrotrimeric structures (the triplexes) that form the intercapsomeric fibers connecting the capsomeres together (Newcomb *et al.*, 1996, Newcomb *et al.*, 1999, Newcomb *et al.*, 1994, Newcomb *et al.*, 1993, Trus *et al.*, 1996, Trus *et al.*, 1995, Zhou *et al.*, 1994).

The tegument component of the HSV virion is the proteinaceous structure present between the capsid and the viral envelope. Although often referred to as an amorphous layer, the tegument is a highly organized structure capable of self-assembling in the

absence of the capsid (Elliott & O'Hare, 1999, McLauchlan & Rixon, 1992, Elliott, 1995, Zhou *et al.*, 1999). This layer contains a number of viral proteins including the viral transactivator VP16, the virion host shutoff protein (vhs), VP1/2, VP11/12, VP13/14, VP22, the protein kinase product of the UL13 gene, and the product of the US11 gene. In addition to structural roles, these proteins provide important functions at early times during HSV infection. For example, VP22 may be involved in virion disassembly and transport of nucleocapsid to the nuclear pores, VP1/2 has been implicated in the release of viral DNA from the capsid at the nuclear pores, VP16 stimulates the expression of immediate early HSV genes which are essential for lytic HSV replicative cycle, and vhs induces the general degradation of host cell mRNAs leading to preferential translation of viral transcripts (reviewed by Roizman & Sears, 1996). The functions of some of these proteins will be discussed in more detail in subsequent sections.

Encasing the structures described above is a lipid bilayer derived from the host cell. Whether this viral envelope is derived from the nuclear, endoplasmic reticular, or cytoplasmic membranes is a controversial issue that remains to be clarified (discussed in more detail in a subsequent section). The viral envelope contains at least 14 viral proteins of which at least 10 are glycoproteins that form the virion spikes (Stannard *et al.*, 1987, and reviewed by Spear, 1993b). These glycoproteins mediate functions important for the viral productive cycle including receptor recognition and adsorption, fusion of viral and cellular membranes, viral entry, virion assembly, cell-to-cell spread, egress, and immune evasion (reviewed by Spear, 1993b). Of these, glycoproteins gB, gC, gD, gH and gL have been shown to be essential for virus infectivity in cultured cells (Cai *et al.*, 1988,

Forrester *et al.*, 1992, Fuller *et al.*, 1989, Hutchinson *et al.*, 1992, Ligas & Johnson, 1988, Roop *et al.*, 1993).

1.4. Herpes Simplex Virus Lytic Cycle.

Generally, lytic viral infections proceed through multiple steps including attachment of viral particles and entry into host cells, delivery of viral genome to a specific cellular compartment, expression of viral genes and viral genome replication, assembly and egress of progeny particles. This section of the introduction is intended to provide a general overview of these processes as they pertain to HSV-1 infection.

1.4.1. Entry into Host Cells.

Entry of HSV into host cells occurs in two distinct stages, an initial attachment of viral particles to cell surface followed by HSV-specific cellular receptor binding and penetration (reviewed by Spear, 1993a)). Initial attachment of viral particles to cell surface is mediated primarily by interactions between the viral membrane glycoprotein gC and heparan sulfate moieties of cell surface proteoglycans. That heparan sulfate is the cell surface molecule responsible for HSV attachment is illustrated by the observations that loss of or alterations in cell surface heparan sulfate and addition of exogenous heparin (structurally similar to heparan sulfate) result in significant reduction in viral attachment (Gruenheid *et al.*, 1993, Shieh *et al.*, 1992, WuDunn & Spear, 1989). Furthermore, WuDunn and Spear (1989) have demonstrated that intact HSV virions can bind to immobilized heparan, strongly suggesting that the only cell surface molecule

required for initial attachment is heparan sulfate. Three lines of evidence indicate that of all the viral membrane proteins, gC appears to be the principle component involved in initial attachment. First, HSV gC deletion mutants are significantly impaired in the initial attachment step (Herold *et al.*, 1991). Second, gC isolated from infected cells and soluble gC bind heparin affinity columns, and cell surface heparan sulphate (Herold *et al.*, 1991, Svennerholm *et al.*, 1991, Tal-Singer *et al.*, 1995). Third, neutralizing antibodies directed against gC can significantly reduce viral attachment (Fuller & Spear, 1985, Svennerholm *et al.*, 1991). Although the ability of HSV virions lacking gC to attach to cells is largely impaired, attachment still occurred at low efficiency suggested that other viral glycoproteins may bind heparan sulfate in the absence of gC. To this end, Herold and colleagues have demonstrated that in the absence of gC, gB binds heparan sulfate to mediate viral attachment to cell surface with low efficiency (Herold *et al.*, 1994, Herold *et al.*, 1991). Furthermore, the recent demonstration that, in addition to binding to heparan sulfate, gC and gB display low efficiency binding to cell surface dextran sulfate and chondroitin sulfate, may be sufficient to explain the previous observation that cells lacking heparan sulfate are not completely refractory to HSV infection (Banfield *et al.*, 1995, Dyer *et al.*, 1997, Williams & Straus, 1997).

The initial gC (and/or gB) interactions with components of cell surface proteoglycans represent low affinity interaction that presumably brings other viral glycoproteins in close proximity to high affinity cell surface receptors required to mediate viral entry. HSV virions with individual deletions of the gB, gD, gH and gL genes can bind cell surface but are unable to enter into cells suggesting that these proteins are

important for receptor binding and/or penetration into cell. Of these, gD has been shown to be the principle glycoprotein responsible for HSV-specific receptor binding. Several lines of evidence support the involvement of gD in the receptor recognition process. First, HSV virions lacking gD can bind to cell surface but are unable to penetrate (Johnson & Ligas, 1988, Ligas & Johnson, 1988). Second, gD expressing cells become resistant to HSV infection leading to the suggesting that endogenous gD might bind to and saturate cell surface receptors and, as such, interfere with virus binding (Campadelli-Fiume *et al.*, 1988, Johnson & Spear, 1989). Third, the mutations in HSV mutants selected for their ability to infect cells expressing endogenous gD have been mapped to the viral gD gene (Dean *et al.*, 1994). Fourth, inactivated wild type, but not gD deleted HSV virions can inhibit entry of homologous virus (Johnson & Ligas, 1988). Fifth, soluble gD inhibits HSV entry into cells (Johnson *et al.*, 1990, Peng *et al.*, 1998).

Recently, three cell surface molecules that mediate HSV entry, to varying extents, have been identified. These molecules, termed herpesvirus entry mediators (Hve) A, B, and C, were identified by expression cloning of a HeLa cell cDNA library in the HSV resistant Chinese hamster ovary (CHO) cell line. HveA was identified as a member of the TNF/NGF receptor superfamily. Although expression of HveA (also known as HVEM) in CHO and swine testis (ST, also resistant to HSV infection; (Subramanian *et al.*, 1994)) cell lines has been shown to enhance entry of some strains of HSV-1 and HSV-2 into these cells, entry of other strains was not enhanced (Montgomery *et al.*, 1996). This and the finding that HveA can only be detected on the cell surface of activated T-cells indicated that HveA can not be the only HSV receptor inasmuch as HSV

infects a wide range of cells in culture (Montgomery *et al.*, 1996). HveB (also known as poliovirus receptor-related protein 2, Prr2) was isolated based on its ability to render CHO cells susceptible to infection with HSV-1 (KOS)Rid1. This viable gD mutant virus is able to infect cells expressing endogenous gD and its entry into CHO cells was not enhanced by HveA (Warner *et al.*, 1998). HveB, a member of the immunoglobulin superfamily, has been shown to enhance entry of HSV-2, the Rid mutants of HSV-1, but not wild-type HSV-1 (Warner *et al.*, 1998). Again the strain restriction displayed by HveB raised questions about it being the only HSV receptor. HveC, the poliovirus receptor-related protein 1 (Prr1), is also a member of the immunoglobulin superfamily. Expression of HveC cDNA in CHO cells renders these cells susceptible to infection with all HSV strains tested, including wild-type and Rid mutants HSV-1 and HSV-2 strains (Geraghty *et al.*, 1998). Together with the finding that HveC is expressed in a variety of cell lines of epithelial and neuronal origin (HSV infects epithelial cells and establishes latency in neurons), the data suggest that HveC may be the predominant HSV entry mediator (Geraghty *et al.*, 1998). More recently, Cocchi and colleagues have identified another HSV receptor, herpesvirus Ig-like receptor (HIgR), which is identical to HveC in the ectodomain but has a different transmembrane and cytoplasmic region. This molecule, expressed in a wide range of human cells including those of epithelial and neuronal origin, has also been shown to mediate HSV-1 and HSV-2 entry into cells that are normally resistant to HSV entry (Cocchi *et al.*, 1998b). These data clearly indicate that the virus has acquired the ability to use multiple receptors to gain entry into cell, a conclusion consistent with the wide host range and tissue tropism of alphaherpesvirus members. The identification of these receptors has also lent further support to the critical

role that gD plays in viral entry, inasmuch as gD has been shown to directly interact with HveA, HveC, and HIgR (Cocchi *et al.*, 1998a, Cocchi *et al.*, 1998b, Krummenacher *et al.*, 1998, Sarrias *et al.*, 1999, Whitbeck *et al.*, 1997, Willis *et al.*, 1998)).

Binding of gD to cell surface receptor(s) is believed to bring the viral and cellular membranes into close proximity, allowing membrane fusion and capsid penetration to occur. HSV penetration occurs through a pH-independent mechanism suggesting that the primary mechanism of entry is through direct fusion of viral and cellular membranes (Koyama & Uchida, 1987, Rosenthal *et al.*, 1989, Wittels & Spear, 1991). The detection of viral membrane glycoproteins (not intact virions) on the surface of infected cells at early times of infection in the absence of *de novo* viral protein synthesis, lends strong support to virion-cell membrane fusion mode of entry (Para *et al.*, 1980). Although endocytic vesicles containing viral particles can be detected early in infection, Kayoma and colleagues (1987) suggested that this pathway does not lead to virion uncoating. Moreover, Campadelli-Fiume and coworkers have demonstrated that virions present in endocytic vesicles in cells expressing gD are degraded and thus do not lead to productive infection (Campadelli-Fiume *et al.*, 1988). Of the viral glycoproteins, gB, gD, and the gH/gL complex are known to be required for virion-cell membrane fusion (Spear, 1993a).

Following fusion with the cellular membrane, the tegument disassembles and the nucleocapsid is transported to nuclear pores leading to the release of viral DNA into the nucleus. Sodeik and co-workers have, recently, provided striking electron micrographs capturing virions undergoing fusion with cellular membranes, tegument disassembly immediately following entry, transport of nucleocapsids to the nucleus, and empty

capsids at the nuclear envelope (Sodeik *et al.*, 1997). The process of tegument disassembly is not well understood. Morrison and colleagues have recently provided evidence strongly suggesting that phosphorylation of the tegument proteins including VP13/14, VP16 and VP22 *in vitro*, leads to dissociation of these proteins from the tegument (Morrison *et al.*, 1998b). Moreover, this dissociation was severely impaired in virions with inactivated UL13 protein *in vitro* suggesting an involvement of the protein kinase in tegument disassembly (Morrison *et al.*, 1998b). The tegument VP1/2 protein is tightly associated with the nucleocapsid (McNabb & Courtney, 1992b, Zhou *et al.*, 1999) and as such, remains associated with the capsid until it reaches the nuclear envelope (Morrison *et al.*, 1998a, Morrison *et al.*, 1998b, Sodeik *et al.*, 1997, Zhou *et al.*, 1999). Once the tegument has dissociated, the naked nucleocapsid is transported to the nucleus via the microtubule network (Morgan *et al.*, 1968, Sodeik *et al.*, 1997). Furthermore, Sodeik and colleagues have demonstrated that the capsid is attached to microtubules through the dynein motor and suggested that dynein moves the capsid from the cell periphery to the nuclear envelope (Sodeik *et al.*, 1997). The observations that VP22 is released from the tegument immediately following internalization of virions (Morrison *et al.*, 1998b) and that VP22 reorganizes and stabilizes microtubules (Elliott & O'Hare, 1998) raise the possibility that VP22 plays a role in the transport of the nucleocapsid to the nucleus.

Once at the nuclear pore, the viral DNA is released from the nucleocapsid and delivered into the nucleus where transcription, replication, and assembly take place. Release of viral DNA from the nucleocapsid appears to be mediated by the tegument

VP1/2 protein. This is evident by the observation that infection with the HSV-1(HFEM)*ts*B7 temperature sensitive mutant leads to accumulation of DNA containing nucleocapsids at the nuclear pores at non-permissive temperature and viral DNA can only be released into the nucleus following incubation at the permissive temperature (Batterson *et al.*, 1983). The *ts* lesion in this mutant was subsequently mapped to the UL36 orf encoding VP1/2 implicating this protein in this process (Batterson *et al.*, 1983, McNabb & Courtney, 1992a, McNabb & Courtney, 1992b). This is consistent with the observations that VP1/2 remains associated with the capsid until it reaches the nuclear envelope where it would be required to release the DNA into the nucleus (Morrison *et al.*, 1998a, Morrison *et al.*, 1998b, Zhou *et al.*, 1999).

1.4.2. Initiation of Viral Gene Expression.

In the nucleus, the viral DNA circularizes through interaction of terminal repeat sequences (Challberg & Kelly, 1989). The DNA localizes to a finite number of subnuclear locations associated with the nuclear matrix (de Bruyn Kops & Knipe, 1988, de Bruyn Kops & Knipe, 1994). These "replication compartments" have been shown to correspond to the periphery of nuclear domain (ND) 10, which are nuclear nodes of unknown function (Lukonis & Weller, 1997, Maul *et al.*, 1996). Both viral transcription and viral DNA replication occur at the same replication compartments at early times after infection, but at later times the number of replication compartments dedicated exclusively to replication increases (Phelan *et al.*, 1997). Generally, viral gene expression proceeds through a highly regulated cascade involving the sequential expression of the immediate early (IE), early (E), and late (L) kinetic classes of viral genes (Honess & Roizman,

1974). The IE gene products are primarily regulatory proteins required for induction and maintenance of expression of both E and L genes. The products of the E genes are proteins involved in DNA replication and L gene products are mostly structural proteins and proteins involved in virion assembly and egress.

Although transfection of naked viral DNA into cells results in infectious progeny virions, it is drastically inefficient when compared to the yield of infection of cells with intact virions (Gao *et al.*, 1994, Graham *et al.*, 1973, Sheldrick *et al.*, 1973). This observation underscores the important role that virion associated proteins play during infection. Two virion proteins that act immediately after entry are the VP16 transactivator which is responsible for stimulating IE gene expression, and the protein responsible for shutoff of host cell protein synthesis, vhs. The function of these proteins is discussed in more detail below (VP16) and in a subsequent section (vhs).

HSV genes are transcribed by the host cell RNA polymerase II (Beck & Millette, 1982, Preston & Newton, 1976, Rice *et al.*, 1994) and the lytic cycle begins with the expression of the IE genes in the absence of viral DNA replication or *de novo* viral protein synthesis (Batterson & Roizman, 1983, Honess & Roizman, 1974). In addition to their expression immediately following delivery of viral DNA into the nucleus, IE genes are further characterized by having one or more copies of the *cis*-acting element, 5'-TAATGARATTC-3' (where R is a purine residue), in their promoter (Mackem & Roizman, 1982a, Mackem & Roizman, 1982b). VP16 released from the tegument in the cytoplasm migrates to the nucleus to stimulate the expression of the IE genes in the absence of any other viral proteins (Batterson & Roizman, 1983, Campbell *et al.*, 1984,

Pellett *et al.*, 1985, Post *et al.*, 1981). Although the VP16 induced transactivation of the IE gene is strictly dependent on the presence of the TAATGARATTC sequence (Kristie *et al.*, 1989, Preston *et al.*, 1984), VP16 alone interacts with this element weakly (Kristie *et al.*, 1989, Kristie & Sharp, 1990). However, recognition of this element is dramatically enhanced upon formation of VP16-cellular protein complex (Kristie & Roizman, 1987, McKnight *et al.*, 1987, O'Hare & Goding, 1988, O'Hare *et al.*, 1988, Preston *et al.*, 1988). VP16 has been shown to directly bind the cellular proteins Oct-1 (a transcription factor) and HCF (poorly understood protein) through its N-terminal ca. 400 amino acid domain (Freiman & Herr, 1997, Gerster & Roeder, 1988, Goto *et al.*, 1997, Greaves & O'Hare, 1989, Haigh *et al.*, 1990, Johnson *et al.*, 1999, Katan *et al.*, 1990, Kristie *et al.*, 1989, Kristie & Roizman, 1988, Kristie & Sharp, 1990, La Boissiere *et al.*, 1999, Liu *et al.*, 1999, Stern *et al.*, 1989, Wilson *et al.*, 1997, Xiao & Capone, 1990). The importance of this complex in VP16-induced transcriptional activation is illustrated by the observation that mutations in VP16 that abolish Oct-1 binding lead to significant reduction in transcriptional activation (Ace *et al.*, 1988, Ace *et al.*, 1989, Spector *et al.*, 1990, Spector *et al.*, 1991). Interestingly, La Boissiere and colleagues have recently demonstrated that co-expression of HCF and VP16 resulted in the nuclear accumulation of VP16 (which lacks nuclear localization signal) suggesting that HCF is the nuclear importer of VP16 (La Boissiere *et al.*, 1999).

VP16-transcription factor complexes mediate activation of IE genes by placing VP16 at the promoter where it can influence transcriptional events. Transcriptional activation is mediated by the acidic C-terminal ca. 80 amino acid domain (CTD) of VP16

which has been shown to be sufficient to activate transcription in yeast when linked to a heterologous DNA binding domain (Sadowski *et al.*, 1988, Triezenberg *et al.*, 1988a, Triezenberg *et al.*, 1988b). Indeed, mutations in the CTD of VP16 drastically reduce its ability to induce IE gene expression at low MOI (Smiley & Duncan, 1997, Tal-Singer *et al.*, 1999). That lytic cycle occurs in cells infected with VP16 CTD mutants (Smiley & Duncan, 1997) or mutants that fail to interact with Oct-1/HCF (Ace *et al.*, 1988, Ace *et al.*, 1989), albeit at a reduced rate, indicates that VP16 transactivation of IE genes is not essential for virus replication. However, VP16 null mutants are not viable and Weinheimer and colleagues showed that the defect in VP16 null mutants is at the assembly stage (Weinheimer *et al.*, 1992). Therefore, in addition to the role that VP16 plays in stimulating IE gene expression, the protein plays an essential role in the assembly/maturation of progeny virions (Ace *et al.*, 1988, Ace *et al.*, 1989, Weinheimer *et al.*, 1992).

The mechanism by which VP16 induces transcriptional activation is not clearly understood. The CTD of VP16 has been show to interact directly with TFIIB, the TATA-binding protein subunit of TFIID, and the TFIIH components of the RNA polymerase II holoenzyme (Goodrich *et al.*, 1993, Gupta *et al.*, 1996, Ingles *et al.*, 1991, Lin *et al.*, 1991, Stringer *et al.*, 1990, Xiao *et al.*, 1994). It is generally accepted that interaction of components of the polymerase with transcriptional activators results in stabilization of the interaction of the polymerase holoenzyme with DNA and stimulation of open complex conformation of the DNA at the promoter leading to enhanced transcription initiation.

1.4.3. Immediate Early Genes.

The net effect of the VP16 activity at early times during infection is the efficient expression of five IE proteins: infected cell protein 0 (ICP0), ICP4, ICP22, ICP27, and ICP47. Of these, ICP4 and ICP27 are essential during lytic HSV cycle (DeLuca *et al.*, 1985, DeLuca & Schaffer, 1985, Everett, 1986, McCarthy *et al.*, 1989, Preston, 1979, Rice *et al.*, 1989, Sacks *et al.*, 1985, Watson & Clements, 1980). Although non-essential, ICP0 appears to stimulate the expression of genes belonging to all three kinetic classes and deletion of ICP0 results in HSV mutants that are impaired in virus production (Everett, 1984, Harris *et al.*, 1989, Leib *et al.*, 1989, Sacks & Schaffer, 1987, Stow & Stow, 1986). ICP22 is dispensable but contributes to efficient expression of late HSV genes in a cell dependent manner (Post & Roizman, 1981, Sears *et al.*, 1985). The remaining IE protein, ICP47, is the only IE gene product that is not involved in regulation of gene expression. ICP47 has been shown to associate with the transporter associated with antigen presentation (TAP) and inhibit antigenic peptide transfer to the MHC class I molecule leading to their retention in the endoplasmic reticulum (Ahn *et al.*, 1996, Fruh *et al.*, 1995, Hill *et al.*, 1995, Tomazin *et al.*, 1996, Tomazin *et al.*, 1998, York *et al.*, 1994). As such, ICP47 prevents presentation of HSV antigenic peptides on the surface of infected cells thus protecting these cells from cytotoxic T-cell response.

ICP4 is a major HSV regulatory phosphoprotein that is required during all stages of HSV infection (Watson & Clements, 1980). Transient transfection assays revealed that ICP4 has the capacity to transactivate gene expression from a variety of HSV and cellular promoter (Cheung *et al.*, 1997, Everett, 1984, Everett, 1985, O'Hare & Hayward,

1985a, O'Hare & Hayward, 1985b, Paterson & Everett, 1988a, Shepard & DeLuca, 1989, Xiao *et al.*, 1997). Within the context of viral infection, ICP4 deletion or temperature sensitive mutants are non-viable due to defects in the expression of E and L genes (DeLuca *et al.*, 1985, DeLuca & Schaffer, 1985, Dixon & Schaffer, 1980, Preston, 1979, Watson & Clements, 1978). In addition to its essential role in transactivation of E and L gene expression, ICP4 acts to negatively regulate IE gene expression as evident by the observation that ICP4 null mutants over express IE genes (DeLuca *et al.*, 1985, Dixon & Schaffer, 1980, Michael & Roizman, 1989, Roberts *et al.*, 1988). ICP4 transactivation and repression functions depend on its ability to interact with DNA since mutations in the ICP4 DNA binding domain result in loss of transactivation function (Paterson & Everett, 1988b, Shepard & DeLuca, 1989, Shepard & DeLuca, 1991). Although an ICP4 consensus binding site has been identified in some promoters (Faber & Wilcox, 1986, Michael *et al.*, 1988), a large number of ICP4 responsive cellular and viral genes do not contain this sequence indicating that ICP4 is capable of binding DNA at sequences other than consensus (Gu & DeLuca, 1994, Imbalzano *et al.*, 1990, Smiley & Duncan, 1992, Smiley *et al.*, 1992).

The mechanisms by which ICP4 mediates transactivation and repression are not well understood. The ICP4 promoter contains a consensus ICP4 binding site that overlaps with the transcription start site (Faber & Wilcox, 1988, Kristie & Roizman, 1986, Muller, 1987). Mutational analysis revealed that ICP4 mediated repression of its own gene is dependent on the position and orientation of this site, leading to the hypothesis that ICP4 binding at this site might prevent the transcription machinery from

binding the promoter and thus repressing transcription initiation (Gu *et al.*, 1995, Leopardi *et al.*, 1995, Michael & Roizman, 1989, O'Hare & Hayward, 1987, Paterson & Everett, 1988b, Roberts *et al.*, 1988). ICP4 has been shown to interact with a number of cellular factors involved in transcription initiation including TATA-binding protein (TBP), TFIID, TFIIB, and TAF250 (Carrozza & DeLuca, 1996, Gu & DeLuca, 1994, Gu *et al.*, 1995, Paterson & Everett, 1988b, Smith *et al.*, 1993). These proteins may be involved in the ICP4 mediated regulation of gene expression. To this end, the formation of an ICP4-TBP-TFIIB complex has shown to be involved in repression (Kuddus *et al.*, 1995) whereas the formation of ICP4-TFIID, through interaction of ICP4 and TAF250, is involved in the transactivation (Carrozza & DeLuca, 1996). The possible involvement of other cellular proteins, including HMG-1 and NFkB, in HSV gene expression has been suggested but their roles have yet to be clearly demonstrated (Panagiotidis & Silverstein, 1999, Patel *et al.*, 1998).

The other essential immediate early gene product, ICP27, does not function directly as an activator or repressor of gene expression at the transcriptional level. Instead, it has been shown to act post-transcriptionally by altering the cellular mRNA processing machinery (Sandri-Goldin, 1994, Sandri-Goldin & Mendoza, 1992, Smith *et al.*, 1992) resulting in the accumulation of unspliced transcripts in the nucleus of infected cells (Hardy & Sandri-Goldin, 1994). This is significant in light of the fact that the great majority of HSV transcripts do not contain introns and therefore are not subject to the block at the processing step (reviewed by Sandri-Goldin, 1998b). ICP27 has recently been shown to shuttle between the nucleus and cytoplasm of infected cells (Phelan &

Clements, 1997, Sandri-Goldin, 1998a, Soliman *et al.*, 1997). It is through this function and its ability to directly bind RNA (Ingram *et al.*, 1996, Sandri-Goldin, 1998a) that ICP27 has been implicated in the efficient transport of intronless HSV mRNAs from the nucleus to the cytoplasm. In addition, ICP27 has been shown to enhance expression of genes with weak 3' polyadenylation signals by stabilizing 3' ends and increasing the efficiency of 3' polyadenylation of their mRNAs (Brown *et al.*, 1995, McGregor *et al.*, 1996, Sandri-Goldin & Mendoza, 1992). This is significant in that late HSV mRNAs are known to contain weak 3' processing signals (McGregor *et al.*, 1996). Therefore, ICP27 increases the efficiency of viral gene expression by reducing the amount of competing cellular mRNAs (Hardwicke & Sandri-Goldin, 1994) and increasing the level of polyadenylated viral mRNA in the cytoplasm of infected cells (Sandri-Goldin, 1998a, Soliman *et al.*, 1997).

ICP0 is a promiscuous transactivator that has been shown to augment transcription from all viral and cellular promoters tested in transfection assays (Cai & Schaffer, 1992, Chen & Silverstein, 1992, Everett, 1984, O'Hare & Hayward, 1985a, Quinlan & Knipe, 1985). HSV mutants lacking functional ICP0 fail to enter lytic cycle at low multiplicity of infection (Cai & Schaffer, 1991, Everett, 1989, Stow & Stow, 1986). The genomes of these mutants remain in a quiescent state in the nuclei of infected cells and can be reactivated upon superinfection with another virus expressing ICP0, stressing the importance of ICP0 in initiating HSV lytic infection (Harris *et al.*, 1989, Harris & Preston, 1991, Samaniego *et al.*, 1998). Although the mechanism by which ICP0

augments gene expression in not known, Jordan and Schaffer have demonstrated that it functions at the transcriptional level (Jordan & Schaffer, 1997).

HSV infection of permissive cells leads to alterations in the nuclear architecture. Most prominent is the disruption of ND10 nuclear domains shortly after infection (Everett & Maul, 1994, Maul & Everett, 1994, Maul *et al.*, 1993). ND10 nuclear domains are structures of unknown function that exist 5 to 30 times in most cell types (reviewed by Maul, 1998). The HSV ICP0 protein has been shown to localize to ND10 domains and induce their disruption at early times post infection (Everett & Maul, 1994, Maul & Everett, 1994, Maul *et al.*, 1993). Indeed, mutations that affect the biological function of ICP0 (transactivation) also affect its ability to interact with and disrupt ND10 domains (Ishov & Maul, 1996, Lukonis & Weller, 1997, Maul *et al.*, 1996, Uprichard & Knipe, 1997). As mentioned above, incoming viral genomes are translocated to sites at the periphery of ND10 domains which then develop into viral transcription and replication compartments (Lukonis & Weller, 1997, Maul *et al.*, 1996, Uprichard & Knipe, 1997). At these sites, HSV genomes are silenced or repressed in the absence of functional ICP0 leading to the suggestion that ICP0 activates viral gene expression by abrogating cell-mediated repression of input HSV genomes (Everett *et al.*, 1998b). Interestingly, ND10 domains increase in number and size following treatment of cells with interferon leading to the suggestion that ICP0 may function by circumventing a cellular, interferon-induced antiviral response pathway (Guldner *et al.*, 1992, Maul *et al.*, 1995).

ICP0 interacts with the herpesvirus-associated ubiquitin-specific protease (HAUSP) located at ND10 domains and mutations that affect ICP0's ability to bind HAUSP also affect its ability to stimulate lytic cycle (Everett *et al.*, 1997, Meredith *et al.*, 1995, Meredith *et al.*, 1994). Ubiquitin-specific protease's normally function by removing ubiquitin adducts from proteins to protect them from proteasome mediated degradation. It is possible that by interacting with HAUSP, ICP0 may eliminate its protective ability leading to changes in protein stability in infected cells. To this end, ICP0 has been shown to induce the degradation of high molecular weight isoforms of PML and SP100 (major constituents of ND10 domains) in a ubiquitin-specific proteasome-dependent process that is directly linked to the disruption of ND10 domains (Everett *et al.*, 1998a, Everett *et al.*, 1998b). Interestingly both of these proteins are modified by covalent linkage to the Small Ubiquitin-like Modifier peptide, SUMO-1 (Chelbi-Alix & de The, 1999, Muller *et al.*, 1998, Sternsdorf *et al.*, 1997). Moreover, Mullar and Dejean have recently demonstrated that ICP0 specifically abrogates SUMO-1 modification of PML and SP100 and that this activity is directly related to its ability to disrupt ND10 domains (Muller & Dejean, 1999). Together, these findings indicate that the ability of ICP0 to induce viral genomes into the productive cycle is directly linked to its ability to alter protein stability and nuclear architecture. These alterations may enhance HSV replication by inducing the assembly of a nuclear environment in which the viral gene transcription can occur or, as mentioned above, bypassing a cellular antiviral response mechanism.

The HSV ICP22 protein is the least understood of the five immediate early gene products. It is a predominantly nuclear protein that contains extensive post-translational modifications including phosphorylation by viral UL13 and US3 protein kinases and nucleotidylylation by casein kinase II (Blaho *et al.*, 1993, Leopardi *et al.*, 1997, Mitchell *et al.*, 1997, Mitchell *et al.*, 1994, Post & Roizman, 1981, Purves *et al.*, 1993, Purves & Roizman, 1992). In transient transfection assays, ICP22 appears to downregulate the expression of a reporter gene under the control of HSV immediate early promoters independent of UL13 function (Prod'hon *et al.*, 1996). The requirement for ICP22 during HSV productive cycle appears to cell type-dependent. ICP22 is dispensable for viral replication in Vero and HEp-2 cell and ICP22 mutants replicate as efficiently as wild type virus in these cell lines. In contrast, ICP22 mutants grow poorly in RAT-1, BHK, and resting HEL cell lines (Poffenberger *et al.*, 1993, Post & Roizman, 1981, Purves *et al.*, 1993, Sears *et al.*, 1985). Infection of restrictive cell lines with ICP22 mutants leads to reduction in the level of ICP0 immediate early and a subset of late gene products (Purves *et al.*, 1993). This reduction correlates with reduced mRNA levels indicating that the effect occurs at the transcriptional level (Purves *et al.*, 1993, Rice *et al.*, 1995). Rice and co-workers have demonstrated that HSV infection leads to rapid alteration in the phosphorylation status of RNA polymerase II (the enzyme used for HSV transcription) (Rice *et al.*, 1994). This HSV-induced depletion of hypo- (IIa) and hyperphosphorylated (IIo) forms and accumulation of novel intermediately phosphorylated (IIi) form of the enzyme, is dependent on the presence of functional ICP22 and UL13 protein kinase proteins (Long *et al.*, 1999, Rice *et al.*, 1995). Together with the observation that IIi is the most transcriptionally active form of the enzyme in infected cells, these data provide

direct evidence that ICP22 is involved in the regulation of gene expression at the transcriptional level (Long *et al.*, 1999, Rice *et al.*, 1995, Spencer *et al.*, 1997). Therefore, the modification of RNA polymerase II by ICP22 and UL13 may favor transcription of viral mRNA in restrictive cells. Long and colleagues have hypothesized that in permissive cells, existing hyperphosphorylated forms of the polymerase may functionally substitute for IIi and therefore, eliminate the requirement for functional ICP22 (Long *et al.*, 1999).

In addition to their individual effects, ICP0, ICP4, and ICP27 have been shown to cooperatively regulate viral gene expression at the transcriptional and post-transcriptional levels. It is now well established that ICP0 and ICP4 act synergistically to activate gene expression (Everett, 1984, Everett, 1988, Gelman & Silverstein, 1985, O'Hare & Hayward, 1985a). Consistent with this synergism, Yao and Schaffer have shown that ICP0 and ICP4 are capable of physically interacting with one another (Yao & Schaffer, 1994). ICP4 transactivation activity has also been shown to be modulated by its physical interaction with ICP27 (O'Hare & Hayward, 1985b, Panagiotidis *et al.*, 1997, Samaniego *et al.*, 1995, Sekulovich *et al.*, 1988, Su & Knipe, 1989). These functional and physical interactions result in sub-cellular redistribution of the immediate early proteins in transfected and infected cells (Gelman & Silverstein, 1986, Knipe & Smith, 1986, Mullen *et al.*, 1995, Zhu *et al.*, 1994, Zhu *et al.*, 1996, Zhu & Schaffer, 1995). Thus, the activities of immediate early gene products appear to be regulated by a complex process involving protein-protein interactions and sub-cellular localization leading to induction of early and late HSV gene expression and the production of progeny virions.

1.4.4. Early Genes and Viral DNA Replication.

Synthesis of IE proteins leads to the induction of HSV early (E) gene expression, the products of which are involved in viral DNA synthesis. Seven of the HSV E gene products are necessary and sufficient for DNA replication. These proteins were identified by their ability to support replication of an HSV origin of replication (ori) containing plasmid DNA in transient transfection assays or when co-expressed in insect cells by recombinant baculoviruses (Challberg, 1986, Stow, 1992). Moreover, mutations in any of these proteins have been shown to result in defective virus blocked at the DNA replication step of the lytic cycle. These proteins are: DNA polymerase (UL30), DNA polymerase processivity factor (UL42), the ICP8 ssDNA binding protein (UL29), DNA helicase-primase or primasome complex (UL5/UL8/UL52), and the origin binding protein (UL9) (reviewed by Boehmer & Lehman, 1997). In addition, HSV encodes at least six other E genes who's products are not essential for DNA replication. These are: uracil N-glycosylase (UL2), alkaline endo-exonuclease (UL12), deoxyuridine triphosphatase (UL50), thymidine kinase (UL23), and ribonucleotide reductase (UL39/UL40). These accessory proteins carry out functions that enhance viral DNA replication including, DNA repair, processing of replication intermediates, and nucleotide metabolism in infected cells. In addition to the virally encoded enzymes, HSV replication also utilizes some of the components of the cellular replication machinery such as DNA ligase and topoisomerase II (Dabrowski *et al.*, 1994, and reviewed by Boehmer & Lehman, 1997).

The accumulation of sufficient quantities of HSV E gene products leads to the induction of viral DNA replication in the nucleus of infected cells. The HSV genome contains three origins of replication, oriL located between UL29 and UL30, and diploid oriS located in the repeated sequences at the ends of the short arm (Stow, 1982, Weller *et al.*, 1985). Interaction of the replication machinery with any of these ori sites results in initiation of bi-directional DNA replication. As mentioned in a previous section, viral DNA adopts a circular form upon entry into the nucleus of infected cells and endless forms of newly synthesized viral genomes prevail until very late stages of the replicative cycle (Becker *et al.*, 1978a, Becker *et al.*, 1978b, Challberg & Kelly, 1989, Davison & Wilkie, 1983, Jacob *et al.*, 1979). These observations, together with the demonstration that replication of a plasmid containing an HSV ori site in cells coinfected with HSV produced large head-to-tail plasmid concatemers, lead to the suggestion that replication proceeds by a rolling-circle mechanism (Stow, 1982). Indeed, digestion of large viral DNA replication intermediates with restriction enzymes that cleave once per genome yields genome unit-length products demonstrating the concatemeric arrangement of replication intermediates (Severini *et al.*, 1994, Zhang *et al.*, 1994). Electron microscopic analysis revealed that HSV replication intermediates are much more complex structures containing highly branched DNA molecules (Friedmann *et al.*, 1977, Shlomai *et al.*, 1976) that can not result from rolling-circle replication alone. That replication intermediates are highly branched has been confirmed using pulse-field and two-dimensional gel electrophoresis analysis (Severini *et al.*, 1994, Severini *et al.*, 1996). These data have led to the suggestion the branched DNA molecules arise as a result of replication by rolling-circle mechanism and recombination events occurring at the same

time. Restriction enzyme analysis of replication intermediates revealed that individual genomes in the network of replication intermediates exist in all possible UL/US orientation (Slobedman *et al.*, 1999, Zhang *et al.*, 1994). Since inversion of the UL and US arms of the genome relative to each other occurs via homologous recombination between the UL and US flanking repeat regions (Hayward *et al.*, 1975, Smiley *et al.*, 1990, Smiley *et al.*, 1981), these data are consistent with the involvement of recombination in the replication process. Although the replication mechanism has yet to be clearly defined, replication leads to the accumulation of complex DNA intermediates that are then resolved into unit length genomes and packaged into pre-formed capsid in the nucleus of infected cells.

1.4.5. Late Genes, Virion Assembly, and Egress.

HSV late (L) genes encode structural proteins and proteins involved in the assembly and egress of progeny virions. Late genes can be subdivided into two classes, early late (EL) and delayed late (DL), depending on their requirement for DNA replication. While the expression of DL genes is strictly dependent of the onset of viral DNA replication, EL genes are expressed prior to DNA replication but their expression is dramatically enhanced by the onset of viral DNA replication.

Accumulation of L gene products leads to the assembly of HSV procapsids in distinct nuclear compartments referred to as assemblons (Desai & Person, 1998, Ward *et al.*, 1996). As stated in a previous section, the HSV-1 capsid, approximately 125 nm in diameter, is composed of 162 capsomeres: 150 hexons and 12 pentons. The pentons are

located at the vertices while the hexons are located at the edges and faces of the capsid shell (Roizman & Sears, 1996). VP5 (major capsid component; product of the UL19 gene) is the structural subunit of both hexon (composed of 6 VP5 molecules) and penton (composed of 5 VP5 molecules) capsomeres (Newcomb *et al.*, 1993, Schrag *et al.*, 1989). In addition, mature HSV capsids also contain three minor proteins: VP19C (UL38), VP23 (UL18), and VP26 (UL35). The VP26 protein binds to the outer tip of each of the hexon-associated VP5 protein subunit to stabilize the capsid structure. One VP19C and two VP23 proteins assemble into hetrotrimeric structures (the triplexes) that form the intercapsomeric fibers connecting the capsomeres together and thus stabilizing the capsid (Newcomb *et al.*, 1996, Newcomb *et al.*, 1999, Newcomb *et al.*, 1994, Newcomb *et al.*, 1993, Trus *et al.*, 1996, Trus *et al.*, 1995, Zhou *et al.*, 1995, Zhou *et al.*, 1994).

Analysis of procapsid assembly in insect cells infected with baculoviruses expressing the various structural proteins and *in vitro* using extracts from these cells has revealed that the process requires VP5, VP23, VP19C, VP26, VP21 and VP24 (UL26), and VP22a (UL26.5) proteins (Newcomb *et al.*, 1996, Newcomb *et al.*, 1994, Thomsen *et al.*, 1995, Trus *et al.*, 1995). The requirement for these minimal assembly proteins has, more recently, been confirmed in an *in vitro* assembly system using pure proteins (Newcomb *et al.*, 1999). The product of the UL26 gene is a protease that cleaves itself to generate VP21 and VP24. This protease also cleaves the product of UL26.5 (pre-VP22a) to yield VP22a. Pre-VP22a, VP21, and VP24 form a protein scaffold around which the shell of the procapsid self-assembles. This shell, initially composed of VP5 (hexons and pentons) bound to the C-termini of the scaffold proteins (Newcomb *et al.*, 1999, Thomsen

et al., 1995, Zhou *et al.*, 1998) is unstable and becomes stable once VP19C and VP23 (triplexes) along with VP26 are recruited and assembled into the structure (Hong *et al.*, 1996, Newcomb *et al.*, 1996, Thomsen *et al.*, 1995). The scaffold proteins VP21 and pre-VP22a are then cleaved by the maturational protease product of the UL26 gene near their C-termini and removed from the capsid (Addison *et al.*, 1990, Church & Wilson, 1997, Preston *et al.*, 1992, Rixon & McNab, 1999).

Following assembly and maturation of the capsid, newly synthesized highly branched viral DNA is resolved into unit-length genomes and packaged into the pre-formed empty capsids in a process that is coupled to the extrusion of the scaffolding proteins. DNA resolution and packaging require a number of viral proteins including the products of UL6, UL12, UL15, UL17, UL25, UL28, UL32, UL33 ((Martinez *et al.*, 1996, Taus *et al.*, 1998 and reviewed by Roizman & Sears, 1996)). With the exception of the alkaline nuclease product of UL12, the function of these proteins remains unknown. Alkaline nuclease possesses both an endo- and exonuclease activities and was initially proposed to function as the resolvase involved in cleaving complex replication intermediates into unit-length genomes (Shao *et al.*, 1993, Weller *et al.*, 1990). More recently, Goldstein and Weller showed that this protein does not function as a resolvase, rather they suggest that this protein may function at the nicks and gaps in the DNA repair and processing pathways (Goldstein & Weller, 1998). Cleavage of concatemeric replication intermediates occurs at *cis*-acting sequences (pac sites) within the *a* sequence of the viral DNA (Deiss *et al.*, 1986, Deiss & Frenkel, 1986, Stow *et al.*, 1983, Varmuza & Smiley, 1985). Viral DNA is spooled into pre-formed capsids until a second *a*

sequence that has the same polarity is encountered, then the DNA is cleaved again and a full length genome is enclosed in the capsid (reviewed by Roizman & Sears, 1996)).

The next stages in the virion maturation process are the acquisition of the tegument layer and envelope. As mentioned in a previous section, the tegument component of the virion is a highly organized structure capable of self-assembling independent of the capsid and virion maturation (McLauchlan & Rixon, 1992, Rixon *et al.*, 1992, Szilagyi & Cunningham, 1991). The site of tegument assembly and acquisition is controversial in the literature. Ward *et al.* have reported that the tegument proteins US11 and VP16 localize to sites near the inner nuclear membrane at late times after infection of Vero cells. These sites partially overlap with sites of capsid assembly leading to the conclusion that tegument acquisition occurs near the inner nuclear membrane (Ward *et al.*, 1996). This is consistent with the previous demonstration of thickened patches of inner nuclear membrane, presumably due the presence of tegument components and viral membrane glycoproteins, at the site of initial virion envelopment (reviewed by Roizman & Sears, 1996). In contrast, Elliott and O'Hare, more recently, demonstrated that the tegument proteins VP22 and VP16 colocalize to the cytoplasmic compartment in infected and transfected cells suggesting that tegument acquisition occurs at a stage downstream of capsid translocation through the nuclear membrane (Elliott & O'Hare, 1999, Elliott *et al.*, 1995).

As with the site of tegument acquisition, the site of virion envelopment is a highly contentious issue. It is well established that mature, DNA containing capsids are initially enveloped by budding through the inner lamellae of the nuclear membrane and enveloped

virions accumulate in the perinuclear space between the inner and outer nuclear membrane (Compton & Courtney, 1984b, Darlington & Moss, 1968, Schwartz & Roizman, 1969, Torrisi *et al.*, 1992). Subsequent events in HSV egress involving the translocation of virions from the perinuclear space to the extracellular environment are not well understood. In one model for HSV egress, enveloped virions pass through the endoplasmic reticulum/Golgi apparatus secretory pathway resulting in the release of mature virions into the cytoplasm in membrane bound vesicles which, in turn, fuse with the cellular membrane releasing infectious virions to the extracellular environment (Campadelli-Fiume *et al.*, 1991, Compton & Courtney, 1984a, Johnson & Spear, 1982, Torrisi *et al.*, 1992). An alternative model proposes that enveloped virions in the perinuclear space become de-enveloped by fusing with the outer nuclear membrane and re-enveloped at the rough endoplasmic reticulum and/or Golgi apparatus as they are transported to the extracellular space (Browne *et al.*, 1996, Cheung *et al.*, 1991, Elliott & O'Hare, 1999, Gershon *et al.*, 1994, Jones & Grose, 1988, Stackpole, 1969, van Genderen *et al.*, 1994). Regardless of the mechanism, maturation and egress of infectious HSV particles from infected cells requires a number of virally encoded proteins including UL20 and UL11 gene products, gD, gH and gK proteins (Baines & Roizman, 1992, Baines *et al.*, 1991, Browne *et al.*, 1996, Campadelli-Fiume *et al.*, 1991, Jayachandra *et al.*, 1997). The precise functions that these proteins play at the various stages of egress are not understood.

1.5. Virion Induced Host Shutoff.

In addition to the alteration to cellular structures and mRNA processing machinery mentioned above, HSV infection also leads to inhibition of host cell macromolecule synthesis. Radioactive nucleotide incorporation assays have revealed that DNA synthesis in HSV infected cells sharply declines over the first 5 hr of infection then increases from 5 hr to late times post-infection, a period corresponding to active viral DNA synthesis (Fenwick & Walker, 1978, Lehtinen *et al.*, 1989, Newton *et al.*, 1962, Roizman & Roane, 1964). Host RNA synthesis in HSV infected cells declines to ca. 50 % of that in uninfected cells at early times post-infection (Aurelian & Roizman, 1965, Fenwick & Walker, 1978, Preston & Newton, 1976, Roizman *et al.*, 1965).

Smiley and colleagues have constructed an HSV recombinant carrying an intact copy of the rabbit β-globin gene (including promoter) inserted at the tk locus of HSV-1 KOS strain. Infection of differentiated MEL cells with this recombinant virus led to repression of the cellular β-globin gene to greater than 90 % of uninfected controls and activation of the viral copy of β-globin with early kinetics (Smibert & Smiley, 1990, Smiley *et al.*, 1987). This led the authors to suggest that the differentiation between cellular and viral promoters is a sequence-independent process that may be mediated by RNA polymerase II modifications, alteration in chromatin structure, or compartmentalization of viral genome within the nucleus (reviewed by Smiley *et al.*, 1991). Recently, Spencer and co-workers have presented data correlating the HSV-induced depletion of the hyperphosphorylated form of RNA polymerase II (IIo) to repression of cellular mRNA synthesis (Spencer *et al.*, 1997). The net effect of inhibition

of cellular macromolecule synthesis is the diversion of the cellular synthetic machinery to the production of viral components.

HSV infection also leads to inhibition of cellular protein synthesis, disaggregation of pre-formed polyribosomes, and degradation of preexisting cellular mRNA at early times after infection (Fenwick & Clark, 1982, Fenwick & Everett, 1990a, Fenwick & Everett, 1990b, Fenwick & McMenamin, 1984, Fenwick & Owen, 1988, Fenwick & Walker, 1978, Jones *et al.*, 1995, Nishioka & Silverstein, 1977, Nishioka & Silverstein, 1978, Oroskar & Read, 1987, Oroskar & Read, 1989, Read & Frenkel, 1983, Read *et al.*, 1993, Roizman *et al.*, 1965, Schek & Bachenheimer, 1985, Smibert *et al.*, 1992, Sydiskis & Roizman, 1966, Sydiskis & Roizman, 1967, Sydiskis & Roizman, 1968). Four lines of evidence indicate that these effects (collectively referred to as host shutoff) are caused by a component of the infecting virion and, as such, do not require *de novo* synthesis of viral polypeptides. First, infection of cells with UV inactivated HSV results in shutoff of host cell protein synthesis and disaggregation of polysomes at early times post-infection (Fenwick & Clark, 1982, Fenwick & McMenamin, 1984, Fenwick & Owen, 1988, Fenwick & Walker, 1978, Schek & Bachenheimer, 1985, Strom & Frenkel, 1987). Second, HSV infection of enucleated cytoplasts (lack nuclei, can not support HSV gene expression) leads to rapid shutoff of protein synthesis (Fenwick & McMenamin, 1984, Fenwick & Walker, 1978). Third, infection of cells with HSV in the presence of transcriptional or translational blockers, which preclude viral gene expression, leads to shutoff of cellular protein synthesis (Fenwick & McMenamin, 1984, Fenwick & Walker, 1978, Hill *et al.*, 1985, Schek & Bachenheimer, 1985, Strom & Frenkel, 1987). Fourth,

infection of cells with HSV-1 or HSV-2 L-particles (lack capsid and DNA; can not produce viral products) leads to shutoff of host cell protein synthesis (McLauchlan *et al.*, 1992). The HSV-induced host shutoff function presumably allows the cellular translation machinery to be available for the translation of viral mRNAs.

Analysis of the HSV-1 (HFEM)tsB7 temperature-sensitive mutant led to the suggestion that the shutoff of host cell protein synthesis occurs in two distinct phases; early shutoff that is independent of *de novo* viral gene expression and late shutoff that is caused by a newly synthesized viral protein(s). As mentioned in a previous section, this mutant can not grow in permissive cells at the non-permissive temperature due to a lesion in ICP1/2 rendering the virus unable to release its DNA into the nucleus. In addition, this mutant is unable to induce host shutoff at the non-permissive temperature (Fenwick & Clark, 1982, Knipe *et al.*, 1981). Fenwick and Clark (1982) have isolated a revertant of this mutant that is able to grow but not induce early host shutoff at the non-permissive temperature. It did, however, induce reduction in host cell protein synthesis at late times post-infection at the non-permissive temperature indicating that late shutoff required *de novo* viral protein synthesis (Fenwick & Clark, 1982)).

Little is known about HSV-induced late shutoff of protein synthesis. As mentioned above it requires the prior expression of viral genes and occurs independently of early shutoff. The HSV IE protein ICP27 has been shown to contribute to late shutoff through its ability to reduce the level of cellular mRNAs in the cytoplasm of infected cells at late times. ICP27 interferes with the cellular mRNA processing and transport pathways leading to the retention of cellular and IE viral mRNAs containing introns in

the nucleus and the efficient transport of intronless E and L viral mRNAs to the cytoplasm of infected cells (Hardwicke & Sandri-Goldin, 1994, Hardy & Sandri-Goldin, 1994, Phelan *et al.*, 1996, Soliman *et al.*, 1997, and reviewed by Sandri-Goldin, 1998b).

Read and Frenkel (1983) isolated a series of viable host shutoff defective HSV mutants (termed vhs, for virion host shutoff). Despite their inability to induce early shutoff, these mutants were able to induce late shutoff of host cell protein synthesis confirming the two phases of host shutoff employed by HSV (Read & Frenkel, 1983). Furthermore, one of these (vhs1) and other vhs mutants have been subsequently shown to lack the ability to induce translational shutoff, disaggregation of polysomes, and mRNA degradation (Fenwick & Everett, 1990a, Kwong & Frenkel, 1987, Kwong *et al.*, 1988, Oroskar & Read, 1989, Strom & Frenkel, 1987) suggesting that these processes are linked. It is not clear, however, whether mRNA degradation is up- or downstream of translational arrest. Schek and Bachenheimer (1985) reported a greater inhibition of protein synthesis than can be explained by loss of mRNA occurs in HSV-1 (KOS) infected Vero cells. Moreover, HSV-1 (F) induces polysome disaggregation before mRNA degradation can be detected in infected MEL cells (Nishioka & Silverstein, 1978). These data raise the possibility that mRNA degradation occurs as a consequence of translational shutoff.

Marker rescue analysis of the vhs1 mutant and analysis of HSV-1/HSV-2 recombinants resulted in mapping the vhs function to the UL 41 gene of HSV (Fenwick & Owen, 1988, Kwong *et al.*, 1988, McGeoch *et al.*, 1988). This assignment was directly confirmed by experiments showing that (i) targeted disruption of the HSV-1

(Smibert & Smiley, 1990) and HSV-2 (Fenwick & Everett, 1990a) UL41 genes results in a vhs null phenotype and (ii) transfer of the UL41 allele from HSV2, which displays a more robust shutoff function than HSV1 (Powell & Courtney, 1975), to HSV1 results in an HSV recombinant with a strong shutoff phenotype (Fenwick & Everett, 1990b)

The UL41 gene has the capacity to encode an unmodified 55 kDa protein (McGeoch *et al.*, 1988) and *in vitro* translation of an mRNA mapping to the UL41 locus yielded a protein of ca. 58 kDa (Anderson *et al.*, 1981). Smibert and colleagues (1992) identified a 58 kDa phosphoprotein in HSV-1 infected cells using an antiserum raised against a synthetic peptide corresponding to amino acids 333 to 347 of the predicted sequence. That this protein was the product of UL41 was demonstrated by Western blot analysis of extracts from cells infected with HSV mutants in which the UL41 gene was disrupted yielding truncated proteins (Smibert *et al.*, 1992). As well, the authors demonstrated that this protein, synthesized with late kinetics, is present in purified virions consistent with its ability to induce host shutoff in the absence of *de novo* protein synthesis. These findings were later confirmed by Read and colleagues using antisera raised against a bacterially expressed UL41-LacZ fusion protein (Read *et al.*, 1993). Within the virion, the vhs protein resides in the tegument as illustrated by the observation that chemically de-enveloped L-particles (non-infectious enveloped particles lacking capsid and DNA components) contain vhs protein (McLauchlan *et al.*, 1992). Despite an 87% identity between the HSV-1 (489 aa) and HSV-2 (492 aa) vhs proteins (Everett & Fenwick, 1990), the HSV-2 protein displays a much stronger shutoff than that of HSV-1. The biochemical basis for this difference is not understood.

In addition to degradation of cellular mRNAs, vhs destabilizes viral mRNAs belonging to all kinetic classes of HSV genes (Krikorian & Read, 1991, Kwong & Frenkel, 1987, Oroskar & Read, 1987, Oroskar & Read, 1989, Strom & Frenkel, 1987). Indeed, infection of cells with vhs mutants results in not only continued host cell protein synthesis but also accumulation of viral IE and E transcripts and delayed onset of L gene expression (Kwong & Frenkel, 1987, Oroskar & Read, 1987, Read & Frenkel, 1983, Strom & Frenkel, 1987) arguing that the shutoff activity plays a role in the regulation of viral mRNA half-lives. Such a function would, thereby, allow for sharp transitions between the temporal classes of viral gene expression by more tightly coupling the rate of viral mRNA synthesis to their cytoplasmic levels. This is in accord with vhs's function in enhancing virus replication as evident by the observations that vhs mutants suffer a ca. 5 to10-fold reduction in virus yield in tissue culture (Read & Frenkel, 1983, Smibert & Smiley, 1990) and display severe defects in causing disease and establishing/reactivating from latency in the mouse model (Leib *et al.*, 1999, Strelow & Leib, 1995).

The indiscriminate nature of the vhs-induced mRNA decay seems at odds with the ability of the virus to carry out productive infection. However, Fenwick and colleagues provided strong evidence that expression of a viral E (or DE) proteins resulted in stabilization of mRNAs and reduction in the vhs-dependent inhibition of protein syntheses (Fenwick & Clark, 1983, Fenwick & Owen, 1988). This observation established that HSV encodes E or DE function(s) that downregulates vhs activity at intermediate times post-infection allowing viral mRNAs to accumulate and viral protein synthesis to proceed efficiently after host cell mRNAs have been degraded. Consistent

with this observation, Lam and co-workers have presented strong evidence demonstrating that the viral transactivator VP16 – which has been shown to form a complex with vhs (Smibert *et al.*, 1994) – fulfills this role. They showed that cells infected with HSV-1 mutant lacking VP16 (propagated on VP16 complementing cells) undergo a severe vhs-mediated protein synthesis shutoff and mRNA degradation at intermediate times and that this shutoff was averted by inactivating the UL41gene in the VP16 mutant background (Lam *et al.*, 1996). Furthermore, the authors show that prior expression of VP16 (cells that constitutively express VP16) renders cells resistant to vhs-induced early shutoff (Lam *et al.*, 1996). Together with the observation that low level VP16 expression can be detected as early as 2 hour post-infection (John Capone, McMaster University; unpublished data), these data strongly suggest that VP16 is responsible for dampening vhs activity at intermediate times post-infection. Moreover, newly synthesized VP16 may serve to inactivate newly made vhs protein (both VP16 and vhs are late genes) allowing viral gene expression to occur efficiently at late times post-infection. Consistent with this is the observation that VP16 exists in great excess relative to vhs in the virion tegument (McLauchlan *et al.*, 1992). Therefore, the net effect of vhs activity is to decrease cellular mRNAs in the cytoplasm of infected cells and regulate the half-lives of viral mRNAs. These functions result in increased pools of precursors and translational apparatus available for viral protein synthesis and a sharper transition from IE to E to L classes of viral gene expression, respectively.

An intriguing property of vhs is its ability to induce the degradation of mRNA but spare rRNA (Krikorian & Read, 1991, Kwong & Frenkel, 1987, Oroskar & Read, 1989).

This raises the important question of how does vhs differentiate between these RNAs? Perhaps one or more features common to most mRNAs and lacking from rRNA, such as the 5' cap structure or 3' poly(A) tract, might selectively target mRNA for degradation. Alternatively, the vhs-dependent mRNA degradation activity might specifically target mRNAs by interacting with component(s) of the translation apparatus.

Overton and colleagues (1994) reported that mutations in the UL13 protein kinase of HSV lead to inefficient host shutoff. They showed that while the levels of vhs mRNA and protein were dramatically reduced in UL13 mutant infected cells, the amount of vhs packaged into the mutant virions was the same as that in wild type virus leading to the conclusion that functional UL13 is required for vhs-induced host shutoff (Overton *et al.*, 1994). Although Ng and colleagues (1997) confirmed that UL13 (and ICP22) mutants display reduced host shutoff, these authors showed that the amount of vhs protein was reduced in both infected cells and mutant virions indicating that the reduced host shutoff caused by UL13 and ICP22 mutants is a reflection of reduced amounts of vhs protein in the mutants virions rather than direct involvement of UL13 in the shutoff process. Attempts to resolve this issue in our laboratory have not been informative, inasmuch as UL13 mutants constructed in our laboratory either failed to affect host shutoff, or reduced it only marginally (Shivak and Smiley, unpublished data). Despite the controversial data outlined above, vhs has been definitively shown to be sufficient to induce shutoff of reporter gene expression in transient transfection assays in the absence of any other viral products. Jones et al. (1995) showed that vhs induced the shutoff of β-galactosidase expression when constructs containing these genes were co-transfected into Vero cell and

vhs1 mutation abrogates this effect. The expression of a chloramphenicol acetyl transferase (CAT) reporter gene was similarly inhibited by co-transfected vhs in Vero cells due to vhs-dependent lose of CAT mRNA (Pak *et al.*, 1995).

Although it is clear that vhs is both necessary and sufficient to induce host shutoff and mRNA degradation, the mechanism of these activities occur remains largely unknown. Attempts to better understand the mode of vhs-induced mRNA degradation in infected and transfected cells have not been successful due, in part, to the fact that mRNA degradation intermediates are not detectable in this system. Two vhs activity *in vitro* assays were described prior to the time we started the work described in this thesis. Krikorian and Read (1991) reported that vhs induced mRNA degradation was reproducible in cytoplasmic extracts from HeLa cells infected with HSV-1. They showed that host and viral mRNAs were rapidly degraded in extracts from wild type but not vhs1 mutant HSV-1. Post-polysomal supernatant from wild type but not vhs mutants HSV-1 infected MEL cells was also shown to induce the degradation of polysome associated mRNAs (Sorenson *et al.*, 1991). Unfortunately, these systems did not yield detectable mRNA degradation intermediates and therefore were not suitable for mechanistic analysis of the vhs activity.

The ability of vhs to induce mRNA degradation *in vitro* (Krikorian & Read, 1991, Sorenson *et al.*, 1991) and in transfected cells (Jones *et al.*, 1995, Pak *et al.*, 1995) prompted us to ask if vhs produced in the rabbit reticulocyte lysate (RRL) cell-free *in vitro* translation system retains activity. The results described in this thesis demonstrate that vhs produced in RRL *in vitro* induced translational arrest and degradation of reporter

mRNAs. Moreover, the degradation of mRNA proceeds through detectable and identifiable degradation intermediates. During the course of our investigation, Zelus and co-workers (1996) confirmed our finding that vhs made in RRL displays mRNA degradation activity when made *in vitro* in rabbit reticulocyte lysates. They also showed that vhs protein extracted from purified virions induces mRNA degradation *in vitro* in the absence of any viral and cellular proteins indicating that vhs itself is a ribonuclease (Zelus *et al.*, 1996). The relevance of this report to the data presented in this thesis is discussed in much more detail in chapters 3 and 4. This RRL based quick and reproducible system allowed us to address some of the outstanding issues regarding vhs activity. These included: (i) what features of mRNAs, if any, render them susceptible to vhs-induced degradation, (ii) what is the mode of the vhs-induced mRNA decay (i.e. exo- or endoribonucleolytic mode of degradation), and (iii) what are the ionic and energetic requirements of the mRNA degradation reaction? The results of this work are presented and discussed in much more detail in chapters 3 and 4 of this thesis.

Chapter 2: Materials and Methods.

2.1. Recombinant DNA manipulation protocols.

This section contains a brief description of protocols routinely used in the construction and maintenance of recombinant plasmids. More detailed description of these protocols can be found in Molecular Cloning, a laboratory manual (Sambrook *et al.*, 1989).

2.1.1. Propagation and maintenance of bacterial strains.

The *E. coli* strain DH5α was used for the amplification and maintenance recombinant plasmids unless the plasmid DNA was destined for digestion with an enzyme that is sensitive to *dam/dcm* methylation. In that case, plasmid DNA was prepared in the *E. coli* strain GM48 which is defective in *dam* and *dcm* genes. Bacterial strains were grown and maintained in Luria-Bertani (LB) broth (1% w/v bacto-tryptone, 0.5% w/v yeast extract, 1% w/v NaCl; pH 7.0). Plating was carried out in the same medium supplemented with 2% agar. When bacteria were transformed with plasmid DNA containing ampicillin resistance gene, the culture medium (as above, broth or agar plates) was supplemented with 100 μg/ml ampicillin. Bacterial culture stocks were kept at -70°C in medium containing 10-15% glycerol.

2.1.2. Restriction enzyme digestion and modifying enzyme treatment of DNA.

Restriction and modifying enzymes were purchased from New England Biolabs and were used according the manufacturer's recommendation. Reactions were carried out at the recommended temperatures for periods of 1 to 3 hours. Partial digests were carried out in a large reaction volume and aliquots removed at various time intervals were analyzed by agarose gel electrophoresis to determine the extent of digestion. Typically, the concentration of plasmid DNA in the final reaction volume was in the range of 0.05 μg/μl. Double digestions were carried out in restriction buffer that allowed the best level of activity for both enzymes. Alternatively, the DNA was digested with one enzyme to completion, precipitated with 95% ethanol, resuspended in the appropriate restriction buffer (1X), and digested with the second enzyme.

To dephosphorylate digested DNA termini, 5 units of calf intestinal phosphatase (CIP) were added to the digestion mixtures during the last 15 to 20 minutes of incubation. CIP was inactivated and removed from the reaction mixture by extraction with equal volume phenol:chloroform:isoamylalcohol (25:24:1 volume), followed by extraction with chloroform. The DNA was then recovered by ethanol precipitation. Klenow (the large fragment of the *E. coli* DNA polymerase I) or T4 DNA polymerase were used to convert 5' and 3' overhangs (respectively) to blunt ends. Typically, 1 μl of dNTP mix (stock of 100 mM each), 2.5 to 5 units (2 μl) of enzyme, 3 μl 10X enzyme buffer, and 4 μl of dH_2O were added to a 20 μl restriction digest reaction when digestion was complete. Incubation was then continued at 37°C for additional 30 minutes. The enzymes were then inactivated and extracted as above.

2.1.3. Agarose gel electrophoresis and gel purification of DNA fragments.

DNA samples were prepared for electrophoresis by making the solution 5% DNA loading dye (50% glycerol, 1 μg/ml xylene cyanol, 1 μg/ml bromophenol blue, 1 mM EDTA). Samples were then loaded into the pre-formed wells of a 1% agarose gel containing 1X TAE (40 mM Tris-acetate, 2mM EDTA) and electrophoretic separation was achieved by applying 20 to 75 volts (depending on the run time required) across the gel in 1X TAE running buffer. Once resolution was achieved, DNA fragments were visualized using ultraviolet (UV) light after the gel was briefly stained with ethidium bromide (EtBr).

If a DNA fragment was to be purified from the gel then a gel slice containing that fragment was excised with a razor blade and transferred to a pre-weighed eppendorf tube. QIAEX II DNA extraction reagents were the used to extract the DNA out of the gel as follows: 1) 300 μl of QX1 buffer/ 100 mg of gel and 10 μl of QIAEX beads were added and the mixture was incubated at 50°C for 10 minutes; 2) The beads were pelleted and washed 2 times with 500 μl of QX1 buffer and 2 times with 500 μl of PE buffer; 3) pellet containing bound DNA was then air dried and the DNA was eluted in 10-20 μl of dH_2O.

2.1.4. Ligation of DNA fragments.

T4 DNA ligase was purchased from New England Biolabs and used according the manufacturer's recommendations. Typically, 20 μl reaction contained 1:1 to 1:5 ratio of vector to insert, 0.5 μl of T4 DNA ligase (ca. 40 units), and 2 μl of 10X ligation buffer were incubated for 1 hour at room temperature or overnight at 16°C. Both cohesive and

blunt end ligations were carried out in this way. Ligation reactions were then stored at -20°C until an aliquot was used to transform bacteria.

2.1.5. Preparation of transformation competent *E. coli*.

A 10 μl aliquot of a 1.5 ml overnight culture of DH5α was used to inoculate 50 ml of LB broth. This culture was incubated at 37°C in a culture shaker until cell density reached ca. 0.5 OD_{595} units (typically 5-7 hours). The cells were then pelleted at low speed at 4°C and resuspended in 25 ml of ice-cold MOPS I buffer (50 mM 3-n-morpholinepropanesulfonic acid (MOPS) pH 7, 10 mM RbCl). Following a 2 minute incubation on ice the bacteria were pelleted and resuspended in 23 ml of ice-cold MOPS II buffer (100 mM MOPS pH6.5, 70 mM $CaCl_2$, 10 mM RbCl). Following incubation on ice for 10 minutes, the cells were pelleted and resuspended in 2 ml of ice-cold MOPS II buffer containing 10% glycerol. The suspension was then aliquoted into 100 μl aliquots that were flash frozen in liquid nitrogen and stored at -70°C.

2.1.6. Transformation of competent *E. coli* with plasmid DNA.

Transformation of *E. coli* was carried out by combining 10 to 100 ng of DNA (plasmid DNA or ligation mixture) with 100 μl of competent bacteria on ice for 30 minutes. The suspension was then heat shocked at 42°C for 45 seconds and returned to ice for 2 minutes. Following the addition of 0.9 ml of LB, the mixture was then incubated for 45 minutes to 1 hour at 37°C with shaking. Aliquots of 100 μl were then plated on LB agar plates containing 100 μg/ml ampicillin. Colonies were allowed to develop at 37°C overnight.

2.1.7. Small scale preparation of plasmid DNA.

A single bacterial colony picked from the agar plate was transferred to 2 ml LB containing 100 µg/ml ampicillin. Following an overnight incubation at a 37°C shaker, 1.5 ml of the culture was transferred to an eppendorf tube. The bacteria were pelleted in microfuge and resuspended in 100 µl of P1 solution (50 mM Tris, 10 mM EDTA, 2 mg/ml lysozyme; pH7.5) by vortexing. After a 5 to 10 minute incubation on ice, the suspension was combined with 200 µl of P2 solution (200 mM NaOH, 1% SDS), mixed by gentle inverting, and incubated at room temperature for 5 minutes. 150 µl of P3 (3 M NaOAc, pH 4.8) was then added and, following a 15 minute incubation on ice the suspension was centrifuged in a microfuge for 5 minutes. The clear supernatant containing plasmid DNA was then transferred to an eppendorf tube and combined with 1 ml of 95% ethanol. After a 15 minute incubation at -20°C, plasmid DNA was pelleted, washed with 70% ethanol, dried, and resuspended in 75 µl of dH_2O. An aliquot of the DNA was analyzed be restriction enzyme digestion and agarose gel electrophoresis. The rest of the DNA was stored at -20°C.

2.1.8. Large scale preparation and CsCl purification of plasmid DNA.

Large scale preparation of plasmid DNA was carried out as outlined above except that the volume of culture was scaled up to 1 L and the volume of P1, P2, and P3 were scaled up to 10, 20, and 15 ml respectively. Once plasmid DNA was ethanol precipitated and dried, it was resuspended in total of 10 ml of dH_2O and combined with 10 g of CsCl salt and 0.5 ml of 10 mg/ml EtBr. The mixture was then loaded into a 5/8X3 in. quick

seal tube (Beckman) and, following heat sealing, the tubes were centrifuged in Beckman Vti65.1 rotor at 55,000 rpm and 20°C for 14 to 18 hours. After centrifugation, plasmid DNA (visible to the naked eye) was removed from the tubes using an 18 gage needle attached to a 5 ml syringe. The EtBr was removed from the DNA solution by extraction (3-5 times) with equal volume of isopropanol saturated with CsCl saturated dH_2O. The solution was combined with 2 volumes dH_2O and 6 volumes of ice-cold 95% ethanol. The plasmid DNA was then pelleted at 10,000 rpm for 10 minutes, resuspended on 0.4 ml of dH_2O, combined with 1 ml ice-cold 95% ethanol, and repelleted. The DNA pellet was then washed with 70% ethanol, dried and resuspended in 0.5 ml dH_2O. Following quantification, the plasmid DNA was stored at -20°C.

2.2. Radioactive labeling of DNA.

2.2.1. Random primer labeling of DNA.

Approximately 200 ng of DNA fragment (or intact plasmid DNA) were combined 1 μg of $pd(N)_6$ DNA random primer mixture and 38 μl of dH_2O and the mixture was boiled for 5 minutes. The mixture was then snap cooled at -20°C for 2 minutes, placed on ice and combined with 6 μl of 10X Klenow buffer, 9 μl of dNTP-dCTP mix (a stock of 50 μM of each dNTP except dCTP), 50 μCi α^{32}P-dCTP (10 mCi/ml, 3000 Ci/mmol; NEN), and 6 units of Klenow. The reaction mixture was then incubated at room temperature for 1-2 hours.

Unincorporated nucleotides (including radioactive dCTP) were removed from the reaction mixture by passing over a Sephadex G50 spin-column. The column was

prepared by placing 1 ml of pre-swollen G50 resin (swollen in TE buffer) into a 1 ml syringe that had been sealed with a glass wool plug at the bottom. The syringe was then spun at ca. 1800 Xg in clinical centrifuge to pack the resin. The packing procedure was repeated until approximately 1 ml of packed resin volume was obtained. The DNA mixture was then loaded on top of the pre-spun resin and the column was centrifuged at 1800 Xg for 5 minutes. Oligonucleotides and larger DNA fragments elute from the column in the flow-through fraction while the unincorporated dNTP's remain in the resin bed. The syringe was then disposed of in the appropriate radioactive waste container and the labeled DNA eluent was stored in a lead container at -20°C until needed.

2.2.2. 5' end labeling of DNA oligonucleotides.

All the oligonucleotides (oligos) used in this work were synthesized by the MOBIX central facility (LS building, McMaster University). Approximately 50-100 ng of oligo DNA (1-2 μl) were combined with 3 μl of 10X Kinase buffer, 10 units (1 μl) of T4 polynucleotide kinase, and 50 μCi of γ^{32}P-ATP (10 mCi/ml, 3000 Ci/mmol, NEN). The reaction was made up to 30 μl with dH_2O and incubated at 37°C for 1 hour. As described in the previous section, unincorporated nucleotide was removed by passing over a Sephadex G50 spin column.

Oligos to be used for primer extension analysis were further purified from polyacrylamide gels. Briefly, labeled oligos (30 μl) were combined with 50 μl of 7.5 M NH4OAC, 5 μl of (25 μg) carrier calf thymus tRNA, and 1 ml of ice-cold 95% ethanol. The oligos were then pelleted by centrifugation at 14000 rpm in microfuge for 10 minutes, washed with 70% ethanol, dried, and resuspended in 3 μl of dH2O and 9 μl of

sequencing gel dye (deionized formamide supplemented with 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). The resulting solution was loaded into 3 wells (4 µl each) of a pre-warmed 8% polyacrylamide / 7 M urea sequencing gel and resolved at 100 W for 45 minutes to 1 hour. The gel, attached to one of the glass plates, was marked with fluorescing compound (Glow Bug) at three positions, covered with plastic wrap and exposed to Kodak X-OMAT AR film for 2-3 minutes in the dark room. The developed film was then aligned with the gel using the Glow Bug markers and the position of correct length oligo was identified. A gel slice containing the oligo was excised with a razor blade and incubated in 0.3 ml of elution buffer (300 mM NH4Oac, 10 mM MgOAc, 1 mM EDTA, 0.1% SDS) overnight at 37°C. The gel material was removed from the elution buffer by filtration and the oligo was ethanol precipitated from solution as before, washed with 70% ethanol, dried, resuspended in 50 µl of dH_2O, and stored in a lead container at -20°C.

2.3. Plasmids.

2.3.1. Vhs *in vitro* translation vectors.

A vhs *in vitro* translation vector was constructed by inserting a 1.8 kbp NcoI-EcoRI fragment containing the vhs ORF from pCMVvhs (Jones *et al.*, 1995) between the NcoI and EcoRI sites of pSPUTK (Falcone & Andrews, 1991). The resulting plasmid (pSP6vhs) bears the vhs ORF fused to a modified derivative of the 5' untranslated region of Xenopus laevis β-globin (termed UTK) which has been engineered to contain a consensus Kozak translational initiation signal. This construct bears an SP6 RNA

polymerase promoter immediately upstream of the modified UTR. A control plasmid (pSP6vhs1) bearing the inactivating vhs1 point mutation (Kwong *et al.*, 1988, Read & Frenkel, 1983) was constructed in the same manner, using pCMVvhs1 (Jones *et al.*, 1995) as the source of vhs1 sequences. *In vitro* translation vectors encoding active, double tagged (HA and 8xHIS) versions of vhs were constructed by modifying the previously described plasmids pN138, pN138-HA, pS344, and pS344-HA (Jones *et al.*, 1995). pN138 and pS344 encode active modified versions of vhs that bear in-frame insertions of an XhoI linker following amino acid residues 138 and 344 respectively, driven from the HCMV IE promoter. pN138-HA and pS344-HA were derived from pN138 and pS344 by inserting sequences encoding an influenza virus hemagglutin (HA) epitope at these newly introduced XhoI sites. Analogous plasmids bearing sequences encoding 8 tandem histidine residues (pN138-HIS and pS344-HIS) were generated by inserting the complementary oligonucleotides 5'-TCGACATCATCATCATCATCATCATCA and 5'-TCGATGATGATGATGATGATGATGATG into the XhoI sites of pN138 and pS344. Doubly-tagged (HA/HIS) derivatives were then generated by exchanging appropriate restriction fragments between these plasmids. pN138HA-S344His was constructed by replacing a 646 bp BamHI-EcoRI fragment of pN138-HA with a 673 bp BamHI-EcoRI fragment of pS344-His containing the 8xHIS tag. pN138His-S344HA was constructed by replacing a 646 bp BamHI-EcoRI fragment of pN138-His with a 679 bp BamHI-EcoRI fragment of pS344-HA containing the HA tag. The doubly tagged vhs ORFs were then transferred from these CMV vectors to pSPUTK as described above for pSP6vhs to yield plasmids 1.1vhs (pSPN138HA-S344His) and 2.1vhs (pSPN138His-S344HA). Control

derivatives bearing the vhs1 point mutation (1.1vhs1 and 2.1vhs1) were generated by replacing a 583 bp SmaI-BamHI fragment of 1.1vhs and 2.1 vhs with the same fragment of pCMVvhs1 (Jones *et al.*, 1995).

A pseudorabies virus (PRV) vhs *in vitro* translation vector was generated as follows. pPRV41 (Berthomme *et al.*, 1993) was digested with DraI and EcoRI, and a 1580 nt fragment extending from 20 nt downstream of the vhs initiation codon (the DraI site) into 3' flanking sequences(EcoRI) was purified by gel electrophoresis. This fragment was then ligated to the complementary oligonucleotides 5'-CATGGGGCTCTTTGGCCTTTT and 5'-AAAAGGCCAAAGAGCCCG to regenerate the 5'-most 20 bp of the PRV vhs ORF, and place an engineered NcoI site at the initiation codon. The modified PRV vhs ORF was then inserted between the NcoI and EcoRI sites of pSPUTK, yielding pSPPRVvhs.

2.3.2. *In vitro* transcription plasmids used to generate substrate RNAs.

pSPSR19N contains a complete cDNA encoding the canine signal recognition particle a subunit (SRPα), initiating at an engineered NcoI site, inserted into pSPUTK (Young *et al.*, 1995). pMAC39 contains the bovine preprolactin ORF (PPL) inserted in the same vector (Falcone & Andrews, 1991). pPRL3'UTRpA contains the PPL ORF and 3' UTR, followed by an engineered 35 residue poly(A) tail, inserted into pSPUTK. The poly(A) tail is flanked by a 5' SspI and 3' EagI site. pBlueK(coreD) contains a 4.5 Kbp EcoRI fragment of human SH-2 containing inositol 5' phosphatase (hSHIP) cDNA cloned at the EcoRI site downstream of the T7 promoter of the vector pBluescript (courtesy of Dr. Peter Whyte, McMaster University).

pCITE-1 (Novagen) contains residues 255 to 836 of the EMCV 5' UTR (the IRES element) 9 bp downstream of the T7 RNA polymerase promoter start site. The IRES element is followed by a 1166 bp fragment corresponding to the extreme 5' end of the EMCV ORF. pCITE RI/AvrII (lacking the 5' most 159 bp of the IRES) was constructed by self-ligating EcoRI/AvrII digested pCITE-1 DNA, after filling in the ends with the Klenow fragment of DNA polymerase I. pCITE Msc/RI (lacking all of the IRES) was generated in the same way, using MscI/EcoRI cleaved pCITE-1 DNA. pSEXAI IRES was constructed by ligating a 600 nt EcoRI-MscI fragment of pCITE-1 (bearing the IRES) into the SexA1 site of pCITE Msc/RI (after filling in all of the ends with Klenow). The resulting plasmid contains the EMCV IRES 729 bp downstream of the T7 start site. pSP19StuI IRES was constructed by inserting the 600 EcoRI-MscI fragment of pCITE-1 into the unique StuI site of pSPSR19N (after all ends were made flush). The resulting plasmid bears the EMCV IRES element 1721 bp downstream of the SP6 RNA polymerase start site.

The plasmid pCITE Msc/RI P2 is a pCITE-1 derivative in which the EMCV IRES is replaced with the IRES of poliovirus type 2. It was constructed by exchanging the ca. 600 bp EcoRI-MscI fragment of pCITE-1 with a ca. 600 bp HindIII-MscI fragment from the plasmid pP2CAT (after making all ends flush with Klenow). P2CAT was a generous gift from N. Sonenberg, McGill University. pSexAI P2 was generated by ligating the ca. 600 bp HindIII-MscI fragment from pP2CAT into the SexAI site of pCITE Msc/RI, after repairing the ends with Klenow. The resulting plasmid lacks the EMCV IRES and contains the poliovirus IRES ca. 729 nt from the 5' end of the T7 RNA polymerase transcript.

2.4. *In vitro* transcription.

Transcription reactions were carried out using the Riboprobe *in vitro* transcription system (Promega) according the vendor's protocol. mRNAs destined for *in vitro* translation (vhs, vhs1, PPL and PRVvhs) were generated by transcription of 3-5 mg of supercoiled plasmid DNA (pSP6vhs, pSP6vhs1, pMAC39, and pSPPRVvhs) in a 50 μl reaction for 30 minutes at 30°C using 40 U of SP6 RNA polymerase in the presence of 0.5 mM cap primer $7^{m}G(5')ppp(5')G$ (Pharmacia), 12.5 μM GTP, and 0.25 mM of each of CTP, ATP, and UTP. Following digestion of plasmid DNA with 5U of RQ1 DNase (Promega), the reaction mixture was extracted once with phenol:chloroform:isoamyl alcohol and once with chloroform. The resulting solution was made 2.5 M ammonium acetate and the mRNA was precipitated with 95% ethanol. The mRNA pellet was then washed with 70% ethanol, dried and resuspended in RNase free water.

2.5. Preparation of HeLa cell-free translation extracts.

HeLa cell translation extracts were prepared by the method described by Carroll and Lucas-Lenard (Carroll & Lucas-Lenard, 1993) with the following modifications. (i) HeLa S3 cells were grown in suspension culture in Jokliks-MEM supplemented with 5% fetal bovine serum, 1% vitamin mix (Gibco-BRL), and 1% non-essential amino acids (Gibco-BRL). (ii) 300 μl of the post-mitochondrial supernatant were mixed with 3 μl of 100 mM $CaCl_2$ and 60 U of micrococcal nuclease. Following a 15 minute incubation at room temperature, 10 μl of 100 mM EGTA were added to the nuclease treated lysate, and samples were snap frozen in liquid N2 and stored at –70°C.

2.6. *In vitro* translation.

Approximately 5-10 µg of vhs mRNA was translated in a 50 µl rabbit reticulocyte lysate (RRL; Promega or Novagen) reaction containing 40 µCi of ^{35}S-methionine according to the vendor's protocol. Translation reactions were carried out for 1 hour at 30°C. Blank RRL controls were generated as above except that mRNA was omitted from the translation reactions. Vhs was synthesized in *in vitro* translation extracts derived from HeLa cells by combining 75 µl of nuclease treated extract (above) with 10 µl of 10X HeLa cell E-Mix (20 mM Hepes-KOH pH7.4, 100 mM KOAc, 2.2 mM MgOAc, 2.0 mM DTT, 12.5 mM ATP, 2.5 mM GTP, 375 mM creatine phosphate, 2 mM spermidine, 0.2 mg/ml calf liver tRNA, 0.1 mM amino acids minus methionine, and 62.5 U creatine kinase), 80 µCi ^{35}S-methionine, 80 U of RNasin, and 5-10 µg of capped vhs RNA (total volume of 100 µl). HeLa translation reactions were carried out at 30°C for 1 hour. Samples of the translation reactions were assessed for ^{35}S-methionine incorporation by SDS-PAGE analysis (Laemmli 1970).

2.7. Ribosome depletion.

To remove ribosomes from vhs containing and blank reticulocyte lysate, 200 µl of each reaction mixture was placed in a 200 µl ultracentrifuge tube (Beckman). The tubes were then centrifuged at 30 pounds per square inch in an airfuge at 4°C for 50 minutes. This air pressure setting leads to rotor speeds resulting in 160,000 Xg. The post-ribosomal supernatant was removed into clean tube and the pellet was rinsed with 100 µl of Retic buffer (1.6 mM Tris-OAc pH 7.8, 80 mM KOAc, 2mM MgOAc, 0.25 mM ATP,

0.1 mM DTT). The pellet was then resuspended into 200 μl of Retic buffer by repeatedly pipetting the material up and down until it went into solution (approximately 10 times). Both fractions along with controls (including untreated reactions and reactions placed at 4°C for the duration of the treatment were then tested for vhs-induced RNA degradation activity. The extent of ribosome clearance was monitored by Northern blot analysis using a rabbit 18S rRNA specific probe.

2.8. Depletion of the cap-binding protein eIF4E.

The buffer in 100 μl of 7^{m}G-Sepharose and Sepharose 4B (Pharmacia) beads was exchanged with Retic buffer by pelleting the beads at low speed (as recommended by the vendor), removing the shipping buffer, and resuspending them in 100 μl Retic buffer. The beads were then incubated in Retic buffer on ice for 5 minutes, pelleted, and resuspended in fresh Retic buffer. This process was repeated 5 more times. 50 μl of vhs and blank control lysates were combined with 50 μl of Retic buffer equilibrated 7^{m}G-Sepharose or Sepharose 4B in eppendorf tubes. The mixtures were incubated with end-over-end shaking at 4°C for 2.5 hours. The mixtures were centrifuged at low speed and the supernatant was moved to a clean tube. To insure that no beads were transferred with the supernatant, the material was centrifuged again and the supernatant was removed into a clean tube. The pellet was washed 4X with fresh Retic buffer with shaking and finally resuspended in 50 ml of fresh Retic buffer. All fractions were tested for the extent of eIF4E depletion by Western blot analysis using eIF4E specific antibody provided by Dr. N. Sonenberg (McGill University, Montreal).

2.9. Western blot analysis.

Following resolution on SDS-polyacrylamide gels, proteins were electroblotted onto a nitrocellulose membrane at 100 V in a Biorad gel transfer apparatus at 4°C in transfer buffer (25 mM Tris, 192 mM glycine, 20% Methanol). The membrane was then incubated in block solution (TBS-T (20 mM Tris.Cl pH 7.5, 150 mM NaCl, 0.1% Tween20) containing 5% skim milk powder and 1% BSA. Blocking was carried out at room temperature for 30 minutes to 1 hour. The membrane was then rinsed with TBS-T and incubated in 5 ml of fresh TBS-T containing the appropriate dilution of primary antibody (1:2000 dilution of the α-eIF4E polyclonal antiserum) for 2 hours at room temperature. The membrane was washed 4 X 10 minutes in TBS-T and incubated in 5 ml of TBS-T containing 1:5000 dilution of HP-conjugated secondary antibody for 1 hour at room temperature. The membrane was washed again as above and the protein bands were visualized be chemiluminescence (Dupont NEN) and autoradioagraphy for 10 to 40 seconds.

2.10. Desalting of vhs containing and control RRL.

Vhs containing and control RRL were desalted to remove small molecules from the lysates. This was done by loading 100 μl of each of the vhs containing and control lysates on a 4 ml packed volume of Sephadex G25 in a spin column. The column was prepared as described in sections 2.2.1 and 2.2.2. The G25 (fine) resin was pre-swollen in Retic buffer lacking MgOAc and ATP. Once the samples were loaded on top of the resin bed, the columns (5ml syringes stoppered with glass wool) were centrifuged in a clinical centrifuge, equipped with a swinging-bucket rotor, at approximately 1,800 Xg for

5 minutes at 4°C. The flow through fraction was the collected into clean tubes and tested for vhs-induced mRNA degradation in the presence and absence of added ATP and MgOAc.

2.11. RNA labeling.

2.11.1. Internally labeled RNAs.

Capped, internally labeled reporter RNAs were generated as above (section 2.4), except that the template was linearized at an appropriate site prior to transcription to yield run-off transcript of defined size, and 1 μCi α^{32}P-GTP was added to the transcription reaction. Uncapped, internally labeled reporter RNAs were produced in a similar fashion, except that the cap primer was omitted and the GTP concentration was raised to 0.25 mM. SRPα reporter mRNA was generated using SP6 polymerase and EcoRV-linearized pSPSR19N plasmid DNA as a template to yield a 2.4 kb run-off transcript. The SRPα anti-sense transcript was generated using T7 RNA polymerase and SnaBI-linearized pSPSR19N to yield a 2.2 kb run-off transcript. The SRPα StuI IRES 3 Kb was generated from EcoRV linearized pSP19StuI IRES using SP6 RNA polymerase. HindIII-linearized pBlueK(coreD) plasmid DNA was transcribed using T7 RNA polymerase to yield a 4.5 kb run-off hSHIP transcript. PPL RNA containing a 35 residue poly(A) tail was generated using SP6 polymerase and EagI-linearized pPRL3'UTRpA plasmid DNA. A poly(A)- derivative of the same RNA was generated by linearizing the template with SspI.

Reporter RNAs transcribed from pCITE, pCITE RI/AvrII, pCITE Msc/RI, pCITE Msc/RI P2, pSexAI IRES, and pSexAI P2 were generated using T7 RNA polymerase and EcoNI-linearized plasmid DNA templates to yield run-off transcripts of ca. 2.3, 2.1, 1.7, 2.3, 2.3, and 2.3 Kb, respectively. The Short pCITE RI/AvrII (437 nt) was generated using T7 RNA polymerase and MscI-linearized pCITE RI/AvrII DNA as a template.

2.11.2. Cap labeled RNAs.

Cap labeled reporter RNAs were generated from uncapped unlabeled run-off transcripts using vaccinia virus guanylyltransferase in the presence of α^{32}P-GTP. Approximately 500 ng of RNA in 50 mM Tris-HCl pH 7.9, 1.25 mM $MgCl_2$, 6 mM KCl, 2.5 mM DTT, 0.1 mg/ml RNase free BSA, 1 U/µl RNasin and 0.1 mM S-adenosyl-L-methionine (Sigma) was combined with 1-3 U of guanylyltransferase (Gibco-BRL) and 50 µCi α^{32}P-GTP in a total reaction volume of 30 µl. Following a 45 minute reaction at 37°C, the reaction mixture was extracted once with phenol:chloroform:isoamyl alcohol and once with chloroform, and the RNA was recovered by ethanol precipitation.

2.12. *In vitro* translational shutoff assay.

Equal amounts of capped unlabeled reporter mRNA was added to RRL containing pretranslated effector molecules (PPL, vhs, vhs1, 1.1 vhs, 1.1 vhs1, 2.1 vhs, 2.1 vhs1; sections 2.3.1 and 2.6) at 30°C. Following 60 minutes incubation at 30°C, 5 ml samples of each reaction was combined with equal volume of 2X SDS-PAGE loading buffer (125 mM Tris.Cl pH6.8, 4% w/v SDS, 600 mM 2-b-mercaptoethanol, 5% glycerol, 0.01% w/v bromophenol blue) and boiled for 10 minutes. The samples were then loaded into the

wells of a 12% resolving/5% stacking SDS-polyacrylamide gel. The samples were resolved by applying 75 V across the gel in Laemmli buffer (25 mM Tris, 192 mM glycine, 1% w/v SDS) for approximately 5 hours. The gel was then removed and fixed in 50% methanol/10% acetic acid solution for 45 minutes. The gel was then rinsed in water 3 times and treated with Enlightening (Dupont-NEN) for 45 minutes. The gel was then placed on a Watman paper and dried (80°C and vacuum). The dried gel was directly (without plastic wrap) exposed to Kodak X-OMAT AR film at -70°C to detect the ^{35}S signal.

2.13. *In vitro* RNA degradation assay.

Reporter RNA substrates generated by *in vitro* transcription were added to rabbit reticulocyte or HeLa cell lysates containing pre-translated vhs, and the reaction was incubated at 30°C. Aliquots (5 μl) of reaction mixture were removed at various times and immediately added to 200 μl Trizol (Gibco-BRL) containing 20 μg of carrier *E. coli* tRNA (Sigma). The samples were extracted after the addition of 40 μl of chloroform and the resulting aqueous phase was extracted with chloroform. RNA was recovered by isopropanol precipitation, resuspended in 100 μl of RNase free water and reprecipitated with 95% ethanol. Following a 70% ethanol wash, the RNA pellet was dried and resuspended in RNase free water. The RNA samples were then analyzed by electrophoresis through agarose/formaldehyde or polyacrylamide sequencing gels, or by primer extension.

2.14. Agarose/formaldehyde gel electrophoresis.

RNA samples were resuspended in 4.5 µl of RNase free water, then combined with 2 µl 10X MOPS buffer (200 mM 3-n-morpholino-propanesulfonic acid pH 7.0, 50 mM NaOAc, and 5 mM EDTA), 10 µl of deionized formamide and 3.5 µl of 37% formaldehyde solution. Following a 10 minute incubation at 75-80°C, the solution was combined with 6 µl of RNA loading buffer (50% glycerol, 1mM EDTA, 10 mg/ml xylene cyanol, and 10 mg/ml bromophenol blue) and subjected to electrophoresis through a 1% agarose gel containing 6% formaldehyde. Electrophoresis was carried out in 1X MOPS buffer containing 6% formaldehyde at approximately 5 V/cm for 3 to 4 hr. The gel was then washed in water for 10 minutes, treated with 50 mM NaOH/10 mM NaCl (20 minutes), and neutralized with 100 mM Tris-HCl (20 minutes). RNA was then transferred to a Nytran Plus membrane in 20X SSC (3 M sodium chloride, 0.3 M sodium citrate). Following UV cross-linking (Stratalinker 2400; Stratagene), ^{32}P-labeled RNA fragments were detected by exposure to Kodak X-OMAT AR film at –70°C.

2.15. Northern blot analysis.

Unlabeled RNA fragments were detected by Northern blot analysis (Church & Gilbert, 1984). Briefly, the Nytran Plus membrane was prehybridized in Church buffer (250 mM $NaPO_4$ pH7.2, 7% SDS, 1% BSA, 1 mM EDTA) at 62°C for 1 hour. The prehybridization buffer was removed and replace with 10 ml of Church buffer containing an appropriate DNA probe (as indicated in figure legends; example, a random primer ^{32}P-labeled 400 nt EcoRV-EcoRI fragment of pSPSR19N corresponding to the 3'-most

portion of the SRPα transcript). The probes, random primer ^{32}P-labeled DNA fragment or 5' end ^{32}P-labeled oligo, were boiled for 5 minutes and snap cooled on ice for 2 minutes before they were added to the hybridization buffer. Hybridization was carried out at 62°C for 13-17 hours. The membrane was then washed 2X 10 minutes in 2X SSC, 0.1% SDS, 2X 10 minutes in 0.1X SSC, 0.1 SDS, and subjected to autoradiography using Kodak X-OMT AR film as above.

2.16. Primer extension analysis.

RNA samples were suspended in 10 μl of 10 mM Tris-HCl pH7.9, 1mM EDTA, and 250 mM KCl containing 50,000 Cerenkov cpm of a 5'- ^{32}P-labeled oligonucleotide (5'-GGTGAAGAAGTCGACCATGGTAGGAT-3') complementary to residues 60-84 of the SRPα RNA; AB11388, 5'-CATTCTTCATCATACTTTAGCAGGT-3' complementary to residues 685-710 of pCITE-1 RNA; AB11259, 5'-CCATTAGGCAGGTTATCCTTGGACC-3' complementary to residues 1447-1471 of pSEXAI IRES RNA; and AB11260, 5'-GCAGCTCCCACCTTGTCATCAATGG-3' complementary to residues 2424-2448 of SRPα StuI IRES RNA. After annealing for 1 hour at 65°C, the samples were combined with 25 μl of PE buffer (20 mM Tris-HCl pH 8.7, 10 mM MgCl2, 5 mM DTT, 330 μM of each dNTP, 10 μg/ml actinomycin D and 10 U of SuperScript II (Gibo-BRL) reverse transcriptase) and the extension reaction was carried out for 1 hour at 42°C. Nucleic acids were precipitated with 95% ethanol, washed with 70% ethanol, dried and resuspended in water. These samples were then subjected to sequencing gel electrophoretic analysis.

2.17. Polyacrylamide sequencing gel electrophoresis.

Samples generated from RNA degradation assays (section 2.9) or primer extension analysis (section 2.12) were combined with equal volume of sequencing gel loading dye, heated to 80°C for 2-3 minutes and loaded into the wells of a pre-warmed 8% polyacrylamide/7M urea, 1X TBE sequencing gel. Resolution was achieved by applying 100 W across the gel in 1X TBE running buffer for 1-2 hours depending on the size of the DNA or RNA molecules. At the end of the run, the glass plates (on either side of the gel) were separated so the gel remained attached to one plate. The gel is then lifted onto backing support (Watman 3M paper or spent film), covered with plastic wrap and exposed to Kodak X-OMAT AR film at -70°C.

2.18. RNA and DNA markers.

RNA size markers were generated by *in vitro* transcription using SP6 RNA ploymerase and pSPSR19N DNA linearized with EcoRV, PvuII, SmaI, NruI, or SnaBI to yield run-off transcripts of 2422, 1628, 800, 429 and 298 nt, respectively. DNA size markers were generated by Klenow filling of HpaII-digested pBR322 plasmid DNA in the presence of α^{32}P-dCTP.

CHAPTER 3: The Herpes Simples Virus Vhs Protein Induces Endoribonucleolytic Cleavage of Target RNAs in Cell Extracts.

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Preface.

The data presented in this chapter, published in the Journal of Virology, have been formated to comply with the "sandwich" thesis format. I carried out all the experiments described in this chapter. 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 manuscript. Chris Hayes was included in the authors list because he was the first to characterize the cleavage events that occur at the extreme 5' end of SRPα RNA.

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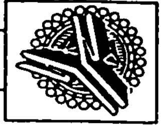

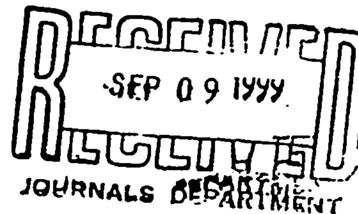

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The Herpes simplex virus vhs protein induces endoribonucleolytic cleavage of target RNAs in cell extracts.

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3.1. Abstract.

The Herpes simplex virus virion host shutoff (vhs) protein (UL41 gene product) is a component of the HSV virion tegument that triggers shutoff of host protein synthesis and accelerated mRNA degradation during early stages of HSV infection. Previous studies have demonstrated that extracts from HSV-infected cells and partially purified HSV virions display vhs-dependent ribonuclease activity, and that vhs is sufficient to trigger accelerated RNA degradation when expressed as the only HSV protein in an *in vitro* translation system derived from rabbit reticulocytes. We have used the rabbit reticulocyte translation system to characterize the mode of vhs-induced RNA decay in more detail. We report here that vhs-dependent RNA decay proceeds through endoribonucleolytic cleavage, is not affected by the presence of a 5' cap or a 3' poly(A) tail in the RNA substrate, requires Mg^{++}, and occurs in the absence of ribosomes. Intriguingly, sites of preferential initial cleavage were clustered over the 5' quadrant of one RNA substrate that was characterized in detail. The vhs homologue of Pseudorabies virus also induced accelerated RNA decay in this *in vitro* system.

3.2. Introduction.

Herpes simplex virus (HSV) is a large enveloped DNA virus that replicates in the nuclei of infected mammalian cells. HSV executes a complex genetic regulatory program during lytic infection of permissive host cells ((16); reviewed in references (37) and (50)). Five immediate early (IE) genes are expressed first, and four of these encode nuclear regulatory proteins that act at transcriptional and post-transcriptional levels to stimulate expression of the viral early (E) and late (L) genes. Expression of the second temporal class of HSV genes, the E genes, leads to synthesis of the seven proteins that comprise the viral DNA replicative machinery. Viral DNA replication then augments expression of the L genes that encode most of the structural components of the virus particle.

The HSV virion contains a variety of regulatory proteins that prime the newly infected cell to support efficient virus replication. These virion-associated regulators are located in the tegument –the space between the viral envelope and the nucleocapsid—, and are therefore presumably delivered into cytoplasm after fusion of the viral envelope with the host cell plasma membrane. The best characterized of these virion regulators is VP16, an abundant tegument protein that stimulates transcription of the five IE genes (reviewed in references (37) and (50)). The tegument also contains vhs, the virion host shutoff protein, which triggers rapid shutoff of host cell protein synthesis, disruption of pre-existing polysomes, and degradation of host mRNAs in the absence of *de novo* viral gene expression (10, 13-15, 18-21, 28-31, 34, 36, 42, 46-49).

Three lines of evidence demonstrate that the vhs protein encoded by HSV gene UL41 is both necessary and sufficient for virion-induced host shutoff. First, Read and Frenkel (34) isolated viable HSV mutants deficient in virion-induced shutoff, and one of these mutations (vhs1) was subsequently mapped to the UL41 open reading frame (21, 26). Confirming this assignment, targeted disruptions of the UL41 gene produce a vhs-deficient phenotype (11, 42). Second, viral recombinants in which the UL41 gene of HSV1 has been replaced by the corresponding gene from HSV2 display the more robust shutoff phenotype characteristic of HSV2 (12). Third, vhs suffices to block reporter gene expression when it is expressed as the only HSV protein in transiently transfected mammalian cells (18, 32). The UL41 gene product has been identified as a 58 kDa phosphoprotein that is packaged into the virion tegument (35, 40).

Vhs destabilizes most, if not all, viral and cellular mRNAs during infection (14, 20, 21, 30, 31, 34, 46). However, rRNA and tRNAs are spared (19, 20, 30, 52), raising the possibility that one or more features common to most mRNAs (such as the 5' cap or 3' poly(A) tail) might play a role in selectively targeting mRNA for degradation. The rapid decline in host mRNA levels triggered by vhs presumably helps viral mRNAs gain access to the cellular translational apparatus. In addition, the relatively short half-life of viral mRNAs contributes to the sharp transitions between the successive phases of viral protein synthesis, by more tightly coupling changes in the rate of synthesis of viral mRNAs to altered mRNA levels (20, 30, 31, 46). These effects likely enhance virus replication, and may account for the finding that vhs mutants display a ca. 10-fold reduction in virus yield in tissue culture (34, 42), and severe defects in the nervous

system of mice (45). Although viral mRNAs belonging to all three temporal classes are significantly destabilized by vhs, Fenwick and colleagues have provided strong evidence that the vhs activity of the infecting virion is partially downregulated by a newly synthesized viral protein, allowing viral mRNAs to accumulate after host transcripts have been degraded (14). Vhs directly binds to VP16 (41), raising the possibility that VP16 may modulate vhs activity. Consistent with this hypothesis, VP16 null mutants undergo vhs-induced termination of viral protein synthesis at intermediate times postinfection (23).

Although the mechanism of vhs action has yet to be completely defined, the available data strongly suggest that vhs is a ribonuclease that triggers shutoff by degrading mRNA. First, vhs displays weak but significant amino acid sequence homology to the fen-1 family of nucleases (8) that are involved in DNA replication and repair (reviewed in reference (24). Second, vhs-dependent mRNA degradation can be reproduced in cytoplasmic extracts prepared from HSV infected cells (19, 43), and extracts of partially purified HSV virions (52). Moreover, Zelus and colleagues (52) have shown that vhs induces accelerated RNA turnover when it is expressed as the only HSV protein in a rabbit reticulocyte lysate (RRL) *in vitro* translation system. The vhs-dependent ribonuclease activity detected in extracts of partially purified virions is inhibited by anti-vhs antibodies (52), suggesting that vhs is an integral and required component of this nuclease.

We used the rabbit reticulocyte-based vhs activity assay system (52) to further characterize the mode of vhs-induced RNA degradation. We confirmed that vhs induces

accelerated RNA decay in this system, and further showed that it severely inhibits translation of reporter RNAs. RNA decay proceeded through endoribonucleolytic cleavage events, was not affected by the presence of a 5' cap or 3' poly(A) tail, and occurred in the absence of ribosomes. Detailed characterization of the mode of decay of one RNA substrate revealed that the preferred sites of initial cleavage were clustered over the 5' quadrant of the transcript . Finally, we show that the vhs homologue of Pseudorabies virus also induced accelerated RNA decay in this system.

3.3. Materials and Methods.

Plasmids. A vhs *in vitro* translation vector was constructed by inserting a 1.8 kbp NcoI-EcoRI fragment containing the vhs ORF from pCMVvhs (18) between the NcoI and EcoRI sites of pSPUTK (9). The resulting plasmid (pSP6vhs) bears the vhs ORF fused to a modified derivative of the 5' untranslated region of Xenopus laevis β-globin (termed UTK) which has been engineered to contain a consensus Kozak translational initiation signal. This construct bears an SP6 RNA polymerase promoter immediately upstream of the modified UTR. A control plasmid (pSP6vhs1) bearing the inactivating vhs1 point mutation (21, 34) was constructed in the same manner, using pCMVvhs1 (18) as the source of vhs1 sequences. *In vitro* translation vectors encoding active, doubly tagged (HA and 8xHIS) versions of vhs were constructed by modifying the previously described plasmids pN138, pN138-HA, pS344, and pS344-HA (18). pN138 and pS344 encode active modified versions of vhs that bear in-frame insertions of an XhoI linker following amino acid residues 138 and 344 respectively, driven from the HCMV IE promoter. pN138-HA and pS344-HA were derived from pN138 and pS344 by inserting sequences encoding an influenza virus hemagglutinin (HA) epitope at these newly introduced XhoI sites. Analogous plasmids bearing sequences encoding 8 tandem histidine residues (pN138-HIS and pS344-HIS) were generated by inserting the complementary oligonucleotides 5'-TCGACATCATCATCATCATCATCATCA and 5'-TCGATGATGATGATGATGATGATGATG into the XhoI sites of pN138 and pS344. Doubly-tagged (HA/HIS) derivatives were then generated by exchanging appropriate restriction fragments between these plasmids. pN138HA-S344His was constructed by

replacing a 646 bp BamHI-EcoRI fragment of pN138-HA with a 673 bp BamHI-EcoRI fragment of pS344-His containing the 8xHIS tag. pN138His-S344HA was constructed by replacing a 646 bp BamHI-EcoRI fragment of pN138-His with a 679 bp BamHI-EcoRI fragment of pS344-HA containing the HA tag. The doubly tagged vhs ORFs were then transferred from these CMV vectors to pSPUTK as described above for pSP6vhs to yield plasmids 1.1vhs (pSPN138HA-S344His) and 2.1vhs (pSPN138His-S344HA). Control derivatives bearing the vhs1 point mutation (1.1vhs1 and 2.1vhs1) were generated by replacing a 583 bp SmaI-BamHI fragment of 1.1vhs and 2.1 vhs with the same fragment of pCMVvhs1 (18).

A Pseudorabies virus (Prv) vhs *in vitro* translation vector was generated as follows. pPRV41 (3) was digested with DraI and EcoRI, and a 1580 nt fragment extending from 20 nt downstream of the vhs initiation codon (the DraI site) into 3' flanking sequences (EcoRI) was purified by gel electrophoresis. This fragment was then ligated to the complementary oligonucleotides 5'-CATGGGGCTCTTTGGCCTTTT and 5'-AAAAGGCCAAAGAGCCCG to regenerate the 5'-most 20 bp of the Prv vhs ORF, and place an engineered NcoI site at the initiation codon. The modified Prv vhs ORF was then inserted between the NcoI and EcoRI sites of pSPUTK, yielding pSPPRVvhs.

pSPSR19N contains a complete cDNA encoding the canine signal recognition particle a subunit (SRPα), initiating at an engineered NcoI site, inserted into pSPUTK (51). pMAC39 contains the bovine preprolactin ORF (PPL) inserted in the same vector (9). pPRL3'UTRpA contains the PPL ORF and 3' UTR, followed by an engineered 35 residue poly(A) tail, inserted into pSPUTK. The poly(A) tail is flanked by a 5' SspI and

3' EagI site. pBlueK(coreD) contains a 4.5 Kbp EcoRI fragment of human SH-2 containing inositol 5' phosphatase (hSHIP) cDNA cloned at the EcoRI site downstream of the T7 promoter of the vector pBluescript (courtesy of Dr. Peter Whyte, McMaster University).

***In vitro* transcription and RNA labeling.** Transcription reactions were carried out using the Riboprobe *in vitro* transcription system (Promega) according the vendor's protocol. mRNAs destined for *in vitro* translation (vhs, vhs1, PPL and Prv vhs) were generated by transcription of 3-5 *m*g of supercoiled plasmid DNA (pSP6vhs, pSP6vhs1, pMAC39, and pSPPRVvhs) in a 50 *m*l reaction for 30 minutes at 30°C using 40 U of SP6 RNA polymerase in the presence of 0.5 mM cap primer 7^{m}G(5')ppp(5')G (Pharmacia), 12.5 *m*M GTP, and 0.25 mM of each of CTP, ATP, and UTP. Following digestion of plasmid DNA with 5U of RQ1 DNase (Promega), the reaction mixture was extracted once with phenol:chloroform:isoamyl alcohol and once with chloroform. The resulting solution was made 2.5 M ammonium acetate and the mRNA was precipitated with 95% ethanol. The mRNA pellet was then washed with 70% ethanol, dried and resuspended in RNase free water.

Capped, internally labeled reporter RNAs were generated as above, except that the template was linearized at an appropriate site prior to transcription, and 1 *m*Ci a^{32}P-GTP was added to the transcription reaction. Uncapped, internally labeled reporter RNAs were produced in a similar fashion, except that the cap primer was omitted and the GTP concentration was raised to 0.25 mM. SRPα reporter mRNA was generated using SP6 polymerase and EcoRV-linearized pSPSR19N plasmid DNA as a template to yield a 2.4

kb run-off transcript. The SRPα anti-sense transcript was generated using T7 RNA polymerase and SnaBI-linearized pSPSR19N to yield a 2.2 kb run-off transcript. HindIII-linearized pBlueK(coreD) plasmid DNA was transcribed using T7 RNA polymerase to yield a 4.5 kb run-off hSHIP transcript. PPL RNA containing a 35 residue poly(A) tail was generated using SP6 polymerase and EagI-linearized pPRL3'UTRpA plasmid DNA. A poly(A)$^-$ derivative of the same RNA was generated by linearizing the template with SspI. A vhs run-off transcript was generated using SP6 RNA polymerase and EcoRI linearized pSP6vhs.

Cap-labeled reporter RNAs were generated from uncapped unlabeled run-off transcripts using vaccinia virus guanylyltransferase in the presence of a^{32}P-GTP. Approximately 500 ng of RNA in 50 mM Tris-HCl pH 7.9, 1.25 mM $MgCl_2$, 6 mM KCl, 2.5 mM DTT, 0.1 mg/ml RNase free BSA, 1 U/*m*l RNasin and 0.1 mM S-adenosyl-L-methionine was combined with 1-3 U of guanylyltransferase (Gibco-BRL) and 50 *m*Ci a^{32}P-GTP in a total reaction volume of 30 *m*l. Following a 45 minute reaction at 37°C, the reaction mixture was extracted once with phenol:chloroform:isoamyl alcohol and once with chloroform, and the RNA was recovered by ethanol precipitation.

HeLa cell-free translation extracts. HeLa cell translation extracts were prepared by the method described by Carroll and Lucas-Lenard (6) with the following modifications. (i) HeLa S3 cells were grown in suspension culture in Jokliks-MEM supplemented with 5% fetal bovine serum, 1% vitamin mix (Gibco-BRL), and 1% non-essential amino acids (Gibco-BRL). (ii) 300 *m*l of the post-mitochondrial supernatant were mixed with 3 *m*l of 100 mM $CaCl_2$ and 60 U of micrococcal nuclease. Following a

15 minute incubation at room temperature, 10 *m*l of 100 mM EGTA were added to the nuclease treated lysate, and samples were snap frozen in liquid N_2 and stored at –70°C.

***In vitro* translation.** Approximately 5-10 *m*g of vhs mRNA was translated in a 50 *m*l rabbit reticulocyte lysate (RRL; Promega or Novagen) reaction containing 40 *m*Ci of ^{35}S-methionine according to the vendor's protocol. Translation reactions were carried out for 20 minutes (figure 3-1) or 1 hour (all of the remaining experiments) at 30°C. Blank RRL controls were generated as above except that mRNA was omitted from the translation reactions. Vhs was synthesized in *in vitro* translation extracts derived from HeLa cells by combining 75 *m*l of nuclease treated extract (above) with 10 *m*l of 10X HeLa cell E-Mix (20 mM Hepes-KOH pH7.4, 100 mM KOAc, 2.2 mM MgOAc, 2.0 mM DTT, 12.5 mM ATP, 2.5 mM GTP, 375 mM creatine phosphate, 2 mM spermidine, 0.2 mg/ml calf liver tRNA, 0.1 mM amino acids minus methionine, and 62.5 U creatine kinase), 80 *m*Ci ^{35}S-methionine, 80 U of RNasin, and 5-10 *m*g of capped vhs RNA (total volume of 100 *m*l). HeLa translation reactions were carried out at 30°C for 1 hour. Samples of the translation reactions were assessed for ^{35}S-methionine incorporation by SDS-PAGE analysis (22).

Vhs activity assay. Reporter RNA substrates generated by *in vitro* transcription were added to rabbit reticulocyte or HeLa cell lysates containing pre-translated vhs, and the reaction was incubated at 30°C. Aliquots (5 *m*l) of reaction mixture were removed at various times and immediately added to 200 *m*l Trizol (Gibco-BRL) containing 20 *m*g of carrier *E. coli* tRNA (Sigma). The samples were extracted after the addition of 40 *m*l of chloroform and the resulting aqueous phase was extracted with chloroform. RNA was

recovered by isopropanol precipitation, resuspended in 100 *m*l of RNase free water and reprecipitated with 95% ethanol. Following a 70% ethanol wash, the RNA pellet was dried and resuspended in RNase free water. The RNA samples were then analyzed by electrophoresis through agarose/formaldehyde or polyacrylamide sequencing gels, or by primer extension.

Agarose gel electrophoresis and Northern blot analysis. RNA samples were resuspended in 4.5 *m*l of RNase free water, then combined with 2 *m*l 10X MOPS buffer (200 mM 3-n-morpholino-propanesulfonic acid pH 7.0, 50 mM NaOAc, and 5 mM EDTA), 10 *m*l of deionized formamide and 3.5 *m*l of 37% formaldehyde solution. Following a 10 minute incubation at 75-80°C, the solution was combined with 6 *m*l of RNA loading buffer (50% glycerol, 1mM EDTA, 10 mg/ml xylene cyanol, and 10 mg/ml bromophenol blue) and subjected to electrophoresis through a 1% agarose gel containing 6% formaldehyde. Electrophoresis was carried out in 1X MOPS buffer containing 6% formaldehyde at approximately 5 V/cm for 3 to 4 hr. The gel was then washed in water for 10 minutes, treated with 50 mM NaOH/10 mM NaCl (20 minutes), and neutralized with 100 mM Tris-HCl (20 minutes). RNA was then transferred to a Nytran Plus membrane in 20X SSC (3 M sodium chloride, 0.3 M sodium citrate). Following UV cross-linking (Stratalinker 2400; Stratagene), ^{32}P-labeled RNA fragments were detected by exposure to Kodak X-OMAT AR film at –70°C.

Unlabeled SRPα RNA fragments were detected by Northern blot analysis (7). Briefly, the Nytran Plus membrane was prehybridized in Church buffer (250 mM $NaPO_4$ pH7.2, 7% SDS, 1% BSA, 1 mM EDTA) at 62°C for 1 hour. The membrane was then

hybridized to a 400 nt EcoRV-EcoRI fragment of pSPSR19N corresponding to the 3'-most portion of the SRPα transcript. The probe was ^{32}P-labeled by random priming. Hybridization was carried out in Church buffer at 62°C for 13-17 hours. The membrane was then washed 2X10 minutes in 2X SSC, 0.1% SDS, 2 X10 minutes in 0.1X SSC, 0.1 SDS, and subjected to autoradiography.

Primer extension. RNA samples were suspended in 10 *m*l of 10 mM Tris-HCl pH7.9, 1mM EDTA, and 250 mM KCl containing 50,000 Cerenkov cpm of a 5'- ^{32}P-labeled oligonucleotide (5'-GGTGAAGAAGTCGACCATGGTAGGAT-3') complementary to residues 60-84 of the SRPα RNA. After annealing for 1 hour at 65°C, the samples were combined with 25 *m*l of PE buffer (20 mM Tris-HCl pH 8.7, 10 mM MgCl2, 5 mM DTT, 330 *m*M of each dNTP, 10 *m*g/ml actinomycin D and 10 U of SuperScript II (Gibo-BRL) reverse transcriptase) and the extension reaction was carried out for 1 hour at 42°C. Nucleic acids were precipitated with 95% ethanol, washed with 70% ethanol, dried and resuspended in water. The samples were then combined with equal volume of sequencing gel loading buffer, heated to 80°C for 2-3 minutes and resolved on 8% polyacrylamide sequencing gels. The radioactive signal was detected by autoradiography.

Markers. RNA size markers were generated by *in vitro* transcription using SP6 RNA polymerase and pSPSR19N DNA linearized with EcoRV, PvuII, SmaI, NruI, or SnaBI to yield run-off transcripts of 2422, 1628, 800, 429 and 298 nt, respectively. DNA

size markers were generated by Klenow filling of HpaII-digested pBR322 plasmid DNA in the presence of a^{32}P-dCTP.

3.4. Results.

HSV1 vhs induces translational arrest and mRNA degradation *in vitro*. As reviewed in the Introduction, vhs suffices to trigger accelerated RNA degradation of reporter RNAs when it is expressed as the only HSV protein in a rabbit reticulocyte lysate (RRL) *in vitro* translation system (52). Here we report experiments that use this RRL-based *in vitro* system to examine the mechanism of vhs-induced RNA degradation in more detail.

We first established that vhs displays shutoff activity *in vitro* under our experimental conditions. To this end, RRL were programmed with *in vitro* transcripts encoding control bovine preprolactin (PPL), three active forms of vhs (wild-type and two doubly tagged variants, 1.1 vhs and 2.1 vhs), and three inactive derivatives bearing the inactivating vhs1 point mutation (vhs1, 1.1 vhs1, and 2.1 vhs1). Translation was allowed to proceed for 20 minutes in the presence of ^{35}S-methionine, and the lysates were then challenged with a 2.4 kb capped reporter RNA encoding the α subunit of the signal recognition particle (SRPα). Translation was allowed to continue for an additional 60 minutes, and the translation products were analyzed by SDS-polyacrylamide gel electrophoresis (figure 3-1A). As expected, pre-translation of control PPL mRNA had little effect on subsequent translation of the SRPα reporter RNA (compare lane 1 with lane 2). In striking contrast, translation of the reporter transcript was severely inhibited in lysates that contained active vhs (lanes 3, 5, 7). This inhibitory effect was reduced by the vhs1 mutation (lanes 4, 6, and 8), arguing for the biological relevance of the results.

Figure 3-1. HSV1 vhs induces translational arrest and mRNA degradation *in vitro*. (A) Rabbit reticulocyte lysates (RRL) were programmed with the indicated effector mRNAs and translation was allowed to proceed for 20 minutes in the presence of ^{35}S-methionine. Lysates were then challenged with an equal amount of capped SRPα reporter mRNA and the reactions were allowed to continue for an additional 60 minutes. The translation products were resolved on a 12% SDS polyacrylamide gel and the ^{35}S signal was detected by autoradiography using Kodak X-OMAT AR film. PPL: bovine preprolactin; 1.1 and 2.1: doubly tagged active vhs variants; vhs1, 1.1 vhs1 and 2.1 vhs1: inactive vhs point mutant derivatives of vhs, 1.1 and 2.1. (B) RRL were programmed with vhs RNA (lanes marked vhs), vhs1 RNA (lanes marked vhs1), or no RNA (control, lanes marked Retic) and translation was allowed to proceed for 20 minutes. The lysates were then challenged with capped, internally labeled SRPα mRNA. Samples were recovered at indicated times (numbers above lanes, minutes) and the RNA reaction products were resolved on a 1% agarose/6% formaldehyde gel, transferred to Nytran Plus membrane and detected by autoradiography using Kodak X-OMAT AR film.

A.

Effector

M | PPL vhs vhs1 1.1 vhs 1.1 vhs1 2.1 vhs 2.1 vhs1

100 KDa —

- SRPα

69 —

]-vhs

46 —

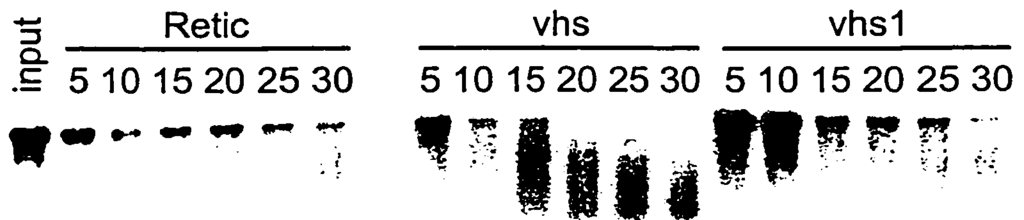
30 —

PPL ⟶

14 —

1 2 3 4 5 6 7 8

B.



To determine if translational arrest was accompanied by accelerated decay of the reporter RNA, the experiment was repeated using capped internally labeled reporter RNA (figure 3-1B). The results indicated that the reporter RNA decayed at an accelerated rate in the presence of wild-type vhs (see also below). These data confirm that vhs induces accelerated RNA degradation in the RRL *in vitro* translation system (52), and that this activity severely inhibits translation of a reporter RNA.

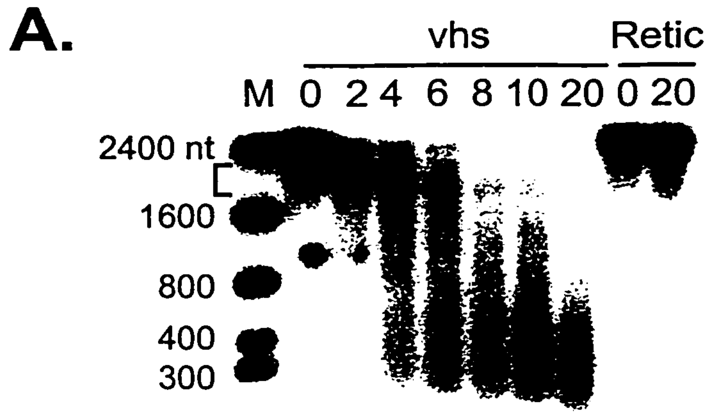
Vhs-induced degradation of SRPα RNA involves early endonucleolytic cleavage events clustered in the 5' quadrant of the transcript. Previous studies have established that vhs induces RNA degradation in both HSV infected cells and *in vitro* systems. However, little information is available about the mode of vhs-induced RNA decay. Zelus and colleagues (52) suggested that vhs extracted from partially purified virions preferentially degrades the 3' end of globin RNA, possibly by targeting sequences within or close to the poly(A) tail. These authors also suggested that RNA degradation likely proceeds through endoribonucleolytic cleavage, although (as acknowledged by the authors) the data advanced to support this conclusion were not definitive. We therefore examined the mode of vhs-induced RNA decay in more detail (figure 3-2).

Internally labeled SRPα RNA gave rise to several discrete high molecular weight intermediates ranging in size from ca. 1800 to ca. 2200 nt, early during the reaction (figure 3-2A (indicated by a bracket), also see figure 3-4A and 3-6B). These intermediates subsequently decayed to lower molecular weight products as the reaction proceeded. In principle, the 1800-2200 nt intermediates could arise from either exonucleolytic decay initiated at one or both ends of the transcript, or by endonucleolytic

cleavage at several sites located close to one or both ends of the 2400 nt substrate RNA. We distinguished between these possibilities by following the fate of the 5' and 3' ends of the transcript during the course of the reaction. To this end, 5' cap labeled SRPα RNA was added to RRL containing pre-translated vhs , and the resulting degradation products were analyzed by agarose/formaldehyde gel electrophoresis and autoradiography (figure 3-2B). The ^{32}P signal from the cap label was then allowed to decay for 6 half-lives, and the membrane was hybridized to a probe corresponding to the 3'-most 400 nt of SRPα RNA (figure 3-2C). The 3' probe revealed a pattern of degradation intermediates similar to that observed with internally labeled RNA (compare panel C with A): discrete fragments of 1800-2200 were observed at early times, and these were reduced in size as the reaction proceeded (figure 3-2C). In contrast, the 5' cap label was recovered in considerably smaller products at the early time points (figure 3-2B). In order to obtain a more accurate estimate of the sizes of these 5' products, RNA was recovered from the gel slices indicated by brackets in figure 3-2B and resolved on an 8% polyacrylamide sequencing gel (figure 3-2D). The 5' fragments recovered from gel slices I and II ranged in size from ca. 200-700 nt and ca. 30-40 nt, respectively. No prominent cap labeled products of higher molecular weight were observed in repeated trials. The 30 - 40 nucleotide fragments accumulated throughout the course of the reaction, while the 200 - 700 nt fragments detected at earlier times decayed as the reaction proceeded (figure 3-2B). Taken in combination, these data exclude the possibility that vhs-induced RNA degradation proceeds exclusively through a 5'-3' or 3'-5' exonucleolytic pathway, and are completely consistent with an endonucleolytic mode of RNA decay. In particular, the early generation of sets of 5' and 3' intermediates that together roughly sum to yield the

Figure 3-2. Analysis of vhs-induced degradation intermediates of SRPα RNA. Internally labeled (A) and cap labeled (B) SRPα mRNAs were added to RRL containing pre-translated vhs (lanes marked vhs) or RRL control (lanes marked Retic). RNA degradation products were recovered at the indicated times (minutes) and analyzed by agarose/formaldehyde gel electrophoresis. (C) The membrane in panel B was hybridized to a ^{32}P-labeled DNA probe corresponding to the 3'-most 400 nt of SRPα RNA (after the radioactive signal from the cap label had been allowed to decay for 6 half-lives). The bound probe was then detected by autoradiography using Kodak X-OMAT AR film. Numbers to the side of panels A, B and C represent the sizes of RNA markers (lanes M) in nucleotides. (D) Cap labeled SRPα RNA was added to RRL-vhs and the RNA degradation intermediates recovered at 10 minutes were resolved on a 1% agarose/6% formaldehyde gel. RNA fragments contained in the gel slices indicated by brackets I and II in panel C were eluted and resolved on an 8% polyacrylamide sequencing gel (lanes I and II) along with the unfractionated products of a vhs reaction on cap labeled SRPα RNA (sampled at 0, 10 and 20 minutes). Numbers to the side of panel D represent sizes of DNA markers in nucleotides.

A.



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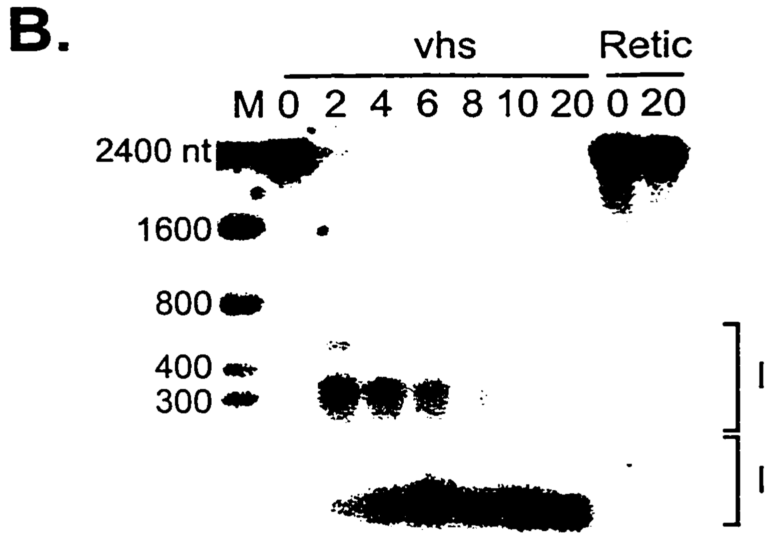

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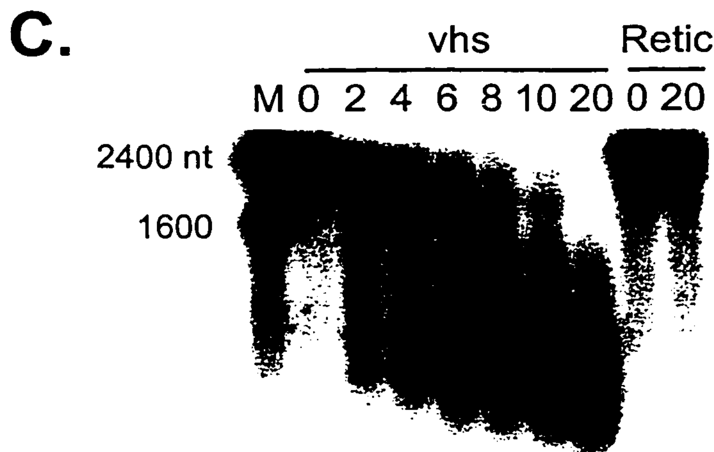

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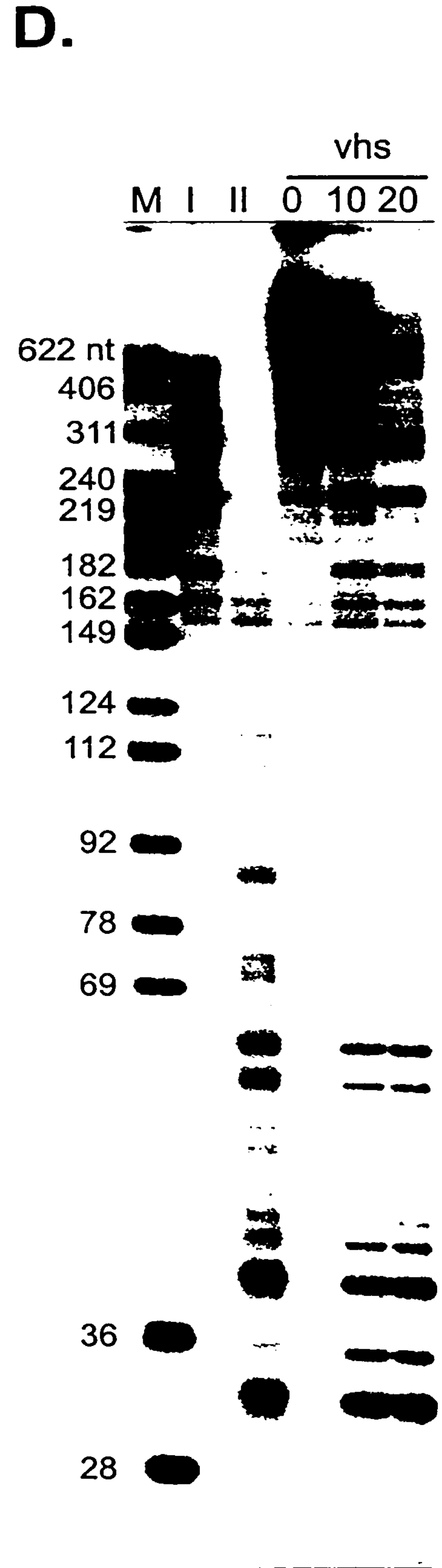
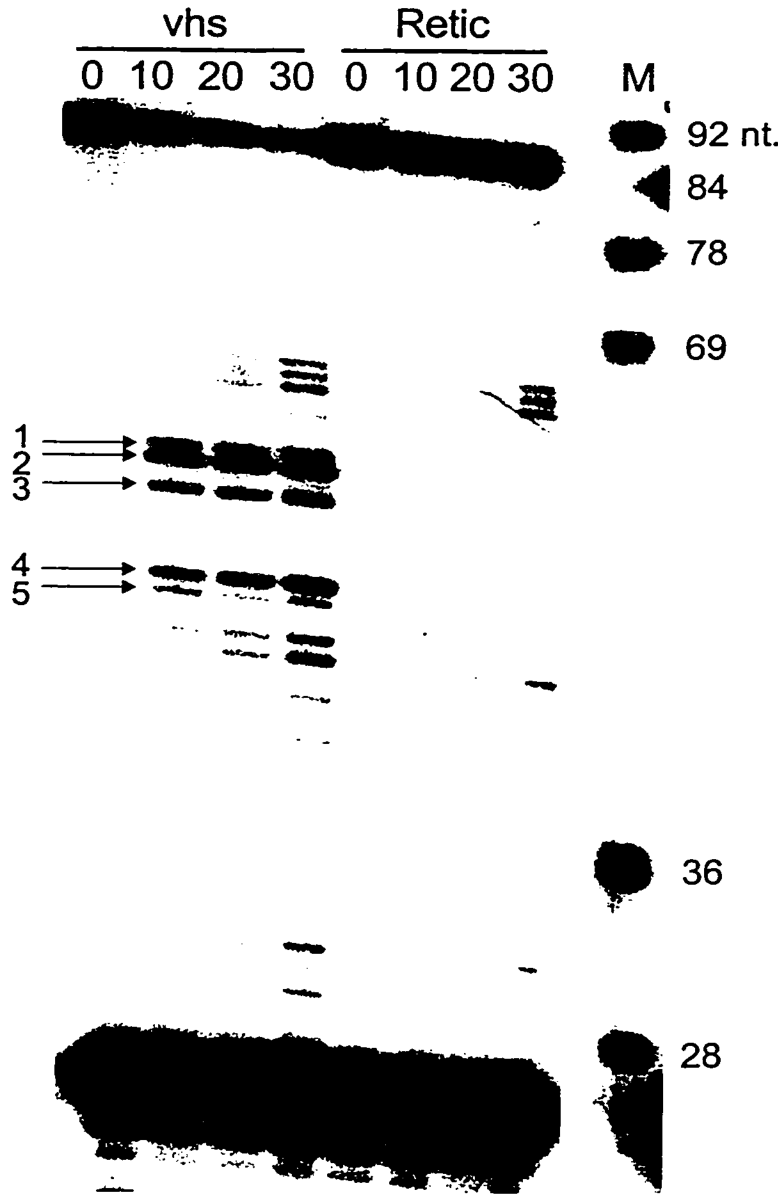
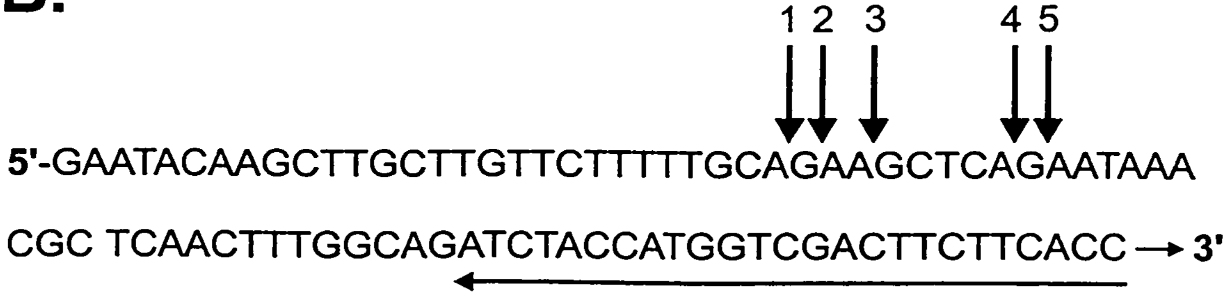


length of the intact substrate (figure 3-2 B and C, 2 minute time point) suggests that a substantial fraction of the RNA molecules are initially cleaved at a limited number of sites clustered in the 5' quadrant of the RNA. The data do not however demonstrate that all molecules are initially cleaved at these positions, and do not exclude the possibility that strong cleavage sites are also located very close to the 3' end of the RNA.

If, as argued above, vhs-induced decay proceeds through endoribonucleolytic cleavage, then novel 5' and 3' RNA termini should be generated at each of the putative sites of endonucleolytic cleavage. We tested this prediction by high resolution analysis of the events at the extreme 5' end of SRPα RNA. As shown figure 3-2D, the four 5'-most vhs-induced 5' limit digestion products generated from cap-labeled SRPα RNA migrate on a sequencing gel with apparent lengths of 30, 32, 38, and 39 nt when measured against DNA size markers. The 30 and 38 nt products were the most prominent amongst these. Taking the 5' cap into account, these data localize putative sites of endonucleolytic cleavage to positions +29, +31, +37, and +38. To test this interpretation, we asked if we could detect the predicted novel 5' termini of the 3' products produced by cleavage at these sites, by primer extension. Unlabeled capped SRPα RNA was added to RRL containing pre-translated vhs, and samples extracted at various times were analyzed by primer extension using a ^{32}P-labeled oligonucleotide complementary to residues 60 to 84 of the SRPα mRNA (figure 3-3A). We detected major primer extension products of 55, 53, and 48 nt and minor products of 56 and 47 nt (indicated by numbered arrows in figure 3-3A). The precise lengths of these products were determined by comparison to a DNA

Figure 3-3. Primer extension analysis of the 5'-most degradation products of SRPα RNA. (A) Unlabeled, capped SRPαRNA was added to RRL-vhs (lanes marked vhs) or RRL control (lanes marked control) and RNA reaction products were recovered at indicated time points (minutes). The RNA reaction products were then analyzed by primer extension using 5' ^{32}P-labeled oligonucleotide complementary to residues 60-84 of the SRPα RNA. Primer extension products were resolved on an 8% polyacrylamide sequencing gel and detected by autoradiography. Numbered arrows indicate the position of primer extension products representing vhs-induced novel 5' ends. Numbers to the side of lane M (marker) indicate the sizes of DNA markers in nucleotides. (B) Sequence of the extreme 5' 84 nt of SRPα RNA. Numbered arrows above the sequence correspond to those in panel A and point to the position of vhs-induced cleavage at the 5' end of SRPα RNA. The arrow under the sequence indicates the position of the oligonucleotide primer used.

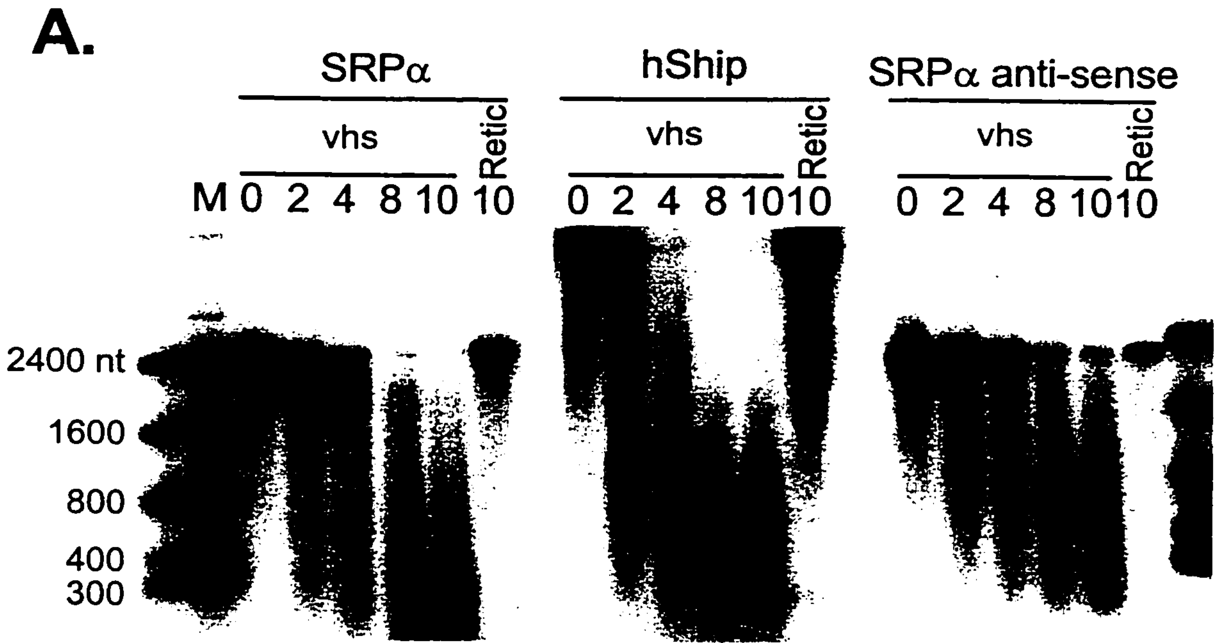

A.
vhs
Retic
0 10 20 30 0 10 20 30
M
92 nt.
84
78
69
36
28
1
2
3
4
5

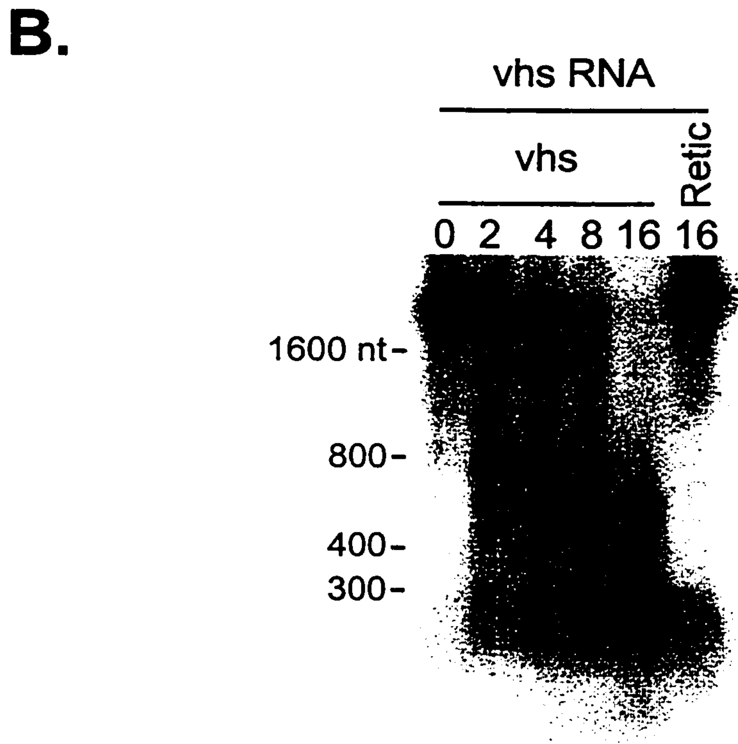

B.
1 2 3 4 5
5'-GAATACAAGCTTGCTTGTTCTTTTTGCAGAAGCTCAGAATAAA
CGC TCAACTTTGGCAGATCTACCATGGTCGACTTCTTCACC 3'

sequencing ladder produced with the same primer (data not shown). These novel 5' ends would correspond to endoribonucleolytic cleavage events at positions +29, +31 and +38 (major) and +28 and +39 nt (minor) on SRPα mRNA. The excellent agreement between the two data sets provides a very strong indication that vhs-induced RNA degradation activity occurs through an endonucleolytic process. The minor discrepancies between the relative abundance of the products detected by these two assays might stem from exonucleolytic fraying of some of the 3' and 5' ends generated by these endonucleolytic cleavages. The positions of the novel 5' ends detected by primer extension are displayed on the sequence of the SRPα transcript in figure 3-3B. Interestingly, all 5 of these 5'-most cleavages occur within GA or AG dinucleotides. Further experiments are required to determine if the vhs-induced activity displays marked sequence specificity.

Vhs induces degradation of a variety of RNA substrates, and displays activity in an *in vitro* translation system derived from HeLa cells. Vhs induces global inhibition of cellular protein synthesis during HSV infection and destabilizes both viral and cellular mRNAs *in vivo* (10, 13-15, 18-21, 28-31, 34, 36, 42, 46-49). To determine if vhs displays a similar lack of selectivity in our *in vitro* system, we compared the overall degradation profiles of internally labeled uncapped *in vitro* transcripts encoding SRPα (2.4 kb), human SH2-containing inositol phosphatase (hSHIP, 4.5 kb), and vhs itself (Figure 3-4). In addition, we tested an anti-sense transcript of the SRPα ORF (SRPα anti-sense; 2.2 kb). These RNA substrates are entirely unrelated in sequence, with the exception that the vhs and the SRPα RNAs share 69 nt at their extreme 5'ends, upstream the respective open reading frames. All of these transcripts were markedly destabilized in

Figure 3-4. Vhs induces degradation of a variety of RNA substrates. (A) Internally labeled SRPα (2.4 kb), hSHIP (4.5 kb), and SRPα anti-sense (2.2 kb) RNAs were added to RRL containing vhs (lanes marked vhs) or RRL control (lanes marked Retic) and samples recovered at indicated times (minutes) were analyzed by agarose/formaldehyde gel electrophoresis as in figure 3-1B. (B) Internally labeled vhs RNA (1.8 kb) was reacted with RRL containing vhs or control RRL and samples recovered at indicated times (minutes) were analyzed as in panel (A).


A.
SRPα
vhs
Retic
M 0 2 4 8 10 10
hShip
vhs
Retic
0 2 4 8 10 10
SRPα anti-sense
vhs
Retic
0 2 4 8 10 10
2400 nt
1600
800
400
300

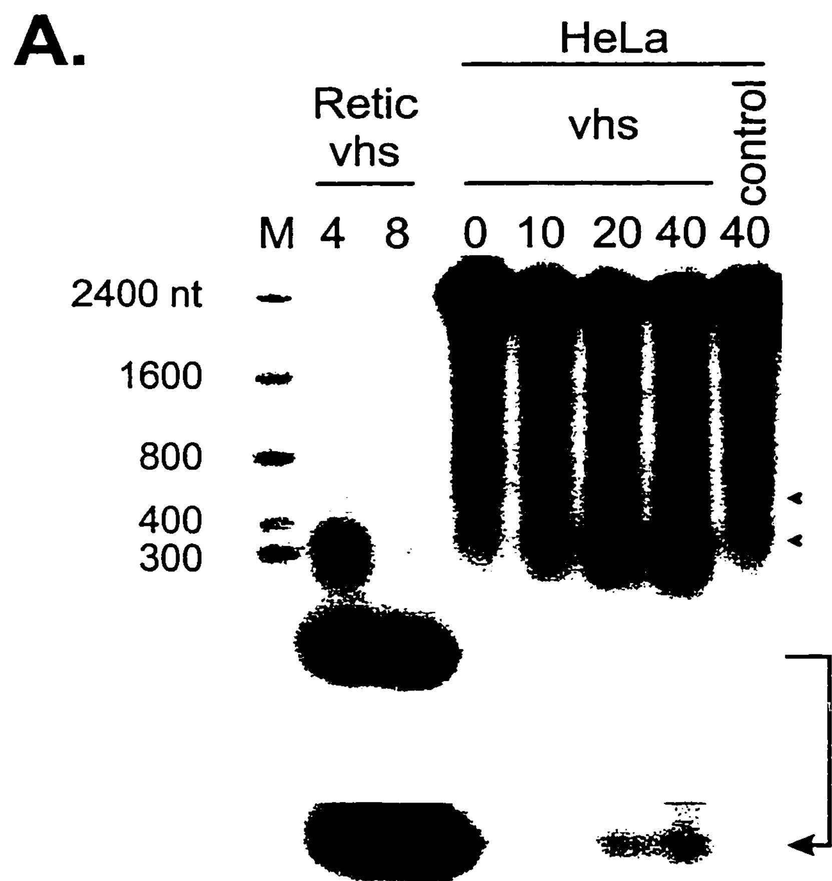

B.
vhs RNA
vhs
Retic
0 2 4 8 16 16
1600 nt-
800-
400-
300-

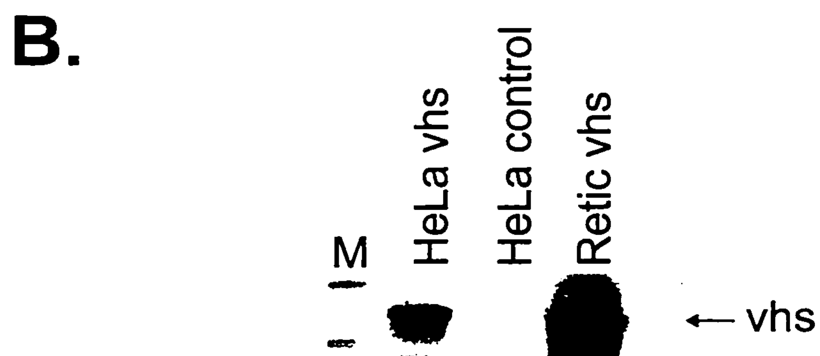
RRL containing pre-translated vhs (figure 3-4). We have not yet characterized the mode of degradation of these additional RNAs in detail, and therefore do not know if they contain preferred sites for initial cleavage such as those detected above in the SRPα transcript.

Read and colleagues have shown that extracts of infected HeLa cells display vhs-dependent RNA destabilizing activity *in vitro* (19). It was therefore of interest to determine if vhs induces accelerated RNA turnover when it is expressed as the only HSV protein in an *in vitro* translation system derived from HeLa cells, and if so, whether the mode of RNA degradation resembles that observed in the RRL system. As shown in figure 3-5A and B, cap labeled SRPα RNA was less stable in HeLa cell translation extracts containing pre-translated vhs than in control extracts lacking vhs. Moreover, some of the vhs-dependent degradation intermediates observed in the HeLa extract displayed electrophoretic mobilities similar to those obtained in the RRL system (indicated by arrowheads). However, the overall rate of vhs-induced RNA decay was substantially lower in the HeLa extract than in the RRL system. This difference is most likely due to the large difference in the amount of vhs protein produced in the two *in vitro* translation systems (figure 3-5D). These data demonstrate that vhs suffices to induce accelerated RNA turnover when it is expressed as the only HSV protein in extracts derived from human cells, and offer a preliminary suggestion that the mode of vhs-induced RNA decay may be similar in the two systems.

Vhs-induced RNA degradation does not require a 5' cap or a 3' poly(A) tail

Figure 3-5. Vhs synthesized in a HeLa cell translation extract induces degradation of SRPα RNA. (A) HeLa cell translation extracts and RRL were programmed with capped unlabeled vhs RNA and translation was allowed to proceed for 60 minutes in the presence of ^{35}S-methionine. Extracts were then challenged with cap labeled SRPα RNA, and samples were recovered at indicated times (minutes). Reaction products were then analyzed by agarose/formaldehyde gel electrophoresis as in figure 3-1B. The bottom of panel A shows an overexposure of the lower portion of the membrane in panel A (indicated by an arrow). Control: HeLa cell extracts lacking vhs RNA. Numbers to the side of panel A indicate the sizes of RNA markers in nucleotides. Filled arrow heads indicate the position of vhs-induced RNA degradation products. (B) Samples of the translation reactions used in panel A were resolved on a 12% SDS polyacrylamide gel and the ^{35}S signal was detected by autoradiography.

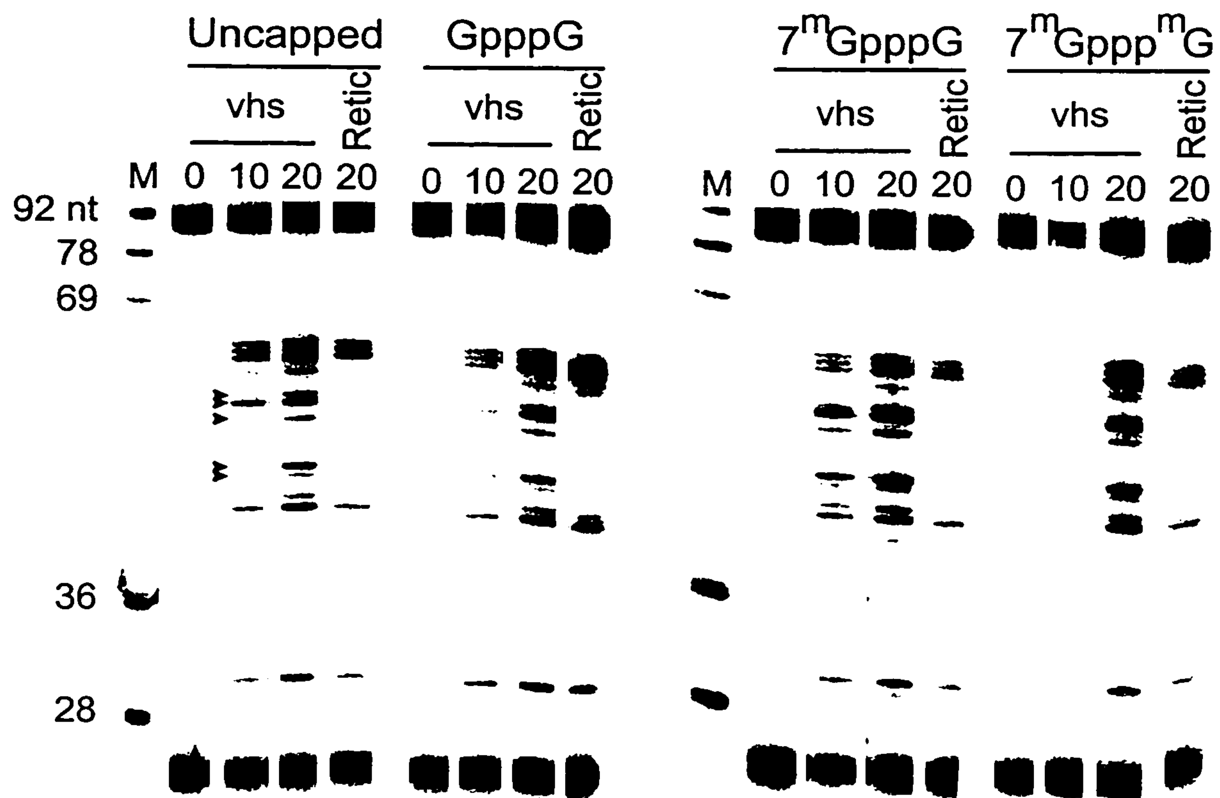

A.
HeLa
Retic
vhs
vhs
control
M 4 8 0 10 20 40 40
2400 nt
1600
800
400
300

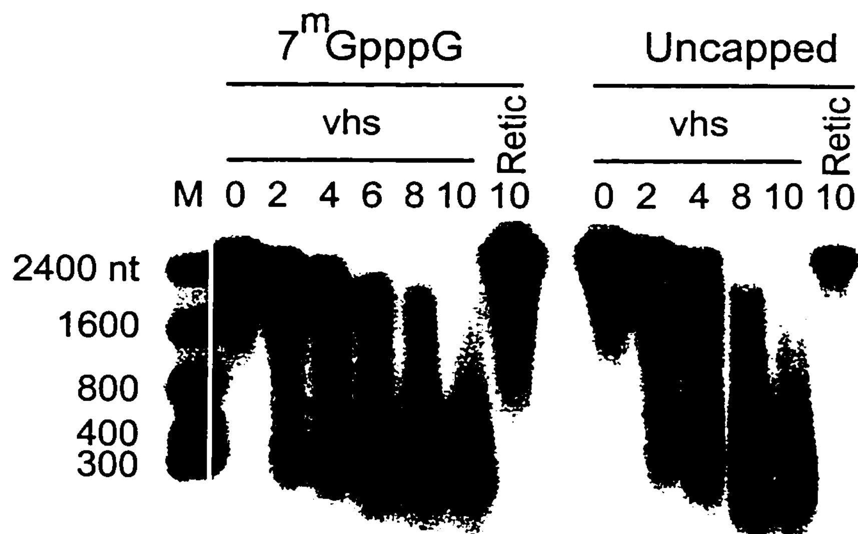

B.
M
HeLa vhs
HeLa control
Retic vhs
← vhs

in the RNA substrate. Although vhs appears to destabilize most if not all mRNAs in infected cells, ribosomal RNA is not degraded (19, 20, 30, 52). This observation raises the possibility that some feature(s) specific to mRNA molecules renders them susceptible to vhs-induced cleavage. Two obvious candidates are the 5' cap structure and 3' poly(A) tract. To investigate whether the presence of a 5' cap influences vhs-induced RNA degradation, we compared the rate and mode of degradation of capped and uncapped SRPα RNA (figure 3-6). In the first experiment, equal amounts of unlabeled SRPα RNA bearing a 5' triphosphate terminus (uncapped), or one of three different cap structures (GpppG, 7^{m}GpppG, and 7^{m}Gppp^{m}G), were added to reticulocyte lysates containing vhs and RNA samples recovered at the indicated times were analyzed by primer extension as in figure 3-3 (figure 3-6A). The results indicated that the presence of a cap structure did not greatly influence the rate of cleavage at the extreme 5' end of the substrate. Note that the level of background vhs-independent cleavages was greater in this experiment than that shown in figure 3-3. We also compared the overall degradation profile of internally labeled uncapped and 7^{m}GpppG capped SRPα RNA (figure 3-6B). Again, the presence of a 5' cap structure did not greatly alter the rate of RNA decay or the nature of the degradation intermediates. Consistent with these findings, we found that vhs activity was not altered when the cap binding protein eIF4E was depleted from the extracts using 7^{m}GTP Sepharose resin (data not shown).

None of the RNA substrates examined above contained a 3' poly(A) tail, indicating that this feature is not required for substrate recognition. To directly assess whether the presence of a 3' poly(A) tract alters the rate of the reaction, we compared the

Figure 3-6. Vhs-induced RNA degradation is cap-independent. (A) Unlabeled SRPα RNAs bearing the indicated 5' cap structures were added to RRL-vhs (lanes marked vhs) and control RRL (lanes marked Retic) and samples were recovered at indicated times (minutes). The RNA reaction products were then analyzed by primer extension using 5' ^{32}P-labeled oligonucleotide complementary to residues 60-84 of the SRPα RNA, as in figure 3-3. Filled arrowheads indicate the mobilities of the vhs-induced products. Numbers to the side of panel A indicate the sizes of DNA markers (M) in nucleotides. (B) Internally labeled capped (7^{m}GpppG) and uncapped SRPα RNAs were added to RRL-vhs (lanes marked vhs) and control RRL (lanes marked Retic). Samples recovered at indicated times (minutes) were then analyzed by agarose/formaldehyde gel electrophoresis. Numbers to the side of panel B indicate the sizes of RNA markers (lane M) in nucleotides.


A.
Uncapped
GpppG
7mGpppG
7mGpppmG
vhs
Retic
M
0 10 20 20
92 nt
78
69
36
28


B.
7mGpppG
Uncapped
vhs
Retic
M 0 2 4 6 8 10 10
0 2 4 8 10 10
2400 nt
1600
800
400
300

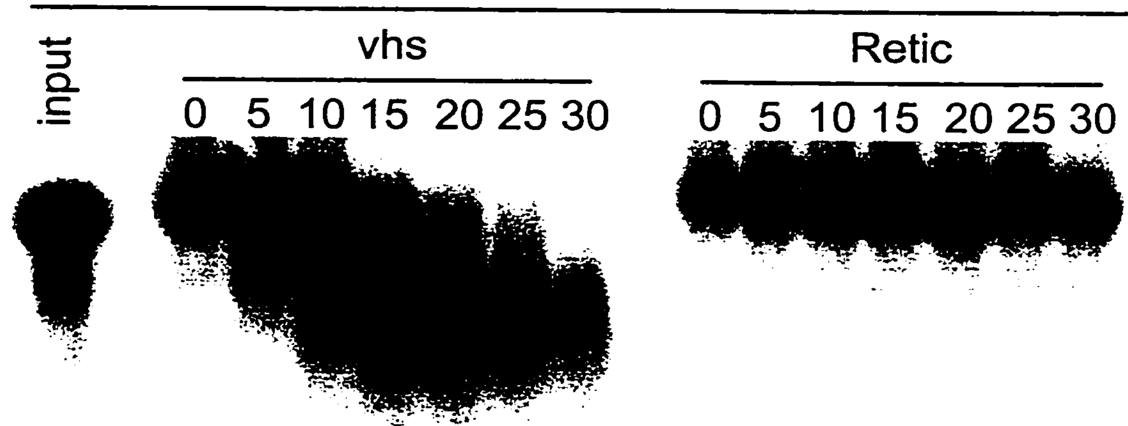
degradation profile of internally labeled uncapped bovine preprolactin RNA containing a 35 residue poly(A) tract (figure 3-7A) to that of a derivative lacking the poly(A) tract (figure 3-7B). The presence of the poly(A) tail had little or no effect on the rate or course of reaction.

Taken in combination, these data demonstrate that neither the 5' cap nor the 3' poly(A) tail detectably influence vhs-induced decay of substrate RNAs in this rabbit reticulocyte lysate-based system.

Vhs-induced RNA degradation occurs in the absence of ribosomes, and requires magnesium. Vhs-induced mRNA decay in HSV1-infected cells occurs in the presence of drugs that block translational initiation and elongation, suggesting that substrate mRNAs need not be engaged in ongoing translation in order to be degraded (13, 39). Furthermore, Sorenson and co-workers (43) reported that vhs-dependent RNA degradation activity partitions with the post-ribosomal fraction of extracts of HSV1 infected cells. To determine if ribosomes are required to recruit vhs activity to substrate RNAs in our *in vitro* system, we removed the ribosomes from the reticulocyte lysate (after translating vhs) by centrifugation at 160,000 Xg for 50 minutes in an airfuge. Northern blot analysis revealed that all of the 18S rRNA was removed from the post-ribosomal supernatant (figure 3-8B), confirming that the procedure effectively depleted the extract of ribosomes. The post-ribosomal supernatant and ribosomal pellet were then assayed for vhs activity, using uncapped internally labeled SRPα RNA as the substrate (figure 3-8A).

Figure 3-7. Vhs-induced RNA degradation is not influenced by a 3' poly(A) tail. Internally labeled bovine preprolactin (PPL) RNA containing (A) or lacking (B) a ~35 A residue poly(A) tail followed by GU was incubated with RRL-vhs (lanes marked vhs) and RRL control (lanes marked Retic) for the indicated times (minutes). RNA reaction products were then analyzed by agarose/formaldehyde gel electrophoresis as in the legend of figure 3-1. Input: untreated RNAs.

A.



B.

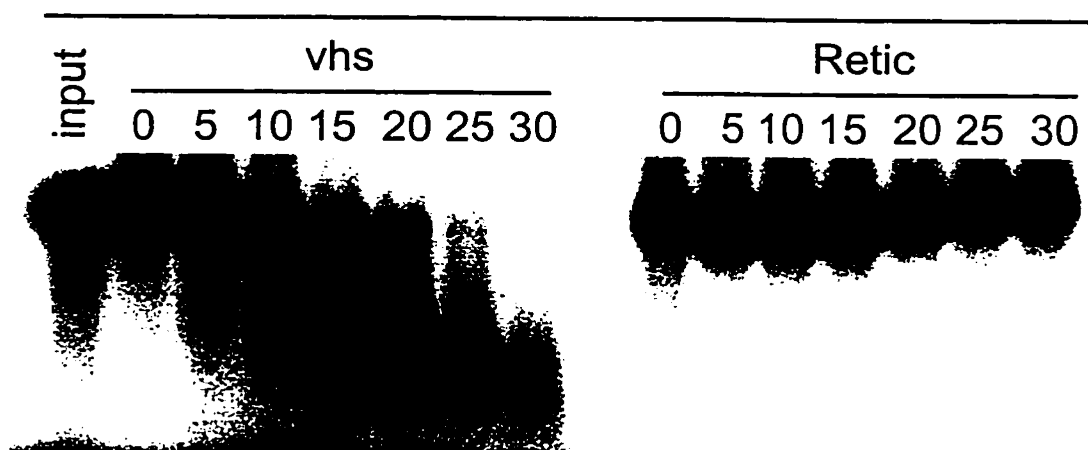
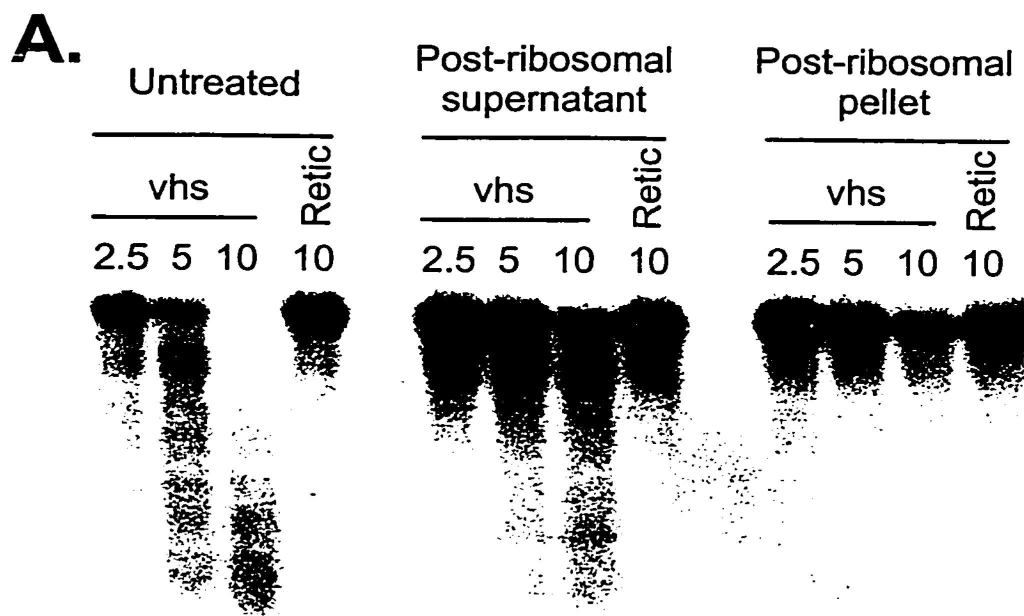
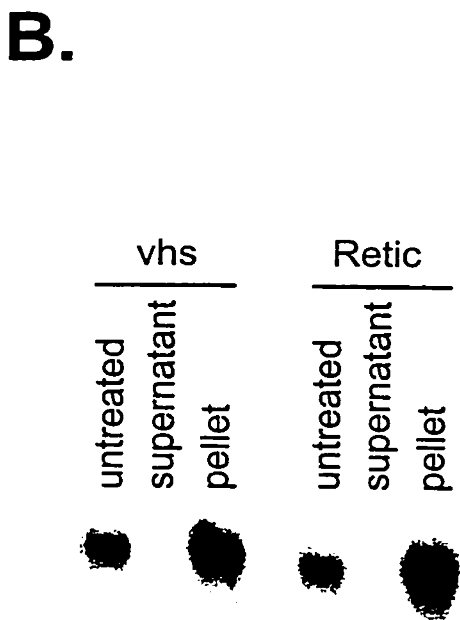
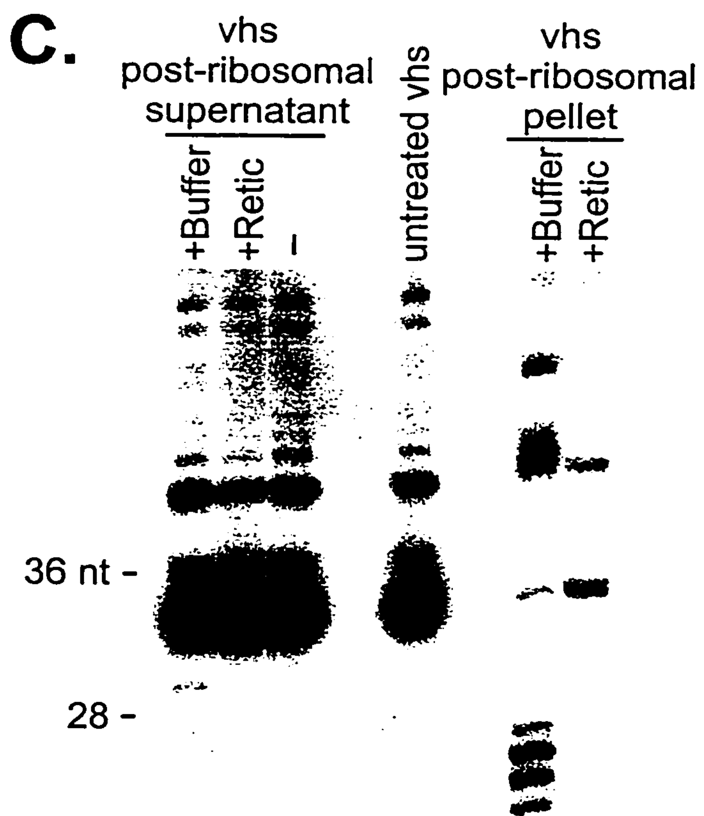


Figure 3-8. Vhs-induced RNA degradation does not require ribosomes. RRL containing (vhs) or lacking (Retic) vhs were centrifuged at 160,000 Xg in an airfuge for 50 minutes at 4°C to pellet the ribosomes. The ribosomal pellet was resuspended in "retic buffer" (1.6 mM TrisOAc pH7.8, 80 mM KOAc, 2 mM MgOAc, 0.25 mM ATP, 0.1 mM DTT). (A) Untreated lysates, post-ribosomal supernatants, and pellets were mixed with internally labeled SRPα RNA, and samples recovered at various times (minutes) were analyzed by agarose/formaldehyde gel electrophoresis as in figure 3-1B. (B) Northern blot analysis of the post-ribosomal supernatant and pellet fractions using a rabbit 18S rRNA specific 5' ^{32}P-labeled oligonucleotide probe. (C) Cap labeled SRPα RNA was added to untreated RRL-vhs, and to post-ribosomal supernatants and pellets that had been mixed with an equal volume of Retic buffer (+buffer) or naive RRL (+Retic). RNA samples were recovered after 10 minutes, then resolved on an 8% polyacrylamide sequencing gel.


A.
Untreated
Post-ribosomal supernatant
Post-ribosomal pellet
vhs
Retic
2.5 5 10 10
2.5 5 10 10
2.5 5 10 10

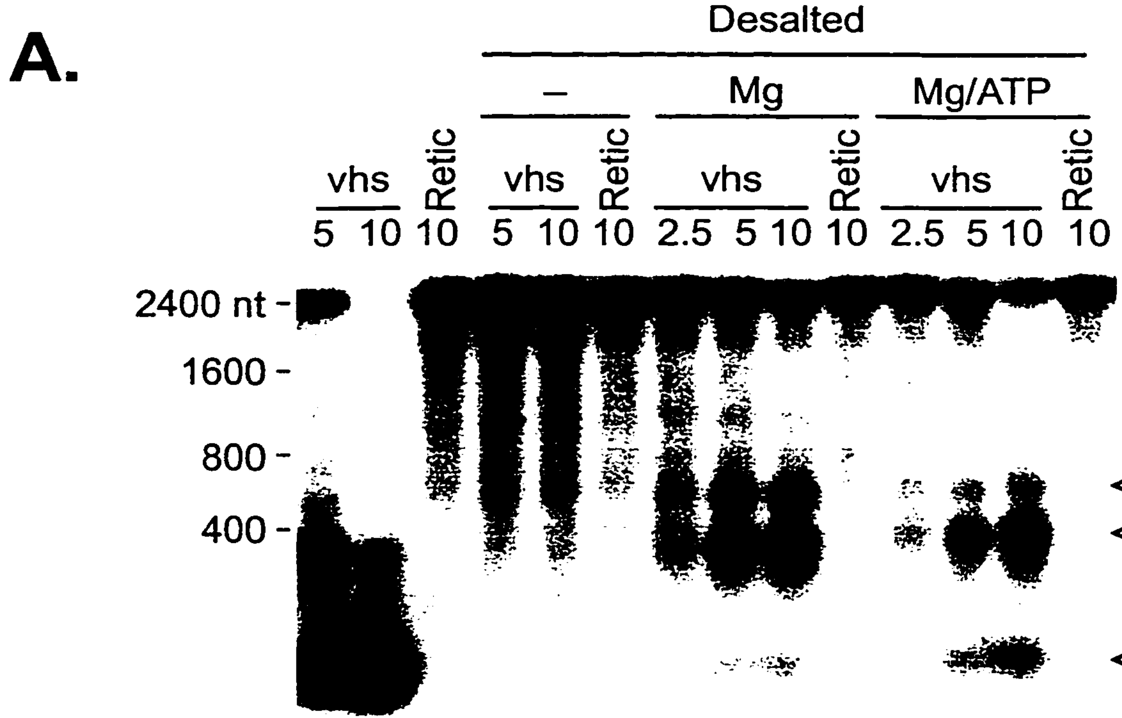

B.
vhs
Retic
untreated
supernatant
pellet
untreated
supernatant
pellet

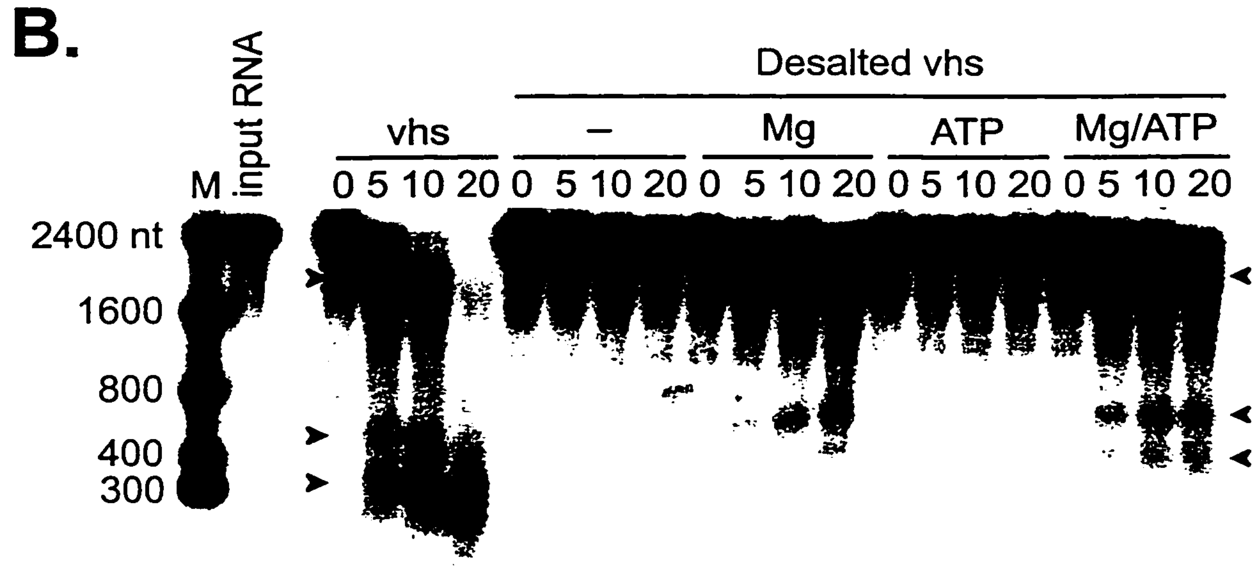

C.
vhs post-ribosomal supernatant
untreated vhs
vhs post-ribosomal pellet
+Buffer
+Retic
-
+Buffer
+Retic
36 nt -
28 -

This experiment indicated that the vhs activity was predominantly associated with the post-ribosomal fraction. This conclusion was confirmed in a experiment where the post-ribosomal supernatant and ribosomal pellet were assayed for activity on 5' cap labeled SRPα RNA, and the reaction products were displayed on an 8% polyacrylamide sequencing gel (figure 3-8C).

Kirkorian and Read (19) reported that the vhs-dependent RNA degradation activity observed in extracts of HSV1 infected cell requires Mg^{++} but not ATP. We examined the ATP and Mg^{++} requirements of the rabbit reticulocyte lysate system. Small molecules were removed from lysates (after translating vhs) by passage over a Sephadex G25 spin column. The resulting desalted extracts were then assayed for vhs activity before and after reconstitution with 0.25 mM ATP and/or 2 mM MgOAc. In the experiment depicted in figure 3-9, cap labeled SRPα (figure 3-9A) and uncapped internally labeled (figure 3-9B) SRPα RNAs were used as substrates for vhs activity in intact, desalted, and desalted/reconstituted extracts. The desalted extracts were devoid of activity, which was partially restored by adding Mg^{++} ions. ATP had no effect by itself (figure 3-9B), but marginally increased the rate of the reaction when it was added in combination with Mg^{++} ions (figure 3-9A, B). The overall reduction in activity after desalting may be due to dilution effects and loss of some of the vhs protein. Alternatively, it is possible that additional co-factors are required for optimal activity. These data demonstrate that the vhs-induced activity requires Mg^{++} ions (as previously reported (19)) and offer a suggestion ATP may accelerate the rate of RNA degradation in the presence of Mg^{++} ions.

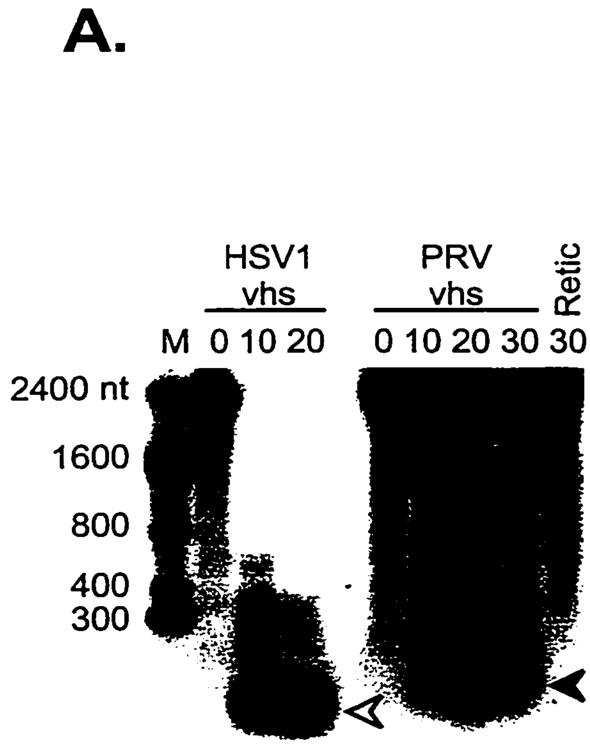
Figure 3-9. Vhs-induced RNA degradation requires magnesium. RRL containing (vhs) or lacking (retic) vhs were desalted on Sephadex G25 spin columns at a ratio of 4 ml of packed resin per 100 *m*l of lysate. The resin was swollen in "retic buffer" (1.6 mM TrisOAc pH7.8, 80 mM KOAc, 0.1 mM DTT), loaded in glass wool plugged 5 ml syringe and prespun for 5 minutes at 4°C and 3000 rpm in a clinical centrifuge equipped with a swinging bucket rotor. Samples of the desalted lysates were then combined with and equal volume of retic buffer containing 4 mM MgOAc (lanes marked Mg), 0.5 mM ATP (lanes marked ATP) or 4 mM MgOAc and 0.5 mM ATP (lanes marked Mg/ATP). Substrate SRPα RNA was then added, and samples withdrawn at the indicated times (minutes) were analyzed by formaldehyde/agarose gel electrophoresis. (A) Analysis of cap labeled SRPα RNA. (B) Analysis of internally labeled SRPα RNAs. Arrowheads indicate the mobilities of some of the vhs-dependent RNA degradation intermediates. Numbers to the side of the panels indicate the sizes of RNA markers (M) in nucleotides.

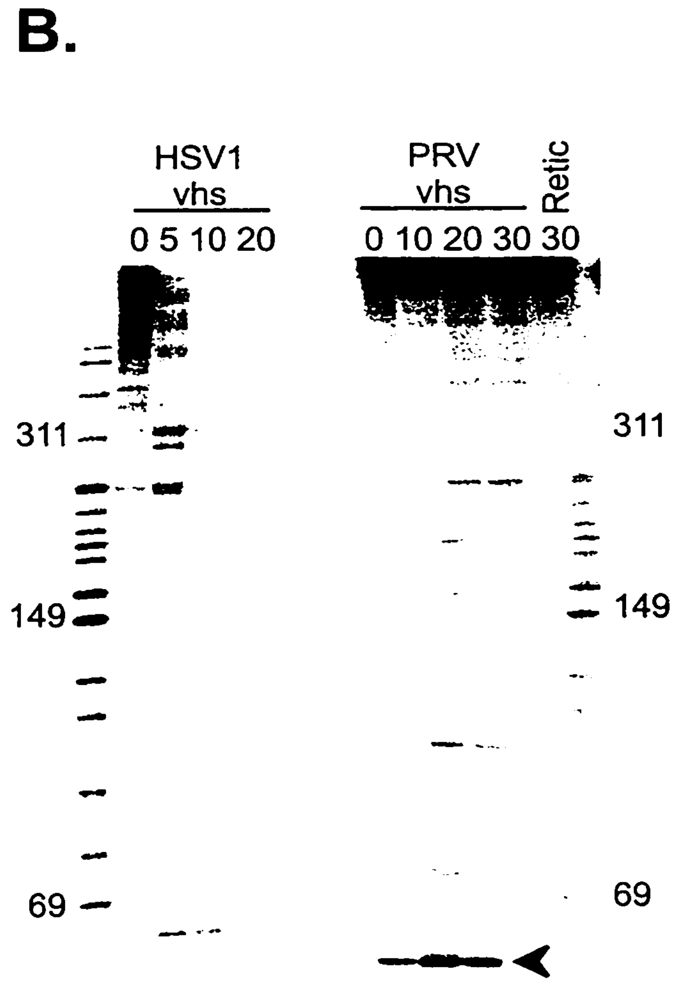

A.
Desalted
–
Mg
Mg/ATP
vhs
Retic
vhs
Retic
vhs
Retic
vhs
Retic
5 10 10 5 10 10 2.5 5 10 10 2.5 5 10 10
2400 nt
1600
800
400

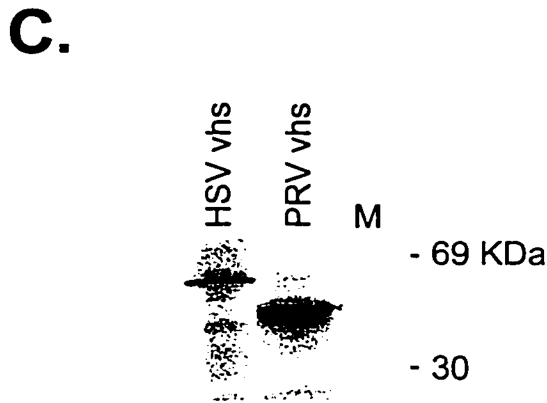

B.
M
input RNA
Desalted vhs
vhs
–
Mg
ATP
Mg/ATP
0 5 10 20 0 5 10 20 0 5 10 20 0 5 10 20 0 5 10 20
2400 nt
1600
800
400
300

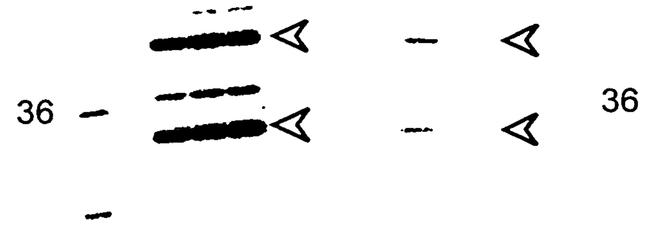
The Pseudorabies virus vhs homologue induces RNA degradation *in vitro*. Homologues of vhs have been found in all of the alphaherpesviruses genomes characterized to date (for example, see reference (3)). However, with the exception of the proteins encoded by HSV1 and HSV2, it is not yet clear if these vhs homologues trigger accelerated RNA turnover. To address this question, we asked if the vhs homologue of Pseudorabies virus (Prv, (3)), displays activity in the reticulocyte lysate system. Cap-labeled SRPα RNA was added to lysates containing pre-translated HSV1 and Prv vhs, and the reaction products were analyzed on an agarose/formaldehyde gel (figure 3-10A), and an 8% polyacrylamide sequencing gel (figure 3-10B). We found that the RNA substrate was destabilized in lysates containing Prv vhs relative to the blank reticulocyte lysate control (figure 3-10A). However, the rate of induced decay was substantially less than that provoked by HSV1 vhs. Inasmuch as the Prv lysate contained at least as much vhs protein as the HSV1 sample (Figure 3-10C), these data may indicates that the Prv vhs homologue displays reduced activity relative to its HSV1 counterpart. Degradation induced by the Prv protein appeared to proceed through intermediates that were, for the most part, different from those induced by the HSV1 vhs (filled versus open arrowheads in figure 3-10A and B). The only common intermediates detected were the 30 and 40 nt 5' fragments which accumulated to a lesser extent in reactions containing Prv vhs (open arrow heads in figure 3-10B).

Figure 3-10. Pseudorabies virus vhs displays RNA degradation activity *in vitro*. RRL were programmed with RNAs encoding HSV1 or Prv vhs and translation was allowed to proceed for 60 minutes. Lysates were then challenged with cap labeled SRPα RNA and samples recovered at indicated times (minutes) were analyzed by electrophoresis through an agarose/formaldehyde gel (A) and an 8% polyacrylamide sequencing gel (B). Numbers to the side of panels indicate the sizes of marker fragments in nucleotides (M; RNA and DNA in panels A and B respectively). Arrowheads indicate the positions of corresponding RNA fragments in the two different gel systems. (C) SDS-Page analysis of the HSV and Prv vhs proteins produced in the translation reactions used in panels A and B.


A.
HSV1
vhs
PRV
vhs
Retic
M 0 10 20 0 10 20 30 30
2400 nt
1600
800
400
300


B.
HSV1
vhs
PRV
vhs
Retic
0 5 10 20 0 10 20 30 30
311
311
149
149
69
69


C.
HSV vhs
PRV vhs
M
- 69 KDa
- 30


36
36

3.5. Discussion.

Previous reports demonstrated that the HSV1 vhs protein is necessary and sufficient to trigger accelerated RNA turnover *in vivo*, and in extracts prepared from mammalian cells and partially purified HSV virions (18, 19, 32, 43, 52). Although highly informative, these studies left many basic questions about the mechanism of vhs action unanswered. In particular, the overall mode of vhs-induced RNA decay had yet to be defined. We therefore conducted a detailed examination of the mechanism of vhs-dependent mRNA degradation, using a rabbit reticulocyte lysate based *in vitro* system (52).

Our data establish that vhs-induced RNA decay proceeds (at least in part) through endoribonucleolytic cleavage events (figures 3-2 and 3-3). The strongest evidence supporting this conclusion emerged from a high resolution analysis of the vhs-dependent events at the extreme 5' end of SRPα RNA, where we were able to detect matching novel 5' and 3' termini at several of the putative sites of endonucleolytic cleavage (figures 3-2 and 3-3). Our conclusion that vhs induces endoribonucleolytic cleavage is in accord with a previous suggestion that the vhs-dependent ribonuclease present in virion extracts acts as an endonuclease (52). However, as acknowledged by the authors, the data presented in that earlier report did not definitively establish this point.

Vhs destabilizes mRNAs but not rRNAs *in vivo* (19, 30, 31, 52), an observation that raises the possibility that one or more feature(s) common to most mRNAs (such as the 5' cap structure or 3' poly(A) tail) might specifically target mRNAs for selective

degradation. However, we found that the *in vitro* reaction was not detectably influenced by the presence of a 5' cap or 3' poly(A) tail in the RNA substrate. These observations indicate that neither of these two characteristic features of mRNAs plays a major role in substrate recognition in our *in vitro* system. Moreover, the majority of the endoribonuclease activity partitioned with the post-ribosomal fraction, arguing against the possibility that ribosomes serve to selectively deliver the nuclease to mRNAs. The cap- and ribosome-independence of our *in vitro* system mirrors earlier data indicating that the vhs-dependent ribonuclease present in virion extracts is cap-independent (52), and that the RNA destabilizing activity detected in extracts of HSV-infected HeLa cells partitions with the post-ribosomal fraction (43). In addition, our observation that poly(A)$^+$ and poly(A)$^-$ RNA substrates are degraded at comparable rates *in vitro* is consistent with an earlier report that histone H3 and H4 mRNAs (which lack poly(A) tails (25)) are destabilized in HSV infected cells (35). Thus, the basis for the apparently selective destruction of mRNAs *in vivo* remains obscure. Perhaps, as suggested by Zelus and co-workers (52), mRNAs are selectively targeted *in vivo* because they are relatively free of secondary structure, and are packaged into ribonucleoprotein structures that are more accessible to nuclease attack than ribosomes.

As reviewed in the introduction, the currently available evidence strongly suggests that vhs is either a ribonuclease, or serves as a required subunit of a ribonuclease that also contains one or more cellular subunits. Our finding that the HSV1 and Prv vhs homologues induce the formation of partially overlapping yet distinct sets of 5' terminal degradation products of SRPα RNA is consistent with this suggestion, as it demonstrates

that the choice of cleavage sites depends on the nature of the vhs protein. A definitive determination of whether or not vhs has ribonuclease activity in the absence of cellular proteins will require purification of biologically active vhs to homogeneity.

Our detailed characterization of the degradation intermediates of SRPα RNA indicated that many of the most prominent sites of initial cleavage induced by HSV1 vhs are clustered over the 5' quadrant of this RNA, in an interval extending from ca. 200-700 nt from the 5' end. The basis for this apparent clustering remains unknown, and it is not yet clear whether many or all RNA substrates display a similar profile. Apparently arguing against this possibility, Zelus and colleagues (52) suggested that the vhs-dependent ribonuclease activity present in virion extracts preferentially cleaves globin mRNA close to the 3' end. High resolution mapping of the cleavage sites at the extreme 5' end of SRPα RNA provided little evidence of sequence specificity, aside from a tendency to cleave between purine residues (figure 3-3). Moreover, the data of Zelus and colleagues (52) suggest that cleavage can also occur within AC and CU dinucleotides. We have recently mapped a number of additional cleavage sites at high resolution (Elgadi and Smiley, in preparation). Of 30 sites analyzed (including the four indicated in figure 3-3), 15 correspond to AG or GA dinucleotides (the others are GC (6), UG (5), GU, UU, UC, CG, and AC (1 each)). Thus, the available data argue that the vhs-induced endoribonuclease displays a relatively relaxed sequence specificity. This suggests that other features, such as RNA secondary structure, may play a major role in defining the sites of preferential cleavage.

Vhs displays limited but significant amino acid homology with the fen-1 family of nucleases (8) that are involved in DNA replication and repair (reviewed in reference (24)). Although fen-1 was initially identified as a DNase, Stevens (44) recently reported that it also cleaves RNA substrates in a cap-independent reaction that requires magnesium. The preferred sites of cleavage in several RNA substrates are confined to the 5'-most 200 nt of the transcript, and are located at the 5' base of predicted stem-loop structures. Based on these results, Stevens (44) proposed that fen-1 loads onto the 5' end of the RNA, then tracks in a 3' direction until it encounters secondary structure elements that trigger cleavage. Similarly, it is possible that the vhs-dependent endoribonuclease loads at one or both ends of the RNA, and migrates until it encounters preferred cleavage determinants. Although the vhs-dependent cleavage sites that we have mapped at the extreme 5' end of SRPα RNA do not obviously correlate with predicted features of RNA secondary structure (data not shown), we have not yet precisely mapped the more prominent sites of initial cleavage that are located ca. 200-700 nt. from the 5' end of the RNA. In this context it is interesting to note that we have recently found that inserting a picornavirus internal ribosome entry site (IRES) at a variety of sites throughout the SRPα transcript provokes novel vhs-dependent endoribonucleolytic cleavage events in the sequences located immediately downstream of the inserted IRES (Elgadi and Smiley, 1999). Inasmuch as IRES elements exhibit extensive secondary structure (27, 33, and reviewed in reference 38), this observation is consistent with (but does not prove) the hypothesis that secondary structure may play a major role in dictating the sites of vhs-induced cleavage.

Our data strongly suggest that vhs-dependent RNA decay is initiated by endoribonucleolytic cleavage, and establish that 5' fragments produced early during the reaction can be re-cleaved by the vhs-dependent endonuclease as the reaction proceeds (figure 3-2). However, they do not exclude the possibility that other cellular endo- and exoribonucleases also contribute the subsequent decay of the primary cleavage products, particularly *in vivo*. Thus, as previously proposed (32, 43, 52), it is possible that vhs achieves translational arrest by endonucleolytic cleavage of mRNAs at limited number of sites, and the products of these initial cleavages are then further processed by existing cellular mRNA surveillance pathways (reviewed in reference (1)). An analogous pathway regulates the stability of human transferrin receptor mRNA and Xenopus Xlhbox2B and Xoo1 mRNAs (4, 5). These mRNAs undergo endonucleolytic cleavage in their 3' UTRs, generating unstable 5' and 3' products that are, likely, further processed by cellular exonucleases. These initiating endonucleolytic cleavages resembles the vhs activity described in this report, in that they do not require a 5' cap structure, 3' poly(A) tail, or ongoing translation. This model might help explain how the limited amount of vhs delivered by the infecting HSV virion is able to trigger global shutoff of host protein synthesis.

The Prv vhs homologue shares most of the amino acid sequences that are conserved among the vhs proteins encoded by alphaherpesviruses (3, 18). Despite this conservation, host shutoff induced by infection with Prv requires *de novo* viral protein synthesis (2, 17), suggesting that Prv vhs is either less active than its HSV1 counterpart, or not packaged into virions. Our finding that Prv vhs induces RNA decay *in vitro*

substantially less efficiently than its HSV1 counterpart strongly argues for the former possibility, and illustrates the sensitivity of our *in vitro* assay.

Our findings provide a glimpse in to the mechanism of vhs activity and begin to define some of the requirements of the vhs-induced RNA degradation reaction. Further studies of the vhs-induced RNA decay pathway may ultimately lead to a better understanding of analogous cellular pathways.

3.6. Acknowledgements.

We thank Joanne Duncan and Carol Lavery for superb technical assistance, David Andrews for many gifts of plasmids and advice on *in vitro* translation systems, Peter Whyte for pBlueK(coreD), Alberto Epstein for pPRV41, and Evan Llewelyn for help constructing the Prv vhs expression vector. This work was supported by a grant from the National Cancer Institute of Canada. JRS was a Terry Fox Senior Scientist of the NCI(C).

3.7. References.

1. **Beelman, C. A., and R. Parker.** 1995. Degradation of mRNA in eukaryotes. Cell. **81**:179-183.

2. **Ben-Porat, T., T. Rakusanova, and A. S. Kaplan.** 1971. Early functions of the genome of herpesvirus. II. Inhibition of formation of cell-specific polysomes. Virology. **46**:890-899.

3. **Berthomme, H., B. Jacquemont, and A. Epstein.** 1993. The pseudorabies virus host-shutoff homolog gene: nucleotide sequence and comparison with alphaherpesvirus protein counterparts. Virology. **193**:1028-32.

4. **Binder, R., J. A. Horowitz, J. P. Basilion, D. M. Koeller, R. D. Klausner, and J. B. Harford.** 1994. Evidence that the pathway of transferrin receptor mRNA degradation involves an endonucleolytic cleavage within the 3' UTR and does not involve poly(A) tail shortening. EMBO J. **13**:1969-1980.

5. **Brown, B. D., I. D. Zipkin, and R. M. Harland.** 1993. Sequence-specific endonucleolytic cleavage and protection of mRNA in Xenopus and Drosophila. Genes & Dev. **7**:1620-1631.

6. **Carroll, R., and J. Lucas-Lenard.** 1993. Preparation of a cell-free translation system with minimal loss of initiation factor eIF-2/eIF-2B activity. Anal Biochem. **212**:17-23.

7. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA. **81**:1991-1995.

8. **Doherty, A. J., L. C. Serpell, and C. P. Ponting.** 1996. The helix-hairpin-helix DNA-binding motif: a structural basis for non-sequence-specific recognition of DNA. Nucleic Acids Res. **24**:2488-2497.

9. **Falcone, D., and D. W. Andrews.** 1991. Both the 5' untranslated region and the sequence surrounding the start site contribute to efficient initiation of translation in vitro. Mol. Cell. Biol. **11**:2656-2664.

10. **Fenwick, M. L., and J. Clark.** 1982. Early and delayed shut-off of host protein synthesis in cells infected with herpes simplex virus. J. Gen. Virol. **61**:121-5.

11. **Fenwick, M. L., and R. D. Everett.** 1990. Inactivation of the shutoff gene (UL41) of herpes simplex virus types 1 and 2. J. Gen. Virol. **71**:2961-7.

12. **Fenwick, M. L., and R. D. Everett.** 1990. Transfer of UL41, the gene controlling virion-associated host cell shutoff, between different strains of herpes simplex virus. J.Gen. Virol. **71**:411-8.

13. **Fenwick, M. L., and M. M. McMenamin.** 1984. Early virion-associated suppression of cellular protein synthesis by herpes simplex virus is accompanied by inactivation of mRNA. J. Gen. Viro. **65**:1225-1228.

14. **Fenwick, M. L., and S. A. Owen.** 1988. On the control of immediate early (alpha) mRNA survival in cells infected with herpes simplex virus. J. Gen. Virol. **69**:2869-77.

15. **Fenwick, M. L., and M. J. Walker.** 1978. Suppression of synthesis of cellular macromolecules by herpes simplex virus. J. Gen. Virol. **41**:37-51.

16. **Honess, R., and B. Roizman.** 1974. Regulation of herpes macromolecular synthesis I. Cascade regulation of synthesis of three groups of viral proteins. J. Virol. **14**:8-19.

17. **Ihara, S., L. Feldman, S. Watanabe, and T. Ben-Porat.** 1993. Characterization of the immediate-early functions of pseudorabies virus. Virology. **131**:437-454.

18. **Jones, F. E., C. A. Smibert, and J. R. Smiley.** 1995. Mutational analysis of the herpes simplex virus virion host shutoff protein: evidence that vhs functions in the absence of other viral proteins. J. Virol. **69**:4863-71.

19. **Krikorian, C. R., and G. S. Read.** 1991. In Vitro mRNA degradation system to study the virion host shutoff function of herpes simplex virus. J. Virol. **65**:112-122.

20. **Kwong, A. D., and N. Frenkel.** 1987. Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs. Proc. Natl. Acad. Sci. USA. **84**:1926-1930.

21. **Kwong, A. D., J. A. Kruper, and N. Frenkel.** 1988. Herpes simplex virus virion host shutoff function. J. Virol. **62**:912-21.

22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **227**:680-685.

23. **Lam, Q., C. A. Smibert, K. E. Koop, C. Lavery, J. P. Capone, S. P. Weinheimer, and J. R. Smiley.** 1996. Herpes simplex virus VP16 rescues viral mRNA from destruction by the virion host shutoff function. EMBO J. **15**:2575-81.

24. **Lieber, M. R.** 1997. The FEN-1 family of structure-specific nucleases in eukaryotic DNA replication, recombination and repair. Bioessays. **19**:233-40.

25. **Marzluff, W. F., and N. B. Pandey.** 1988. Multiple regulatory steps control histone mRNA concentration. Trends in Biochemical Science. **13**:49-52.

26. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69:**1531-74.

27. **Nicholson, R., J. Pelletier, S. Y. Le, and N. Sonenberg.** 1991. Structural and functional analysis of the ribosome landing pad of poliovirus type 2: in vivo translation studies. J. Virol. **65:**5886-94.

28. **Nishioka, Y., and S. Silverstein.** 1977. Degradation of cellular mRNAs during infection by herpes simplex virus. Proc. Natl. Acad. Sci. USA. **74:**2370-2374.

29. **Nishioka, Y., and S. Silverstein.** 1978. Requirement of protein synthesis for the degradation of host mRNA in friend erythroleukemia cells infected with herpes simplex virus type 1. J. Virol. **27:**619-627.

30. **Oroskar, A. A., and G. S. Read.** 1989. Control of mRNA stability by the virion host shutoff function of herpes simplex virus. J. Virol. **63:**1897-1906.

31. **Oroskar, A. A., and G. S. Read.** 1987. A mutant of herpes simplex virus type 1 exhibits increased stability of immediate-early (alpha) mRNAs. J. Virol. **61:**604-606.

32. **Pak, A. S., D. N. Everly, K. Knight, and G. S. Read.** 1995. The virion host shutoff protein of herpes simplex virus inhibits reporter gene expression in the absence of other viral gene products. Virology. **211:**491-506.

33. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature. **334:**320-5.

34. **Read, G. S., and N. Frenkel.** 1983. Herpes simplex virus mutants defective in the virion-associated shutoff of host polypeptide synthesis and exhibiting abnormal synthesis of a (immediate early) viral polypeptides. J. Virol. **46:**498-512.

35. **Read, G. S., B. M. Karr, and K. Knight.** 1993. Isolation of a herpes simplex virus type 1 mutant with a deletion in the virion host shutoff gene and identification of multiple forms of the vhs (UL41) polypeptide. J. Virol. **67:**7149-60.

36. **Roizman, B., G. S. Borman, and M.-K. Rousta.** 1965. Macromolecular synthesis in cells infected with herpes simplex virus. Nature. **206:**1374-1375.

37. **Roizman, B., and A. E. Sears.** 1996. Herpes simplex viruses and their replication, p. 2231-2295. *In* B. N. Fields and D. M. Knipe and P. M. Howley (ed.), Virology, 3rd ed, vol. 2. Lippincott-Raven Press, Philadelphia.

38. **Sachs, A. B., P. Sarnow, and M. W. Hentze.** 1997. Starting at the begining, middle, and end: translation initiation in eukaryotes. Cell. **89:**831-838.

39. **Schek, N., and S. L. Bachenheimer.** 1985. Degradation of cellular mRNAs induced by a virion-associated factor during herpes simplex virus infection of vero cells. J. Virol. **55:**601-610.

40. **Smibert, C. A., D. C. Johnson, and J. R. Smiley.** 1992. Identification and characterization of the virion-induced host shutoff product of herpes simplex virus gene UL41. J. Gen. Virol. **73:**467-470.

41. **Smibert, C. A., B. Popova, P. Xiao, J. P. Capone, and J. R. Smiley.** 1994. Herpes simplex virus VP16 forms a complex with the virion host shutoff protein vhs. J. Virol. **68:**2339-46.

42. **Smibert, C. A., and J. R. Smiley.** 1990. Differential regulation of endogenous and transduced beta-globin genes during infection of erythroid cells with a herpes simplex virus type 1 recombinant. J. Virol. **64:**3882-94.

43. **Sorenson, C. M., P. A. Hart, and J. Ross.** 1991. Analysis of herpes simplex virus-induced mRNA destabilizing activity using an in vitro mRNA decay system. Nucleic Acids Res. **19:**4459-65.

44. **Stevens, A.** 1998. Endonucleolytic cleavage of RNA at 5' endogenous stem structures by human flap endonuclease 1. Biochem. Biophys. Res. Com. **251:**501-508.

45. **Strelow, L. I., and D. A. Leib.** 1995. Role of the virion host shutoff (vhs) of herpes simplex virus type 1 in latency and pathogenesis. J. Virol. **69:**6779-86.

46. **Strom, T., and N. Frenkel.** 1987. Effect of herpes simplex virus on mRNA stability. J. Virol. **61:**2198-2207.

47. **Sydiskis, R. J., and B. Roizman.** 1967. The disaggregation of host polyribosomes in productive and abortive infection with herpes simplex virus. Virology. **32:**678-686.

48. **Sydiskis, R. J., and B. Roizman.** 1966. Polysomes and protein synthesis in cells infected with a DNA virus. Science. **153:**76-78.

49. **Sydiskis, R. J., and B. Roizman.** 1968. The sedimentation profiles of cytoplasmic polyribosomes in mammalian cells productively and abortively infected with herpes simplex virus. Virology. **34:**562-565.

50. **Wagner, E. K., J. F. Guzowski, and J. Singh.** 1995. Transcription of the herpes simplex virus genome during productive and latent infection. Prog. Nucleic Acid Res. Mol. Biol. **51:**123-165.

51. **Young, J. C., J. Ursini, K. R. Legate, J. D. Miller, P. Walter, and D. W. Andrews.** 1995. An amino terminal domain containing hydrophobic and hydrophilic

sequences binds the signal recognition particle receptor alpha subunit to the beta subunit on the endoplasmic reticulum membrane. J. Biol. Chem. **270:**15650-15657.

52. **Zelus, B. D., R. S. Stewart, and J. Ross.** 1996. The virion host shutoff protein of herpes simplex virus type1: messenger ribonucleolytic activity in vitro. J. Virol. **70:**2411-2419.

3.8. Addendum.

3.8.1. Data not shown in chapter 3.

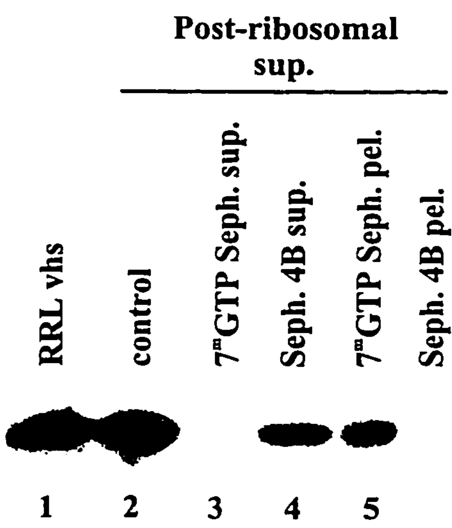
The data described in chapter 3 show that the vhs protein of HSV-1 induces translational arrest and RNA degradation when synthesized as the only HSV protein in RRL *in vitro*. The vhs-induced SRPα RNA decay *in vitro* initiates through 5' cap- and poly(A)-independent endoribonucleolytic cleavage events clustered over the 5' quadrant of the RNA. Cap-independence was demonstrated by comparison of the degradation profiles of capped and uncapped SRPα RNA substrates using Northern blots and primer extension analyses (chapter 3, figure 3-6). The cap independence conclusion was supported by the observation that the vhs-induced RNA degradation was not altered by depletion of the cap-binding protein, eIF4E, from RRL containing vhs. However, the data were not presented in chapter 3. Therefore, the purpose of this section of the addendum is to present these data.

The cap-binding protein was depleted from post-ribosomal supernatant fraction of vhs-containing RRL using 7^{m}GTP-Shepharose resin as described in chapter 2, materials and methods. The extent of eIF4E depletion was monitored by Western blot analysis of the treated and untreated lysate using eIF4E specific polyclonal antiserum. Figure 3-11B shows that eIF4E was effectively depleted (within the constraints of Western blot detection) from lysates, inasmuch as the 7^{m}GTP-Sepharose but not Sepharose 4B supernatant fraction is devoid of detectable eIF4E (figure 3-11B, compare lanes 3 and 4).

Figure 3-11. The cap-binding protein, eIF4E, does not contribute to vhs-dependent RNA degradation *in vitro*. (A) Post-ribosomal supernatants of RRL-vhs were treated with 7^{m}GTP-Sepharose and control Sepharose 4B beads to remove the cap-binding protein. The resulting depleted supernatant along with untreated post-ribosomal supernatant and intact RRL containing vhs were reacted with cap-^{32}P-labeled SRPα RNA substrate. Following a 10 minute incubation at 30°C, the reaction mixtures were extracted once with Trizol, once with chloroform and the RNA was recovered by ethanol precipitation. The RNA was then resolved on a 20% polyacrylamide sequencing gel and the cap label signal was detected by autoradiography using Kodak X-OMAT AR film. (B) Samples of the untreated and depleted lysates used in A were analyzed by Western blot using an eIF4E specific polyclonal antiserum as described in the materials and methods (chapter 2).

A.

B.



Effective eIF4E depletion was also evident by the finding that the protein partitions exclusively with the 7^{m}GTP-Sepharose but not Sepharose 4B bead pellet (figure 3-11B, lanes 5 and 6).

To test for vhs-dependent RNA degradation activity, intact, 7^{m}GTP-Sepharose, and Sepharose 4B depleted supernatants were reacted with equal amounts of cap-labeled SRPα RNA for 10 minutes at 30°C. RNA was then extracted and resolved on a 20% polyacrylamide sequencing gel. The results show that eIF4E depletion did not detectably alter the extent or the profile of vhs-dependent SRPα degradation *in vitro* as determined by the detection of the 5' most vhs-dependent cleavage products (figure 3-11A). These data do not exclude the possibility that trace amounts of the protein (below the lower threshold of Western blot detection) may still exist in the depleted lysates and could contribute to vhs activity. This, however, is unlikely given my previous finding that RNAs need not be capped to be targeted for vhs-dependent degradation (chapter 3 figure 3-6). Therefore, these data strongly support 5' cap independence of the vhs RNA degradation activity in RRL *in vitro*.

3.8.2. Unpublished data relating to chapter 3.

i) Vhs produced in wheat germ extracts does not induce translational arrest or 5' end RNA degradation.

I have shown in chapter 3 that vhs synthesized in two *in vitro* translation systems derived from mammalian cells induces mRNA degradation. It was of interest to determine if vhs translated in extracts derived from wheat germ displays the same

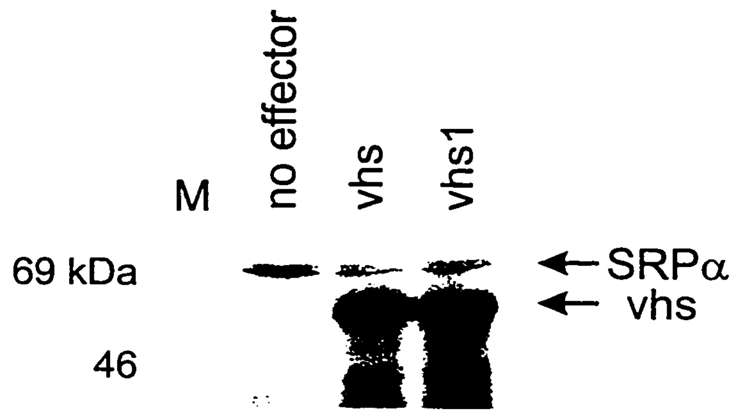
activity. The results shown in figure 3-12 demonstrate that, in contrast to vhs produced in RRL, vhs produced in wheat germ extracts does not inhibit the translation of capped reporter (SRPα) mRNA, since both wild type vhs and the defective vhs1 point mutant affect translation to the same extent. To determine if vhs produced in wheat germ affected mRNA stability I challenged wheat germ extracts containing vhs with cap-labeled SRPα RNA and monitored the appearance of vhs-dependent 5' most RNA degradation products. The data presented in figure 3-12B show that vhs produced in wheat germ extracts does not lead to the accumulation of small RNA degradation products. The gel system used in the analysis has a limited resolution capacity and, therefore, these data do not exclude the possibility that RNA molecules are cleaved internally resulting in products beyond the resolution capacity of this gel system. The most interesting observation to come out of these experiments is that the defect in vhs activity in wheat germ can be partially restored by the addition of naive RRL or post-ribosomal and eIF4E depleted post-ribosomal supernatant of naive RRL, but not by Retic buffer. Together, these data provide a preliminary suggestion that vhs produced in wheat germ is non-functional unless a co-factor present in RRL but not wheat germ is provided, thus arguing that vhs activity requires one or more cofactors present in RRL. Further analysis to assess the stability of RNA in wheat germ extracts using Northern blots will be necessary to clarify this issue.

ii) Vhs proteins from HSV-1 and HSV-2 display the same activity *in vitro*.

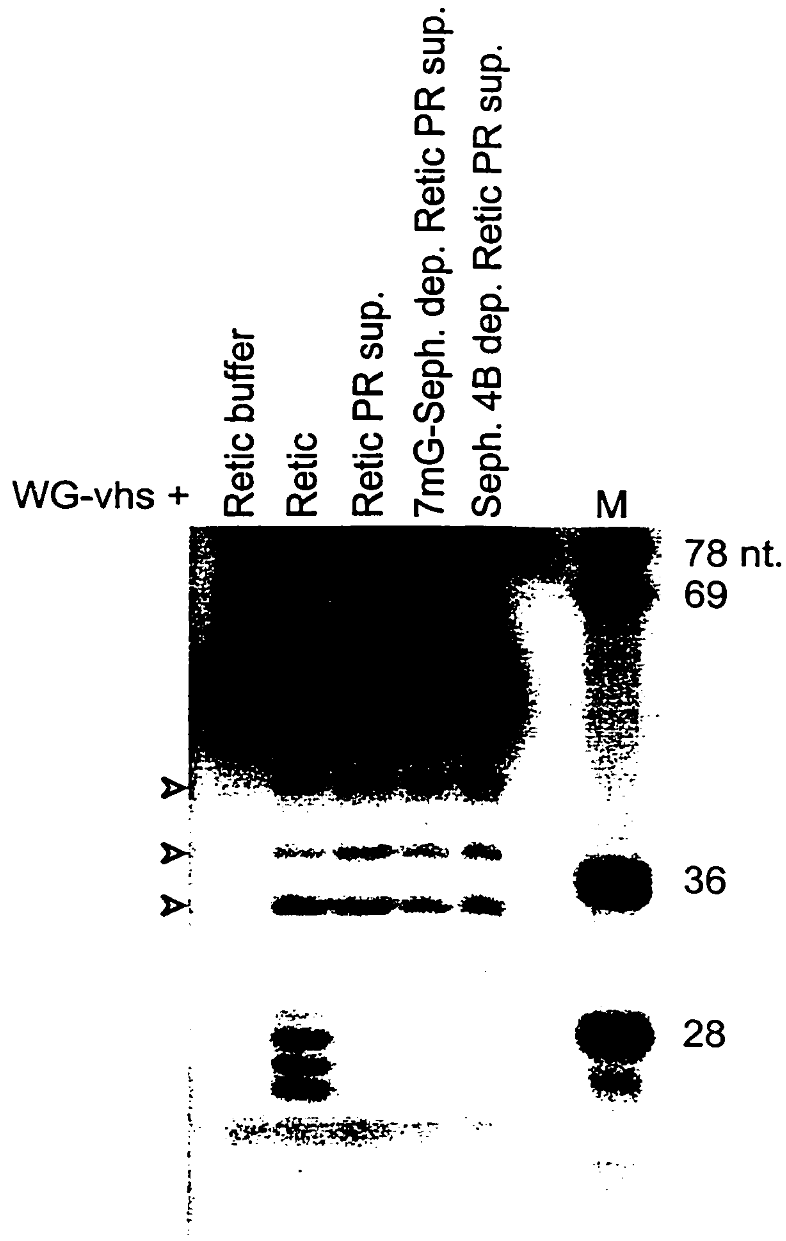
As mentioned in the introduction, the vhs from HSV-2 (T2 vhs) displays much stronger host shutoff phenotype than vhs from HSV-1 (T1 vhs) in infected cells.

Figure 3-12. Vhs produced in wheat germ extracts does not induce translational arrest and RNA degradation. (A) Wheat germ extracts (Promega) were programmed with capped vhs RNA, vhs1 RNA, or no RNA (no effector lane) in the presence of ^{35}S-methionine and translation was allowed to occur at 30°C for 1 hour as recommended by the vendor. The extracts were then challenged with capped SRPα reporter RNA and the reaction was continued for an additional hour. Samples were then analyzed by SDS-PAGE to asses the extent of ^{35}S-methionine incorporation. The ^{35}S signal was detected by autoradiography (materials and methods). Numbers to the left of panel A indicate the size of protein markers in kDa. (B) Wheat germ extracts containing pretranslated vhs were supplemented with an equal volume of Retic buffer, naive RRL, post-ribosomal supernatant of naive RRL, 7^{m}GTP-Sepharose depleted post-ribosomal supernatant of naive RRL, or Sepharose 4B depleted post-ribosomal supernatant of naive RRL and reacted with cap-labeled SRPα RNA for 15 minutes. RNAs extracted from each sample were resolved on a 20% sequencing gel and the cap label was detected by autoradiography. Numbers to the right of panel B indicate the size of DNA markers in nucleotide. Arrow heads indicate the position of vhs-dependent cleavage products.

A.



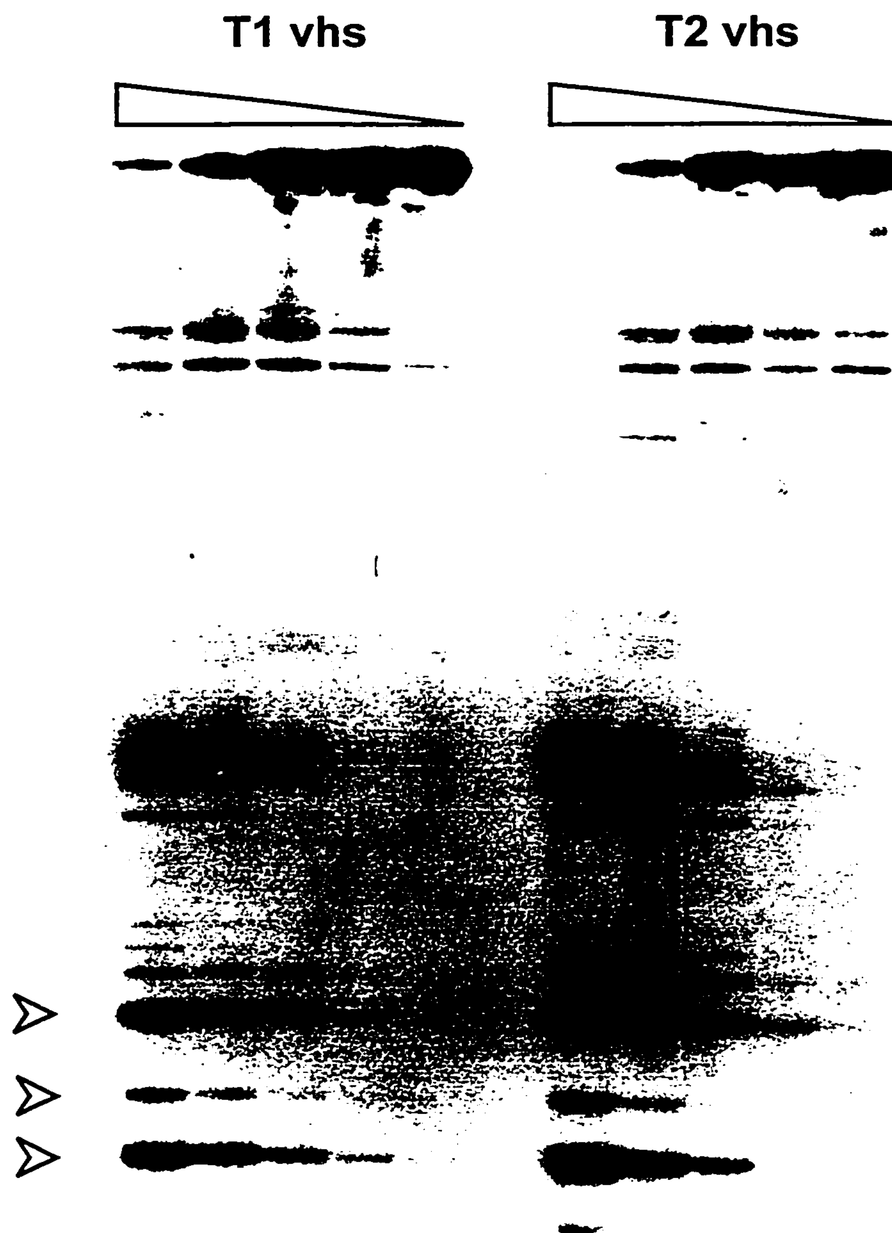
B.



Therefore, it was of interested to determine if this difference in activity can be reproduced *in vitro*. To address this issue, T1 and T2 vhs were translated in RRL in the presence of ^{35}S-methionine for 1 hour. The resulting lysates were serially diluted by 2-fold in naive RRL and reacted with cap-labeled SRPα RNA for 10 minutes at 30°C. The purpose of the serial dilution was to increase the chance of obtaining results where the degradation reaction did not go to completion. The results show that both T1- and T2-induced RNA degradation produce the same 5' most degradation products suggesting that the same mechanism is employed by the two proteins (figure 3-13). Furthermore, taking into consideration the difference in the amount of vhs proteins, there does not appear to be a significant difference between the rate of T1- and T2-induced RNA degradation *in vitro* (figure 3-13). These preliminary data provide the suggestion that other factors may contribute to the difference in the strength of the shutoff functions of HSV-1 and HSV-2 observed during infection. Again these data do not exclude the possibility that the T1 and T2 vhs proteins display a significant rate difference at cleavage sites yielding undetectable products in this resolution system.

Figure 3-13. HSV-1 and HSV-2 vhs proteins induce RNA degradation with similar kinetics *in vitro*. HSV-1 (T1) and HSV-2 (T2) vhs RNAs were translated in RRL *in vitro* as outlined in the materials and methods. Following serial 2-fold dilution in naive RRL, samples of each dilution were combined with cap-labeled SRPα RNA and incubated at 30°C for 10 minutes. (A) RNAs were then extracted and analyzed using a 20% sequencing gel as previously described. (B) The amount of protein in each sample was determined by SDS-PAGE analysis. Arrow heads indicate the position of vhs-dependent degradation products.

A.



B.

CHAPTER 4: Picornavirus IRES elements target RNA cleavage events induced by the herpes simplex virus vhs protein.

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Preface.

The data presented in this chapter, published in the Journal of Virology, have been formated to comply with the "sandwich" thesis format. I carried out all the experiments described in this chapter. 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 manuscript.

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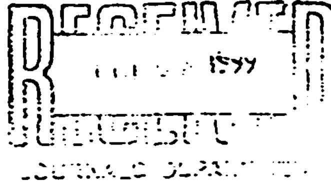

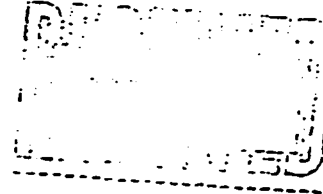

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Picornavirus IRES elements target RNA cleavage events induced by the herpes simplex virus vhs protein.

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4.1. Abstract.

The Herpes simplex virus virion host shutoff (vhs) protein (UL41 gene product) is a component of the HSV virion tegument that triggers shutoff of host protein synthesis and accelerated mRNA degradation during early stages of HSV infection. Vhs displays weak amino acid sequence similarity to the fen-1 family of nucleases, and suffices to induce accelerated RNA turnover through endoribonucleolytic cleavage events when it is expressed as the only HSV protein in a rabbit reticulocyte *in vitro* translation system. Although vhs selectively targets mRNAs *in vivo*, the basis for this selectivity remains obscure, as *in vitro* activity is not influenced by the presence of a 5' cap or 3' poly(A) tail. Here we show that vhs activity is greatly altered by placing an internal ribosome entry site (IRES) from EMCV or poliovirus in the RNA substrate. Transcripts bearing the IRES were preferentially cleaved by the vhs-dependent endoribonuclease at multiple sites clustered in a narrow zone located immediately downstream of the element in a reaction that did not require ribosomes. 'Targeting" was observed when the IRES was located at the 5' end or placed at internal sites in the substrate, indicating that it is independent of position or sequence context. These data indicate that the vhs-dependent nuclease can be selectively targeted by specific cis-acting elements in the RNA substrate, possibly through secondary structure or a component of the translational machinery.

4.2. Introduction.

Many viruses selectively inhibit host cell protein synthesis as a key element of their strategy of reprogramming the cellular biosynthetic machinery to support efficient virus replication. In the best understood cases, picornaviruses employ multiple mechanisms to inactivate the cap-binding translation initiation factor eIF4F, thereby preventing translation of most cellular mRNAs. The eIF4F complex is composed of three proteins: eIF4E (the cap-binding protein), eIF4A (an RNA helicase), and eIF4G (which serves as a scaffold for assembly of the complex). Once bound to the mRNA through the 5' cap structure, eIF4F recruits eIF3, which in turn serves as bridge between eIF4F and the incoming 40S ribosomal subunit (reviewed in reference (24)). Members of the enterovirus, rhinovirus, and aphthovirus genera of the picornaviridae family encode proteases (e.g., the poliovirus $2A^{pro}$ protease) that cleave eIF4G into two fragments (3, 5, 8, 21, 23, 41). The N-terminal fragment contains the eIF4E binding site, while the C-terminal fragment contains the binding sites for eIF4A and eIF3 (27, 40). Thus, these viral proteases uncouple the cap-recognition and ribosome recruitment functions of eIF4F, thereby inhibiting cap-dependent translation. Picornavirus mRNAs escape shutoff by utilizing a cap-independent mode of translation initiation mediated by the C-terminal fragment of eIF4G (48, 55), which binds to the highly structured internal ribosome entry site (IRES) elements found in the 5' UTRs of picornavirus RNAs (28, 29, 53, 54). Recently, Gradi and colleagues have identified an additional mammalian homologue of eIF4G, termed eIF4GII. These authors showed that both eIF4GI (the original eIF4G) and

eIF4GII are cleaved in poliovirus- and rhinovirus-infected cells, and concluded that cleavage of both proteins is required for shutoff of host cell protein synthesis (20, 21, 68).

Picornavirus infection also leads to inactivation of the cap-binding protein (eIF4E) component of eIF4F, by two distinct mechanisms. First, the active phosphorylated form of eIF4E (reviewed in reference (63)) is dephosphorylated, resulting in loss of activity (33). Second, the eIF4E inhibitor 4E-BP1 is also dephosphorylated (18, 19), leading to its activation. Active 4E-BP1 binds to eIF4E and prevents it from interacting with eIF4G, thereby inhibiting cap dependent translation (22, 43, 45). Inactivation of eIF4E seems to be the major mechanism contributing to the host shutoff induced by cardioviruses (exemplified by encephalomyocarditis virus, EMCV), which do not induce cleavage of eIF4G (18, 19, 33). More recently, poliovirus and coxsackievirus have been shown to induce cleavage of the poly(A)-binding protein (PABP) (30, 32). This observation is significant, because PABP directly interacts with components of eIF4F to synergistically stimulate cap-dependent translation ((26) and reviewed in reference (16)). Moreover, PABP has been shown to compensate for partial loss of eIF4E function and stimulate translation initiation of uncapped mRNAs in yeast (56, 71). Therefore, by inactivating eIF4G, eIF4E and PABP, picornaviruses ensure the complete shutoff of cap-dependent translation.

Adenovirus and influenza virus also target eIF4E for inactivation as a component of their host shutoff strategy (10, 25, 76). Adenovirus late mRNAs escape this shutoff by utilizing a mode of translation that is less dependent on intact eIF4F. This is mediated by the tripartite leader (found in the 5' UTR of late mRNAs), which directs efficient

translation initiation in the presence of limiting amounts of functional eIF4F by ribosome jumping (74). Similarly, influenza virus mRNAs have been shown to posses cis-acting sequence elements in their 5' UTRs that direct efficient translation initiation in the presence of limiting amounts of functional eIF4F (17, 52).

Herpes simplex virus (HSV) also causes a dramatic reduction of host cell protein synthesis. Shutoff occurs in two distinct phases that occur at early and intermediate times post-infection respectively (reviewed in reference (11)). The early host shutoff induced by HSV provides a striking contrast to the strategies employed by picornaviruses and adenovirus, as it involves rapid degradation of pre-existing cellular mRNAs rather than alterations to the translational apparatus *per se*. This effect is triggered by the infecting virus particle, and requires the virion host shutoff protein (vhs) product of the HSV gene UL41 (12-15, 31, 34-37, 46, 47, 49, 50, 57, 59, 64, 67, 69, 70). Vhs is a 58 kDa phosphoprotein that is produced late during infection and packaged into the virion tegument (the space between nucleocapsid and viral envelope) (58, 60). Vhs inhibits reporter gene expression in transiently transfected mammalian cells (31, 51) and triggers translational arrest and accelerated degradation of reporter RNAs when it is produced in a rabbit reticulocyte lysate *in vitro* system (7, 75). Thus, vhs suffices to induce shutoff in the absence of other HSV proteins.

Although vhs is not essential for virus replication, vhs mutants display a ca. 10-fold reduction in virus yield in tissue culture (57, 62) and severe defects in the nervous system of mice (66), indicating that vhs plays an important role during the viral life cycle. Vhs presumably helps viral mRNAs compete for the cellular translation

machinery, by reducing the level of cellular mRNAs in the cytoplasm. Vhs also significantly destabilizes HSV mRNAs belonging to all three temporal classes (14, 36, 37, 49, 50, 57, 67), an effect that sharpens the transitions between the successive phases of viral protein synthesis by tightly coupling changes in the rate of mRNA synthesis to altered mRNA levels. Although HSV mRNAs are susceptible to vhs-induced decay, Fenwick and Owen demonstrated that the onset of viral protein synthesis leads to a significant increase in the half-lives of viral mRNAs (14). This observation suggests that a newly synthesized HSV protein(s) partially downregulates the vhs activity of the infecting virion, allowing viral mRNAs to accumulate after host mRNAs have been degraded (14). Vhs binds to the virion transactivator VP16 (61), and VP16 null mutants undergo a severe vhs-induced translational arrest at intermediate times postinfection (39). These data strongly suggest that VP16 plays an important role in the downregulation of vhs activity.

The mechanism of vhs action remains to be precisely defined. 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 (6), and recent studies have shown that human fen-1 cleaves both RNA and DNA substrates (65). These data suggest that vhs may be a ribonuclease. Consistent with this hypothesis, Zelus and colleagues showed that extracts of partially purified HSV virions contain a vhs-dependent ribonuclease activity that is inhibited by anti-vhs antiserum (75). Although these data do not exclude the possibility that this ribonuclease contains one or more cellular subunits, they strongly suggest that vhs is an integral and required component of the enzyme.

We and others have shown that vhs induces translational arrest and degradation of reporter mRNA *in vitro* when it is expressed as the only HSV product in rabbit reticulocyte lysates (7, 75). We further demonstrated that decay occurs through an endoribonucleolytic mechanism that does not depend on a 5' cap or 3' poly(A) tail in the RNA substrate (7). During a survey of the mode of decay of a variety of RNAs in this system, we discovered that picornavirus IRES elements profoundly alter the degradation profile of substrate RNAs. In this communication, we show that the IRES elements of EMCV and poliovirus strongly direct vhs-induced endoribonucleolytic cleavage to sequences located immediately 3' to the IRES. This targeting activity was observed in several distinct sequence contexts, demonstrating that these IRESs serve as movable targeting elements for vhs-dependant cleavage.

4.3. Materials and Methods.

Plasmids. The vhs *in vitro* transcription vector pSP6vhs has been previously described (7). pCITE-1 (Novagen) contains residues 255 to 836 of the EMCV 5' UTR (the IRES element) 9 bp downstream of the T7 RNA polymerase promoter start site. The IRES element is followed by a 1166 bp fragment corresponding to the extreme 5' end of the EMCV ORF. pCITE RI/AvrII (lacking the 5' most 159 bp of the IRES) was constructed by self-ligating EcoRI/AvrII digested pCITE-1 DNA, after filling in the ends with the Klenow fragment of DNA polymerase I. pCITE Msc/RI (lacking all of the IRES) was generated in the same way, using MscI/EcoRI cleaved pCITE-1 DNA. pSEXAI IRES was constructed by ligating a 600 nt EcoRI-MscI fragment of pCITE-1 (bearing the IRES) into the SexA1 site of pCITE Msc/RI (after filling in all of the ends with Klenow). The resulting plasmid contains the EMCV IRES 729 bp downstream of the T7 start site. pSPSR19N contains a cDNA encoding canine signal recognition particle a subunit (SRPα), initiating at an engineered NcoI site, inserted into pSPUTK (9, 73). pSP19StuI IRES was constructed by inserting the 600 nt. EcoRI-MscI fragment of pCITE-1 into the unique StuI site of pSPSR19N (after all ends were made flush). The resulting plasmid bears the EMCV IRES element 1721 bp downstream of the SP6 RNA polymerase start site.

The plasmid pCITE Msc/RI P2 is a pCITE-1 derivative in which the EMCV IRES is replaced with the IRES of poliovirus type 2. It was constructed by exchanging the ca. 600 bp EcoRI-MscI fragment of pCITE-1 with a ca. 600 bp HindIII-MscI fragment from the plasmid pP2CAT (after making all ends flush with Klenow). P2CAT was a generous

gift from N. Sonenberg, McGill university. pSexAI P2 was generated by ligating the ca. 600 bp HindIII-MscI fragment from pP2CAT into the SexAI site of pCITE Msc/RI, after repairing the ends with Klenow. The resulting plasmid lacks the EMCV IRES and contains the poliovirus IRES ca. 729 nt from the 5' end of the T7 RNA polymerase transcript.

***In vitro* transcription and RNA labeling.** Transcription reactions were carried out using the Riboprobe *in vitro* transcription system (Promega) according to the vendor's protocol. Vhs mRNA destined for *in vitro* translation was generated by transcribing 3-5 mg of supercoiled pSP6vhs plasmid DNA in a 50 ml reaction for 30 minutes at 30°C using 40 U of SP6 RNA polymerase in the presence of 0.5 mM cap primer $7^{m}G(5')ppp(5')G$ (Pharmacia), 12.5 mM GTP, and 0.25 mM of each of CTP, ATP, and UTP. Following digestion of plasmid DNA with 5U of RQ1 DNase (Promega), the reaction mixture was extracted once with phenol:chloroform:isoamyl alcohol and once with chloroform. The resulting solution was made 2.5 M ammonium acetate and the mRNA was precipitated with 95% ethanol. The mRNA pellet was then washed with 70% ethanol, dried and resuspended in RNase free water.

Capped, internally labeled reporter RNAs were generated as above, except that the template was linearized at an appropriate site (see below) prior to transcription, and 1 mCi $a^{32}P$-GTP was added to the transcription reaction. Uncapped, internally labeled reporter RNAs were produced in a similar fashion, except that the cap primer was omitted and the GTP concentration was raised to 0.25 mM. Transcription of reporter mRNAs was terminated by adding 20 % RNA loading buffer (50% glycerol, 1mM

EDTA, 10 mg/ml xylene cyanol, and 10 mg/ml bromophenol blue) and immediately loading the sample on a 1% agarose gel in 1X TBE (90 mM Tris-borate, 2 mM EDTA) buffer. Following electrophoresis for 2 hours at approximately 7 V/cm, gel slices containing full length transcripts (detected with UV light after ethidium bromide staining) were excised and equilibrated in 0.5X TBE for 10 minutes. The RNA was then electroeluted from gel slices into a 100 ml 7.5 M ammonium acetate trap in a 6-well v-channel electroeluter (IBI) at 100 volts for 30 minutes. The RNA was then recovered from the salt solution by ethanol precipitation. The RNA pellets were washed 2 times with 70% ethanol, dried, and resuspended in RNase free water.

Reporter RNAs transcribed from pCITE, pCITE RI/AvrII, pCITE Msc/RI, pCITE Msc/RI P2, pSexAI IRES, and pSexAI P2 were generated using T7 RNA polymerase and EcoNI-linearized plasmid DNAs as templates to yield run-off transcripts of ca. 2.3, 2.1,1.7, 2.3, 2.3 and 2.3 Kb, respectively. A shorter transcript of pCITE RI/AvrII (437 nt) was generated using T7 RNA polymerase and MscI-linearized pCITE RI/AvrII plasmid DNA as a template. SRPα and SRPα StuI IRES reporter mRNAs were generated using SP6 RNA polymerase and EcoRV-linearized pSPSR19N and pSP19StuI IRES plasmid DNAs as templates to yield run-off transcripts of 2.4 and 3 kb, respectively.

Cap-labeled reporter RNAs were generated from gel purified uncapped unlabeled run-off transcripts using vaccinia virus guanylyltransferase in the presence of a^{32}P-GTP. Approximately 500 ng of RNA in 50 mM Tris-HCl pH 7.9, 1.25 mM $MgCl_2$, 6 mM KCl, 2.5 mM DTT, 0.1 mg/ml RNase free BSA, 1 U/ml RNasin and 0.1 mM S-adenosyl-L-methionine was combined with 1-3 U of guanylyltransferase (Gibco-BRL) and 50 mCi

a^{32}P-GTP in a total reaction volume of 30 ml. Following a 45 minute reaction at 37°C, the reaction mixture was extracted once with phenol:chloroform:isoamyl alcohol and once with chloroform, and the RNA was recovered by ethanol precipitation.

***In vitro* translation and vhs activity assay.** Approximately 5 mg of vhs mRNA was translated in a 50 ml rabbit reticulocyte lysate (RRL; Promega or Novagen) reaction containing 40 mCi of ^{35}S-methionine according to the vendor's protocol. Translation reactions were carried out for 1 hour at 30°C. Blank RRL controls were generated as above except that mRNA was omitted from the translation reactions. Samples of the translation reactions were assessed for ^{35}S-methionine incorporation by SDS-PAGE analysis (38).

To assay for vhs activity, reporter RNA substrates were added to RRL containing pre-translated vhs and the reactions were incubated at 30°C. Aliquots (5 ml) of reaction mixtures were removed at various times and immediately added to 200 ml Trizol (Gibco-BRL) containing 20 mg of carrier *E. coli* tRNA (Sigma). The samples were extracted after the addition of 40 ml of chloroform and the resulting aqueous phase was re-extracted with chloroform. RNA was recovered by isopropanol precipitation, resuspended in 100 ml of RNase free water and reprecipitated with 95% ethanol. Following a 70% ethanol wash, the RNA pellet was dried and resuspended in RNase free water. The RNA samples were then analyzed by electrophoresis through agarose/formaldehyde or polyacrylamide sequencing gels, or by primer extension.

Agarose gel electrophoresis and Northern blot analysis. RNA samples were resuspended in 4.5 ml of RNase free water, then combined with 2 ml 10X MOPS buffer (200 mM 3-n-morpholino-propanesulfonic acid pH 7.0, 50 mM NaOAc, and 5 mM EDTA), 10 ml of deionized formamide and 3.5 ml of 37% formaldehyde solution. Following a 10 minute incubation at 75-80°C, the solution was combined with 6 ml of RNA loading buffer and subjected to electrophoresis through a 1% agarose gel containing 6% formaldehyde. Electrophoresis was carried out in 1X MOPS buffer containing 6% formaldehyde at approximately 5 V/cm for 3 to 4 hr. The gel was then washed in water for 10 minutes, treated with 50 mM NaOH/10 mM NaCl (20 minutes), and neutralized with 100 mM Tris-HCl (20 minutes). RNA was then transferred to a Nytran Plus membrane in 20X SSC (3 M sodium chloride, 0.3 M sodium citrate). Following UV cross-linking (Stratalinker 2400; Stratagene), ^{32}P-labeled RNA fragments were detected by exposure to Kodak X-OMAT AR film at –70°C.

Unlabeled RNA fragments cross-liked to Nytran Plus membranes were detected by Northern blot analysis (4). Briefly, the membranes were prehybridized in Church buffer (250 mM $NaPO_4$ pH7.2, 7% SDS, 1% BSA, 1 mM EDTA) at 62°C for 1 hour. The membrane in figure 3-1C was hybridized to a 5' ^{32}P-labeled oligonucleotide (AB9899, 5'-CATCATCCTCTCCATCAG-3') complementary to residues 729-746 of the pCITE transcript. The membranes in figure 3-5C and D were hybridized to a 400 nt EcoRV-EcoRI fragment of pSPSR19N corresponding to the 3'-most portion of the SRPα transcript (^{32}P labeled by random priming). Rabbit 18S rRNA was detected with a 5' ^{32}P-labeled oligonucleotide complementary to residues 269 to 299 of the rRNA (AB12084,

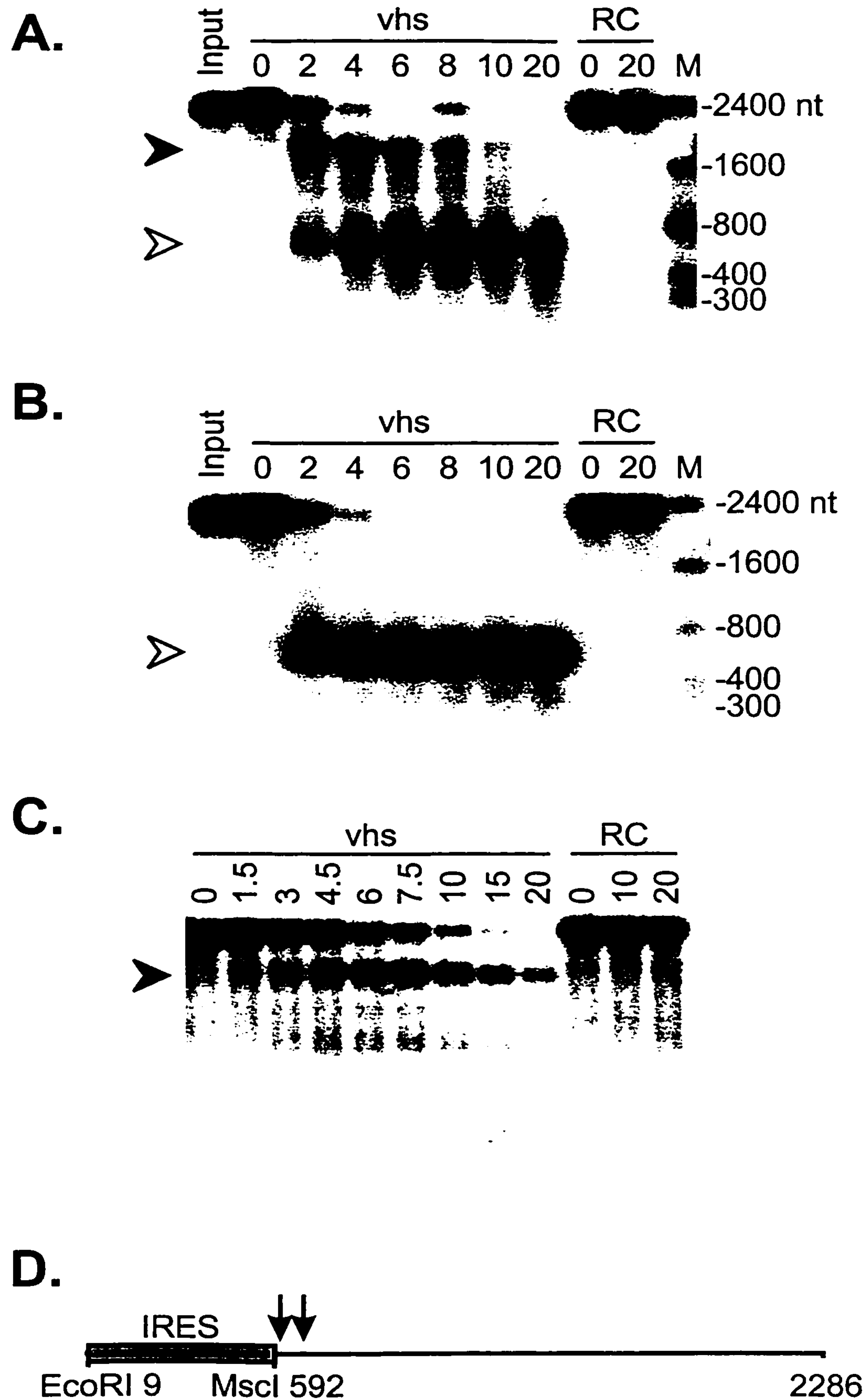
5'-TATCTAGAGTCACCAGAGCCGCCGAGCCCA-3'). Hybridization was carried out in Church buffer at 62°C for 13-17 hours. The membrane was then washed 2X10 minutes in 2X SSC, 0.1% SDS, 2 X10 minutes in 0.1X SSC, 0.1 SDS, and subjected to autoradiography.

Primer extension. RNA samples were suspended in 10 ml of annealing buffer (10 mM Tris-HCl pH7.9, 1mM EDTA, 250 mM KCl) containing 50,000 Cerenkov cpm of an appropriate 5'- ^{32}P-labeled oligonucleotide. The following oligonucleotide primers were used to detect vhs-induced cleavages downstream of the IRES elements: AB11388, 5'-CATTCTTCATCATACTTTAGCAGGT-3', complementary to residues 685-710 of pCITE-1 RNA (figures 4-2C and 4-7B); AB11259, 5'-CCATTAGGCAGGTTATCCTTGGACC-3', complementary to residues 1447-1471 of pSEXAI IRES RNA, figure 4-4; and AB11260, 5'-GCAGCTCCCACCTTGTCATCAATGG-3', complementary to residues 2424-2448 of SRPα StuI IRES RNA (figure 4-6). After annealing for 1 hour at 65°C, the samples were combined with 25 ml of PE buffer (20 mM Tris-HCl pH 8.7, 10 mM MgCl2, 5 mM DTT, 330 mM of each dNTP, 10 mg/ml actinomycin D and 10 U of SuperScript II (Gibo-BRL) reverse transcriptase) and the extension reaction was carried out for 1 hour at 42°C. Nucleic acids were precipitated with 95% ethanol, washed with 70% ethanol, dried and resuspended in water. The samples were then combined with an equal volume of sequencing gel loading buffer, heated to 80°C for 2-3 minutes and resolved on 8% polyacrylamide sequencing gels. The radioactive signal was detected by autoradiography.

4.4. Results.

Preferential vhs-induced cleavage near the 3' boundary of the EMCV IRES. We and others have previously shown that HSV1 vhs triggers endoribonucleolytic cleavage of exogenous RNA substrates when it is produced as the only HSV protein in an *in vitro* translation system derived from rabbit reticulocytes (RRL) (7, 75). During a survey of the mode of decay of a variety of substrate RNAs, we discovered that a 2.3 kb transcript of pCITE-1 (bearing the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) at its 5' end) gave rise to a strikingly simple pattern of early degradation intermediates. As shown in figure 4-1A, capped internally labeled pCITE-1 RNA yielded two prominent products of ca. 1800 and 600 nt. when it was incubated in RRL containing pre-translated vhs (figure 4-1A, filled and open arrowheads respectively). The ca. 600 nt fragment was stable throughout the course of the reaction, while the ca. 1800 nt fragment was subject to further decay. Only the ca. 600 nt fragment was detected when 5' cap labeled RNA was used as the substrate (figure 4-1B), indicating that it is derived from the 5' end of the RNA. Inasmuch as the two fragments roughly sum to yield the size of the intact transcript (ca 2300 nt), these data suggested that vhs-induced endoribonucleolytic cleavage occurs at one or several closely spaced sites located approximately 600 nt from the 5' end of the RNA. Consistent with this interpretation, an oligonucleotide complementary to residues 729-746 of the transcript hybridized to only the larger (3') fragment (figure 4-1C). Similarly, only the larger fragment was detected with a probe corresponding to the 3'-most 253 nt. of the RNA (data not shown).

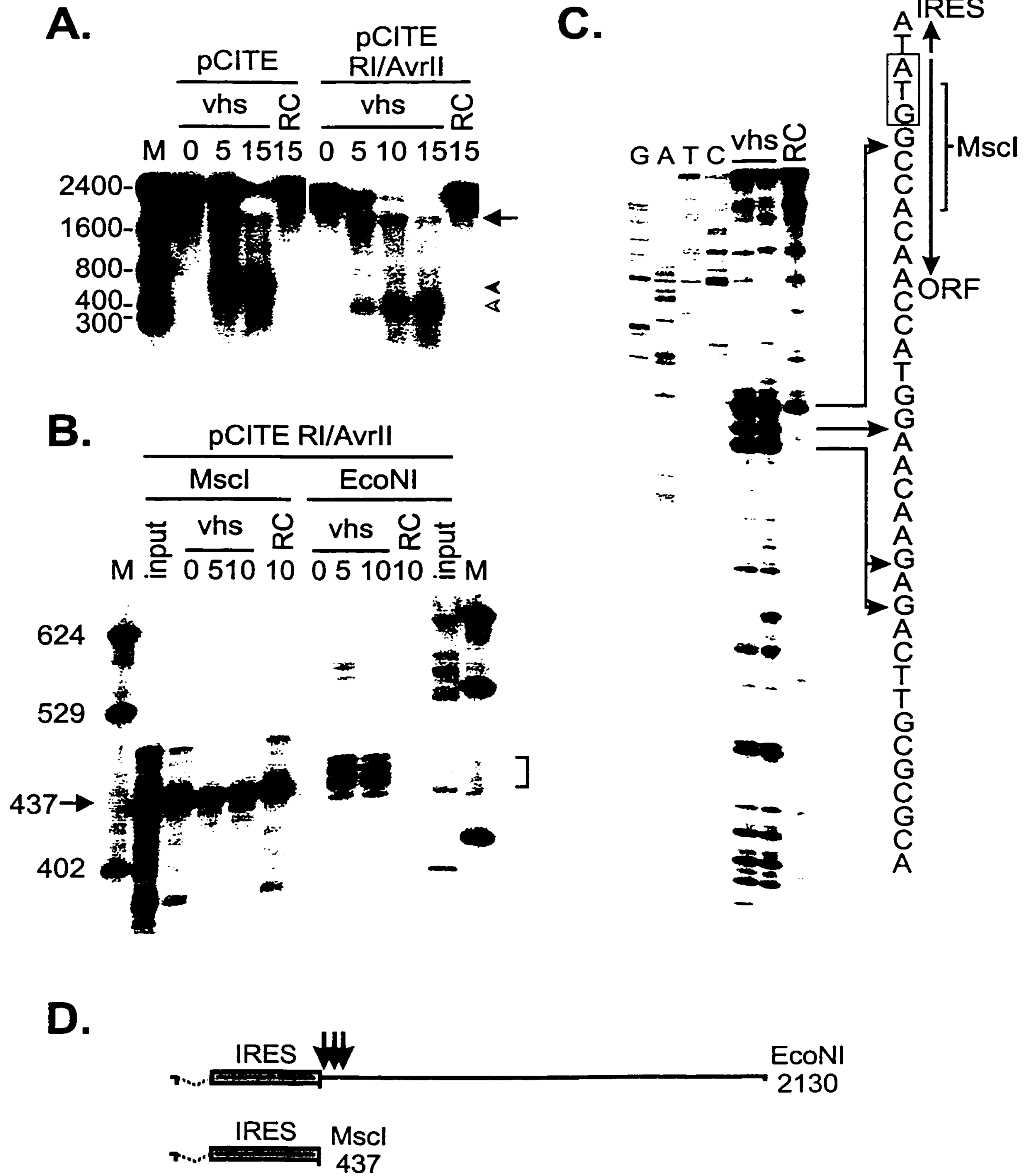
Figure 4-1. Vhs induces preferential endonucleolytic cleavage in the vicinity of the 3' boundary of the EMCV IRES. A 2.3 kb run-off transcript of pCITE-1 was added to rabbit reticulocyte lysates (RRL) containing pre-translated vhs (lanes marked vhs) and control RRL (lanes marked RC), and samples were removed at the indicated times (minutes). RNA was extracted from each sample, then resolved on a 1% agarose/6% formaldehyde gel and transferred to a Nytran Plus membrane. (A), capped internally labeled RNA; (B), 5' cap labeled RNA; (C), capped unlabeled RNA. The radioactive signals in panels A and B were detected by autoradiography. The RNA products in panel C were detected by hybridization to a 5' labeled oligonucleotide probe complementary to residues 729-746 of the pCITE transcript, followed by autoradiography. Numbers to the right of panels A and B indicate the size of RNA markers (lanes marked M) in nucleotides. (D) Diagram of the pCITE transcript indicating the approximate position(s) of the cleavage site(s) (arrows).


A.
Input
vhs
RC
0 2 4 6 8 10 20 0 20 M
-2400 nt
-1600
-800
-400
-300
B.
Input
vhs
RC
0 2 4 6 8 10 20 0 20 M
-2400 nt
-1600
-800
-400
-300
C.
vhs
RC
0 1.5 3 4.5 6 7.5 10 15 20 0 10 20
D.
IRES
EcoRI 9
MscI 592
2286

As shown schematically in figure 4-1D, the prominent cleavage site(s) detected above map to the vicinity of the 3' boundary of the IRES. These data raised the possibility that the IRES element might play a role in targeting vhs-induced cleavage, and indicated that the majority of the IRES sequence is highly resistant to degradation.

Cleavage occurs immediately downstream of the EMCV IRES element. We mapped the location(s) of the prominent early cleavage site(s) more precisely, through high-resolution analysis of the cleavage products. In order to increase the resolution of the experiment, we used a pCITE derivative that lacks the 5' -most 156 nt of the EMCV IRES (pCITE RI/AvrII) to characterize the 5' products. This mutant IRES element retains the ability to promote cap-independent initiation of translation (72). We first established that this transcript is cleaved in the vicinity of the 3' boundary of the IRES in the same fashion as pCITE-1 RNA. Figure 4-2A shows that internally labeled pCITE RI/AvrII RNA gave rise to products of ca. 1800 and 450 nt. The larger fragment co-migrated with the 3' fragment of pCITE-1 RNA (figure 4-2A, arrow), and the estimated size of the smaller product (ca. 450 nt.) approximately corresponds to that of the deleted IRES (437 nt, figure 4-2D). Thus, these data strongly suggested that pCITE RI/AvrII RNA is cleaved at or near the 3' boundary of the IRES. We then used 5' cap labeled pCITE RI/AvrII RNA as a substrate, and analyzed the products on an 8% polyacrylamide sequencing gel. As shown in figure 4-2B, a 2130 nt EcoNI run-off transcript (figure 4-2D) gave rise to four prominent 5' products ranging in size from approximately 437 to

Figure 4-2. The pCITE transcript is cleaved immediately downstream of the IRES. (A) Uncapped internally labeled transcripts of pCITE and pCITE RI/AvrII (lacking the 5' most 159 nucleotides of the IRES) were added to RRL containing vhs and the reaction products were analyzed as in figure 4-1A. The arrow and arrow heads indicate the 3' and 5' degradation products respectively. Numbers to the side of panel A indicate the sizes of RNA markers (lane M) in nucleotides. (B) 5' cap labeled run-off transcripts of pCITE RI/AvrII were added to RRL containing vhs and reaction products extracted at the indicated times (minutes) were resolved on an 8% polyacrylamide sequencing gel. The template DNA was linearized with MscI or EcoN1 prior to transcription, generating run-off transcripts of 437 and 2127 nt respectively. Numbers to the side of panel B indicate the sizes of DNA markers (lane M) in nucleotides. (C) A capped unlabeled EcoN1 run-off transcript of pCITE was incubated for 10 minutes in RRL containing pre-translated vhs, or in control RRL (RC). The RNA was then extracted, and analyzed by primer extension using a 5' labeled oligonucleotide complementary to residues 685-710 of the transcript. The primer extension products were resolved on an 8% polyacrylamide sequencing gel along with a DNA sequencing ladder generated from pCITE-1 DNA using the same primer. The diagram accompanying panel C shows the nucleotide sequence at the IRES/ORF boundary. The EMCV translational initiation codon is indicated by a rectangle, and arrows indicate the MscI cleavage site and the positions of some of the 5' ends generated by vhs-induced cleavage. (D) Structures of the pCITE RI/AvrII run-off transcripts. Dashed lines indicate the extent of the IRES deletion, and arrows indicate the approximate locations of the vhs-dependent cleavage sites.

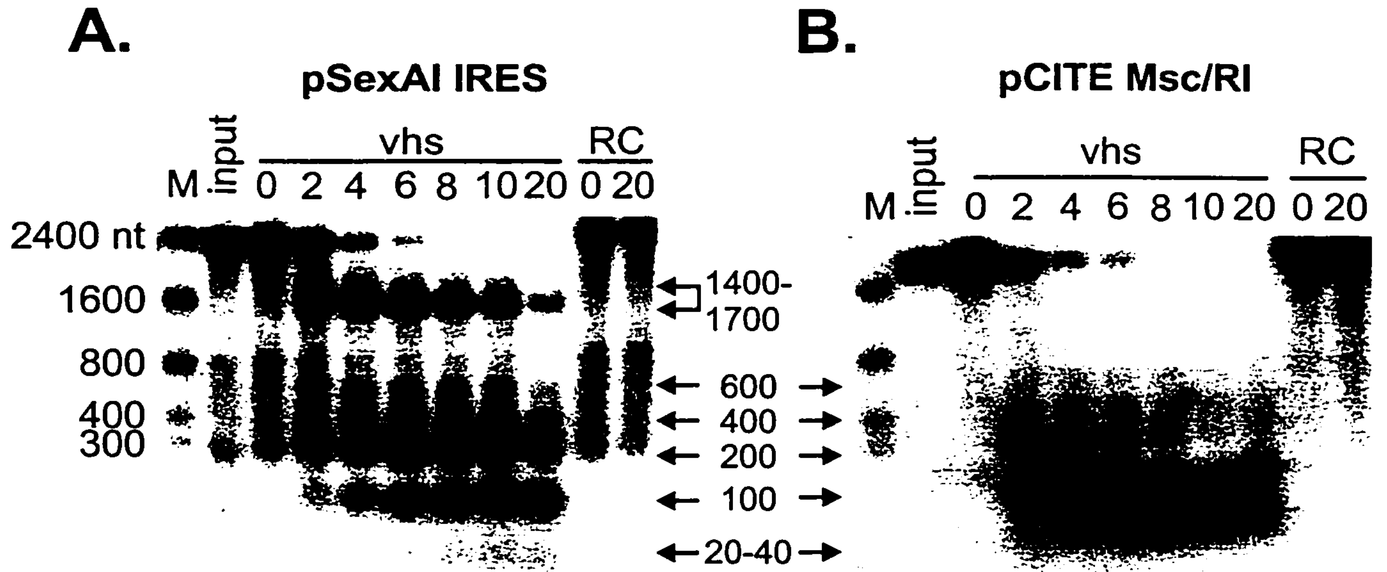

A.
pCITE
pCITE RI/AvrII
vhs
RC
M 0 5 15 15 0 5 10 15 15
2400
1600
800
400
300
B.
pCITE RI/AvrII
MscI
EcoNI
input
M
0 5 10 10
0 5 10 10
624
529
437
402
C.
G A T C
IRES
ORF
MscI
D.
IRES
EcoNI
2130
MscI
437

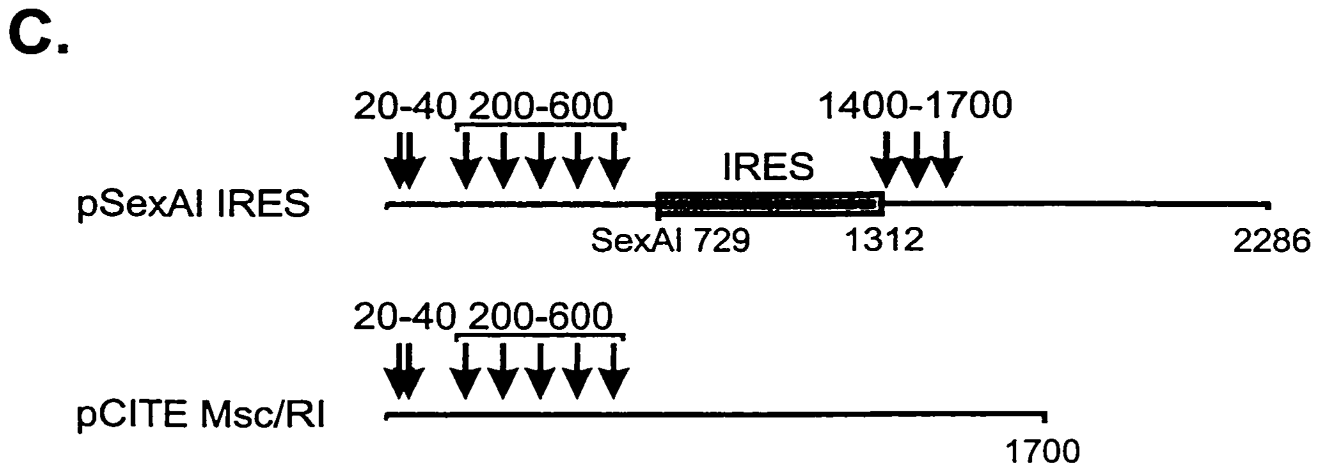
460 nt (indicated by a bracket in figure 4-2B), as well as a fainter ca. 430 band. In contrast, a 437 nt MscI run-off transcript was essentially immune to vhs-induced cleavage. The MscI cleavage site is located 5 nt downstream of the EMCV initiation codon (figure 4-2C), and is operationally considered to mark the 3' boundary of the IRES. Thus, these data indicate that vhs-induced cleavage occurs primarily at several sites located just downstream of the IRES. This conclusion was confirmed by primer extension analysis of the cleavage products of pCITE RNA, using a primer complementary to sequences located 91-115 nt. downstream of the MscI site (figure 4-2C). The results indicated that the majority of the novel vhs-induced 5' ends detected in this region are located 3' to the MscI site. Three of the most prominent of these 5' ends were mapped at the nucleotide level, and are indicated in figure 4-2C. The strong primer extension signal at the MscI site was also observed in RNA samples incubated in control reticulocyte lysate (figure 4-2C, RC), and likely represents pausing of reverse transcriptase due to the extensive secondary structure of the IRES.

The EMCV IRES serves as a movable targeting element for vhs-induced cleavage. The data described above demonstrate that prominent sites of initial vhs-induced cleavage are located just downstream of the EMCV IRES in the pCITE transcript. In principle, this narrow clustering of preferred cleavage sites might depend on the nucleotide sequence of the cleavage sites, the structure or function of the adjacent IRES, or the fact that this region corresponds to the 5' most section of the transcript that is accessible to the vhs-induced endoribonuclease (the IRES itself is highly resistant to cleavage).

As one approach to distinguish between these possibilities, we asked if the EMCV IRES element induced novel vhs-dependent cleavages when it was transplanted to the middle of the pCITE transcript. To this end, we deleted the IRES element from the 5' end to yield construct pCITE RI/MscI, then reinserted the IRES at a SexA1 site located 729 nt into the pCITE RI/McsI RNA (construct pSexA1 IRES, figure 4-3C). 5' cap-labeled pCITE RI/MscI RNA (lacking an IRES) gave rise to a heterogeneous set of 5' products ranging in size from ca. 20 –600 nt. (figure 4-3B). Although we have not yet examined the mode of degradation of this transcript in detail, the pattern of 5' fragments produced is similar to that previously observed with SRPα RNA, which is preferentially cleaved at a variety of sites distributed over the 5' quadrant of the transcript early during the reaction ((7) and see figure 4-5 below). pSexA1 IRES displayed a prominent novel band of ca. 1400 nt., in addition to these smaller 5' products (figure 4-3A). This novel band was quite broad at early times (extending from ca. 1400–1700 nt., figure 4-3A, 2 minute time point), then sharpened into a more discrete signal at ca. 1400 nt. as the reaction proceeded. These data indicate that the internal IRES present in the pSexA1 IRES transcript provokes novel cleavage events in the region extending from ca. 1400-1700 nt. from the 5' end of the RNA. As diagrammed in figure 4-3C, these cleavage sites map at or close to the 3' boundary of the IRES. The intensity of the 1400-1700 nt. signal declined as the reaction proceeded while the 20-600 nt products accumulated, suggesting that the 5' products of the IRES-directed cleavages may be substrates for additional cleavage events. Inasmuch as we were able to easily detect the cleavage sites located 1400-1700 nt from the 5' end of the pSEXA1 IRES transcript using 5' cap-labeled RNA,

Figure 4-3. The EMCV IRES serves as a movable targeting element for vhs-induced RNA cleavage. 5' cap-labeled EcoN1 run-off transcripts of pSexAI IRES (A) and parental pCITE Msc/RI (B) were added to RRL containing vhs (lanes marked vhs) and control RRL (lanes marked RC), and samples removed at the indicated times (minutes) were analyzed by agarose/formaldehyde gel electrophoresis as in figure 4-1. Arrows and associated numbers indicate the position and size (in nucleotides) of the vhs-dependent RNA cleavage products. Numbers to the left of panel A indicate the sizes of RNA markers (lanes M) in nucleotides. (C) Diagram showing the structures of the two transcripts, and the approximate locations of the vhs-induced cleavages. The shaded box represents the EMCV IRES.


A.
pSexAI IRES
M
input
vhs
RC
0 2 4 6 8 10 20 0 20
2400 nt
1600
800
400
300
1400-
1700
600
400
200
100
20-40
B.
pCITE Msc/RI
M
input
vhs
RC
0 2 4 6 8 10 20 0 20


C.
20-40 200-600
1400-1700
IRES
pSexAI IRES
SexAI 729
1312
2286
20-40 200-600
pCITE Msc/RI
1700

the data displayed in figure 4-3B argue that the most prominent sites of initial cleavage of the pCITE RI/MscI RNA are confined to the 5' quadrant of this transcript.

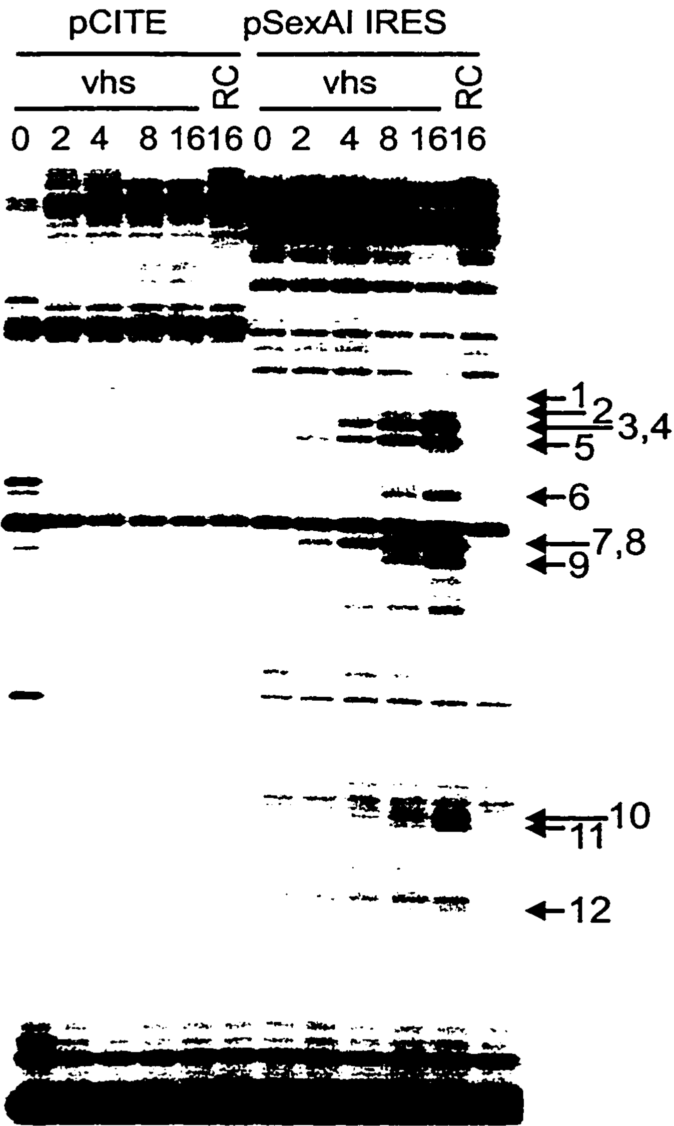
As shown in figure 4-2, pCITE RNA is cleaved just downstream of the IRES, within the 3' flanking sequences. To determine if this is also the case with the pSEXAI IRES transcript, we mapped some of the cleavage sites by primer extension using a primer complementary to residues 1447-1471 of the RNA (figure 4-4). pSexA1 IRES RNA displayed a variety of vhs-dependent novel 5' ends in the region examined (figure 4-4A). The precise positions of some of these were determined by resolving the primer extension products beside a DNA sequencing ladder generated from pSexAI IRES DNA using the same primer (data not shown, diagrammed in figure 4-4B). The majority of the new 5' ends were located downstream of the 3' boundary of the IRES. Only one very faint band (labeled 1) mapped within the IRES itself. Although pCITE RNA has exactly the same nucleotide sequence as the pSEXA1 IRES transcript downstream of site 2, prominent vhs-induced cleavages were not observed in this region of pCITE RNA.

Taken in combination, these data indicated that the internal IRES present in the pSEXAI IRES RNA provoked novel vhs-induced RNA cleavage events in the 3' flanking sequences. In this sense, the IRES behaved as a movable targeting element for vhs-induced cleavage.

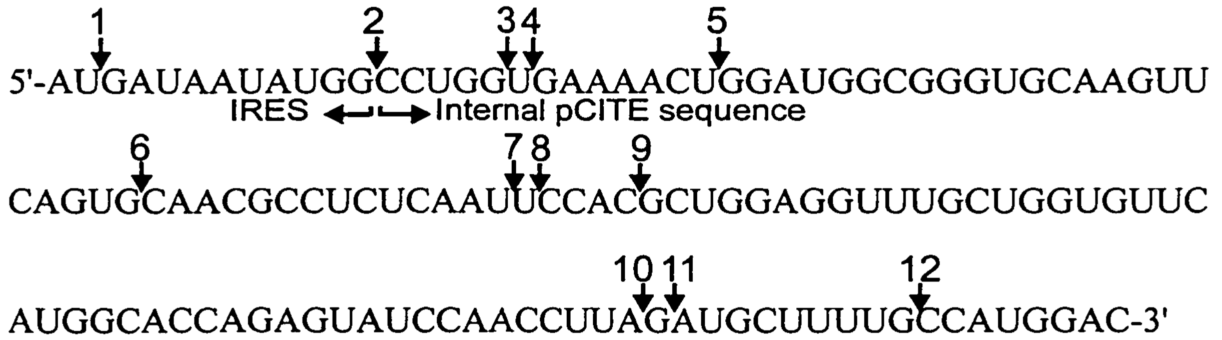
We tested the generality of this finding, by asking if the EMCV IRES similarly altered the degradation pattern of the entirely unrelated RNA encoding SRPα (figure 4-5). We inserted the IRES at the StuI site located at residue 1721 of the transcript,

Figure 4-4. Vhs-induces cleavage downstream of an internally located EMCV IRES. (A) Uncapped unlabeled transcripts of pCITE and pSexAI IRES were added to RRL containing vhs (lanes marked vhs) and control RRL (lanes marked RC) and samples were removed at the indicted times (minutes). Following extraction, the reaction products were analyzed by primer extension using a 5' labeled oligonucleotide complementary to residues 1447-1471 of the pSexAI IRES RNA (and residues1438-1462 of the pCITE transcript). Primer extension products were analyzed on an 8% polyacrylamide sequencing gel. Numbered arrows indicate primer extension products resulting from vhs-induced cleavage. (B) Diagram showing the nucleotide sequence at the 3' boundary of the IRES in pSexAI IRES RNA. Numbered arrows correspond to those in panel A, and indicate the location of the novel 5' ends produced by vhs-induced cleavage.

A.



B.



generating construct SRPα StuI IRES (figure 4-5E). SRPα and SRPα StuI IRES RNAs were then 5' cap-labeled, and added to RRL containing pre-translated vhs. As previously reported, SRPα RNA gave rise to 5' fragments ranging in size from ca. 30 to 700 nt. (figure 4-5A), reflecting the clustering of the preferred sites of initial cleavage over the 5' quadrant of this RNA (7). SRPα StuI IRES RNA displayed these same products, as well as an additional ca. 2300 nt. fragment that was not observed with SRPα RNA (figure 4-5B). The size of this novel 5' fragment agreed well with that predicted for cleavage at the 3' boundary of the inserted IRES (figure 4-5E). Such cleavage events would also generate novel 3' fragments of ca. 700 nt. We tested for these by hybridizing the membranes used in figures 4-5A and B to a probe for the extreme 3' end of the RNA (after allowing the 5' cap-label to decay for six half lives). The results clearly indicated that SRPα StuI IRES RNA gave rise to the predicted set of novel ca. 700 nt 3' fragments (figure 4-5D). As previously reported, SRPα RNA generated a heterogeneous set of early 3' products ranging in size from 1800-2200 nt. (figure 4-5C), reflecting the 5' clustering of initial cleavage events on this transcript (7). Analogous high molecular weight 3' products were also observed with SRPα StuI IRES RNA (ca. 2400-2700 nt., figure 4-5D). Taken in combination, these data indicate that SRPα StuI IRES RNA is cleaved over its 5'-most 600 nt in the same fashion as SRPα RNA, and additionally cleaved downstream of the inserted IRES. The two sets of cleavage events appear to occur independently.

Figure 4-5. The EMCV IRES targets vhs-induced cleavage of SRPα mRNA. Cap labeled SRPα (A) and SRPα StuI IRES (B) RNAs were added to RRL containing vhs (diluted 1:4 in naive RRL; lanes marked vhs) and control RRL (lanes marked RC) and RNA extracted from samples removed at the indicated times (minutes) was analyzed by agarose/formaldehyde gel electrophoresis as in figure 4-1. (C) and (D) The membranes displayed in panels A and B respectively were hybridized to a probe corresponding to the 3' most 400 nt of SRPα RNA (after the radioactive signal from the cap label had been allowed to decay for more than 6 half-lives). Arrows and associated numbers indicate the position and sizes of the vhs-induced cleavage intermediates. Numbers to the left of panel A and right of panel B indicate the sizes of RNA markers (lanes M) in nucleotides. (E) Diagram showing the structures of both transcripts, with the approximate location of vhs-induced cleavages indicated by arrows. The shaded rectangle represents the EMCV IRES.

A.
SRPα
vhs
RC
M 0 2 4 6 8 10 10
2400 nt
1600
800
400
300

B.
SRPα StuI IRES
vhs
RC
0 2 4 6 8 10 10 M
2300
2400 nt
1600
800
400
300

700
550
350
30-40

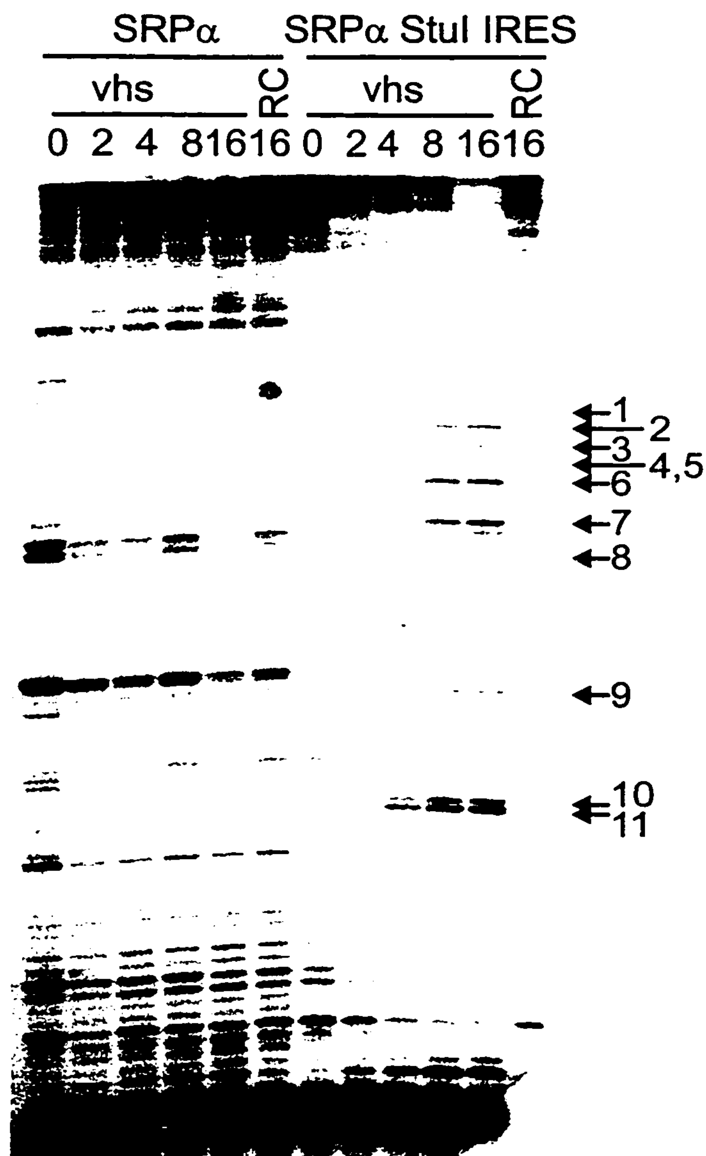
C.
SRPα
vhs
RC
M 0 2 4 6 8 10 10
2400
1600

D.
SRPα StuI IRES
vhs
RC
0 2 4 6 8 10 10 M
2800-
2400
700

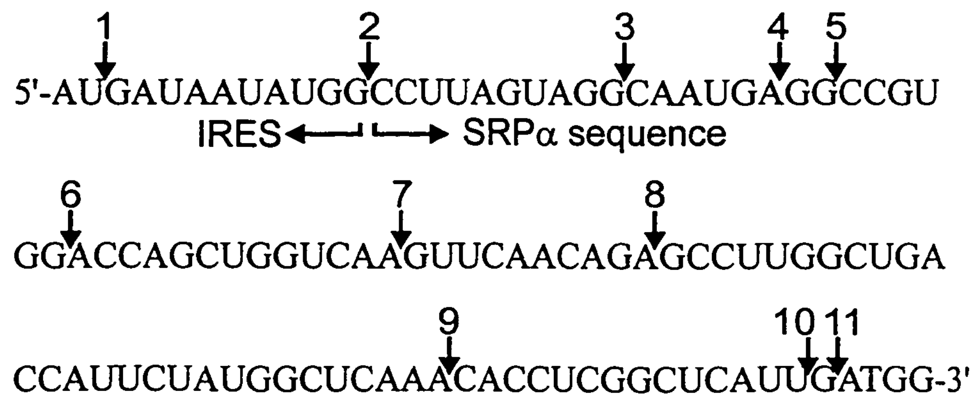
E.
20-30 200-700
2300-2400
IRES
3' Probe
SRPα
StuI IRES
StuI 1721
2304
3000
20-30 200-700
3' Probe
SRPα
2400

Figure 4-6. The EMCV IRES targets cleavage to 3' flanking SRPα sequences. (A) Uncapped unlabeled SRPα and SRPα StuI IRES RNAs were combined with RRL containing vhs (lanes marked vhs) and control RRL (lanes marked RC) and samples were withdrawn at the indicated times (minutes). RNAs were then extracted and analyzed by primer extension using a 5' labeled oligonucleotide that anneals to residues 1841-1865 of SRPα RNA (and residues 2424-2448 of the SRPα StuI IRES transcript). Numbered arrows indicate the position of novel 5' ends resulting from vhs-induced RNA cleavage. (B) A diagram showing the nucleotide sequence at the 3' boundary of the IRES in SRPα StuI IRES RNA. Numbered arrows correspond to those in panel A and indicate the locations of the vhs-induced cleavage sites.

A.



B.



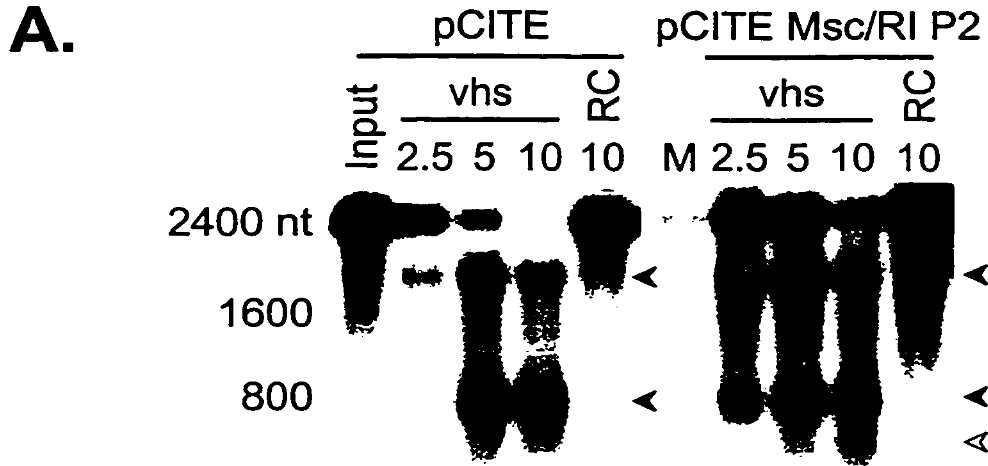
In this case as well, primer extension analysis using a primer complementary to residues 2424 to 2448 of the SRPa StuI IRES transcript revealed that the cleavage events induced by the IRES occurred in the 3' flanking sequences (figure 4-6A). Only one weak cleavage site was detected within the IRES element (site 1). Interestingly, both this site and the site at the junction between the IRES and SRPα sequences (site 2) are the same as those observed with pSexAI IRES (compare figures 4-6B and 4-4B).

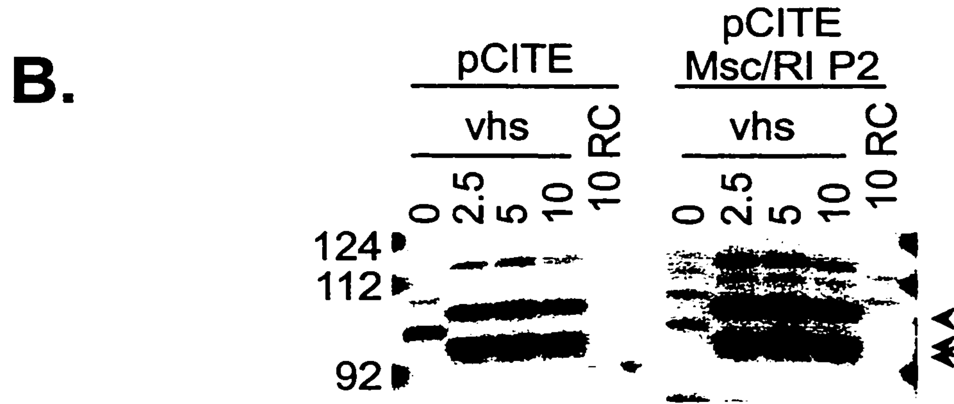
Taken in combination, these data clearly show that the EMCV IRES targets vhs-dependent endoribonucleolytic cleavage events to 3' flanking sequences. This activity is independent of sequence context, and operates when the IRES is placed at a variety of locations in the substrate RNA.

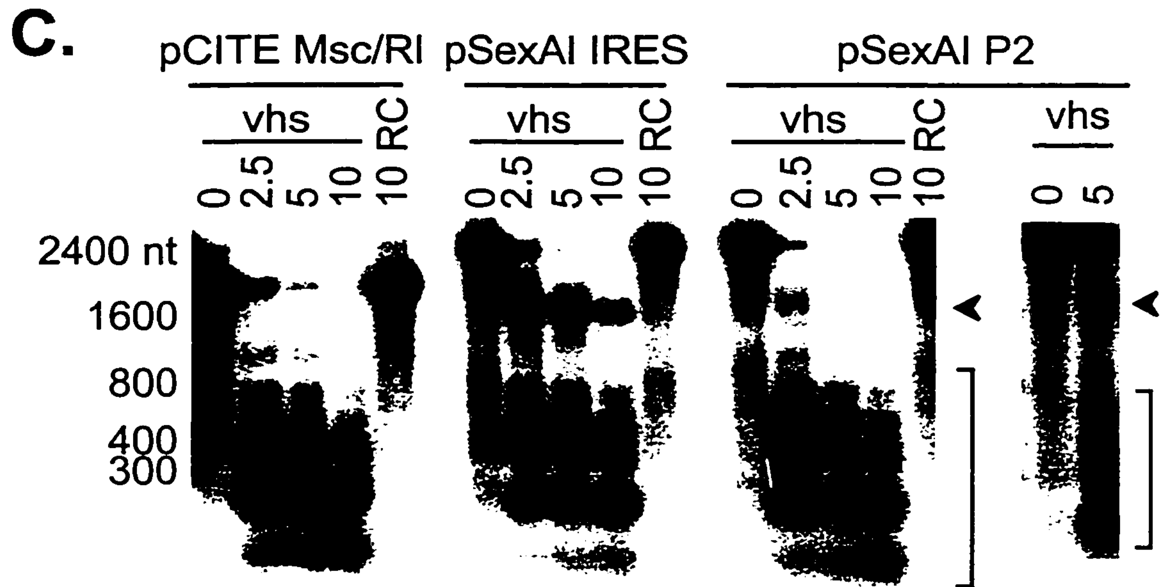
The poliovirus IRES also serves as a movable vhs targeting element. We asked if the unrelated poliovirus IRES also acts to target vhs-dependent cleavage events. To this end, we constructed pCITE derivatives in which the EMCV IRES element in pCITE and pSexAI IRES was replaced with a 600 nt fragment corresponding to the polio virus type 2 IRES, yielding pCITE Msc/RI P2 and pSexAI P2 respectively.

Internally labeled pCITE Msc/RI P2 RNA gave rise to ca. 1800 and 600 nt. products at early times (Figure 4-7A, filled arrow heads). These co-migrated with those generated from pCITE RNA, and therefore likely correspond to the 3' and 5' fragments produced by cleavage near the 3' end of the poliovirus IRES, respectively. pCITE Msc/RI P2 RNA also produced a ca. 450 nt fragment (figure 4-7A, open arrow head) which we have yet to definitively identify. Primer extension revealed that the IRES-

Figure 4-7. The poliovirus IRES serves as a moveable targeting element for vhs-induced RNA cleavage. (A) Uncapped internally labeled pCITE and pCITE Msc/RI P2 RNAs were reacted with RRL containing vhs and control RRL (RC) and samples removed at indicated times (minutes) were analyzed by agarose/formaldehyde gel electrophoresis as in figure 4-1. pCITE Msc/RI P2 is a derivative of pCITE in which the EMCV IRES is replaced by the IRES of poliovirus type 2. (B) Uncapped unlabeled pCITE and pCITE Msc/RI P2 RNAs were added to RRL containing vhs and control RRL. RNAs extracted from samples removed at indicated times (minutes) were analyzed by primer extension using a 5' labeled oligonucleotide that anneals to residues 685-710 of pCITE RNA (and residues ca. 685-710 of pCITE Msc/RI P2 RNA). The arrow heads indicate the position of the novel 5' ends generated by vhs-induced cleavage. These correspond to the 3 primer extension products indicated in figure 4-2C. The fainter band running just above the 112 nt marker is the reverse transcriptase pause site at the MscI site of pCITE (figure 4-2C). Numbers to the left indicate the sizes of DNA markers in nucleotides. (C) Cap labeled run-off transcripts of the indicated plasmids were combined with RRL containing vhs (lanes marked vhs) and control RRL (lanes marked RC) and samples removed at indicated times (minutes) were analyzed by agarose/formaldehyde gel electrophoresis as in figure 4-1. pCITE Msc/RI lacks an IRES, while pSexAI IRES and pSexAI P2 bear the EMCV and poliovirus type 2 IRES elements respectively, inserted at the SexAI site of pCITE Msc/RI. Arrow heads in panels A and C indicate vhs-induced cleavage intermediates. Numbers to the left of panels A and C indicate the sizes of RNA markers (lanes M) in nucleotides.


A.
pCITE
pCITE Msc/RI P2
Input
vhs
RC
2.5 5 10 10
M 2.5 5 10 10
2400 nt
1600
800


B.
pCITE
pCITE Msc/RI P2
vhs
RC
0 2.5 5 10 10
124
112
92


C.
pCITE Msc/RI
pSexAI IRES
pSexAI P2
vhs
RC
0 2.5 5 10 10
0 5
2400 nt
1600
800
400
300

directed cleavages occurred at exactly the same sites downstream of the IRES element in both RNAs (figure 4-7B, arrow heads), strongly suggesting that both elements function in the same way to target vhs-dependent cleavage events.

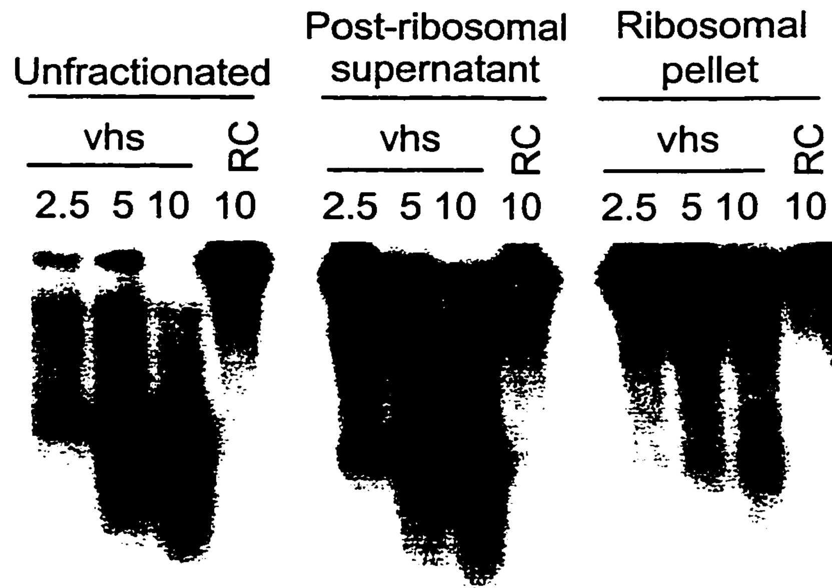
Taken in combination, these data indicate that the polio virus IRES targets vhs-dependent cleavage to 3' flanking sequences when it is placed at the 5' end of the pCITE transcript. To determine if the polio virus IRES retains this activity when it is placed at an internal site, we compared the degradation profiles of cap labeled pSexAI P2 RNA to that of the pSexAI IRES transcript (figure 4-7C). As described above, pSexAI IRES RNA gives rise to a novel 1400 nt 5' fragment representing cleavage downstream of the internal IRES, in addition to the 20-600 nt products observed with the parental pCITE Msc/RI construct (figure 4-7C). pSexAI P2 RNA also gave rise to the ca. 1400 nt fragments, albeit at a much lower levels than pSexA1 IRES (figure 4-7C, arrow head). These data therefore indicate that the poliovirus IRES displays weak but detectable targeting activity when it is located at an internal site.

The IRES targeting function does not require ribosomes. Vhs-induced RNA degradation occurs in the presence of agents that block translational initiation and elongation in HSV1 infected cells (13, 59, 67), and the RNA destabilizing activity present in extracts of infected cells partitions with the post-ribosomal fraction (64). These data indicate that an RNA need not be actively translated in order to be degraded. Consistent with this conclusion, we have shown that vhs-dependent degradation of SRPα does not require ribosomes in the RRL *in vitro* system (7). As described above, the IRES-dependent cleavage events that we have described in the present communication appear

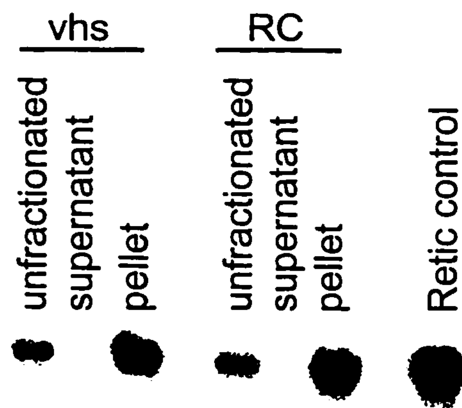
to occur independently of those that occur over the 5' quadrant of SRPα and pCITE RI/MscI RNA (figures 4-3 and 4-5). This observation, and the fact that IRES elements serve to recruit translation initiation components and ribosomes to mRNAs, prompted us to ask if ribosomes are required for the IRES to target vhs-dependent cleavage events to 3' flanking sequences (figure 4-8). To answer this question, vhs was first generated in RRL , then the ribosomes were removed from the lysate by ultracentrifugation. The extent of ribosomal clearance was verified by assaying the post-ribosomal supernatant for rabbit 18S rRNA by Northern blot hybridization (figure 4-8B). We found that the post-ribosomal supernatant retained the ability to cleave internally labeled pCITE RNA downstream of the EMCV IRES, while the ribosomal pellet displayed substantially less activity. Inasmuch as the post-ribosomal fraction was devoid of detectable 18S rRNA, these data indicate that ribosomes are not required for the targeting activity of the IRES.

Figure 4-8. IRES-targeted cleavage occurs in the absence of ribosomes. Vhs was translated in RRL, then ribosomes were removed by ultracentrifugation as previously described (7). (A) The post-ribosomal supernatant and ribosomal pellets were combined with uncapped internally labeled pCITE RNA, and samples recovered at various times (minutes) were analyzed by agarose / formaldehyde gel electrophoresis as in figure 4-1. RC: blank RRL control. (B) Northern blot analysis of the post-ribosomal fractions and controls using a 5' ^{32}P-labeled oligonucleotide probe complementary to rabbit 18S ribosomal RNA.

A.



B.



4.5. Discussion.

Currently available data indicate that vhs selectively targets mRNA *in vivo* and *in vitro* (7, 34, 49, 50, 75). However, the basis for this apparent selectivity remains obscure. We and others have previously demonstrated that the *in vitro* vhs-induced RNA cleavage activity is independent of the 5'cap structure and 3' poly(A) tail (7, 75), excluding the two most obvious structural features that distinguish mRNAs from other cytoplasmic RNA species. Thus, uncovering the basis for substrate recognition by the vhs-dependent endoribonuclease is of considerable interest. The experiments described in this report demonstrate that RNA substrates bearing the EMCV IRES are preferentially cleaved by the vhs-dependent endoribonuclease at multiple sites clustered in a narrow zone located immediately downstream of the IRES. This selective 'targeting" of cleavage events was observed when the IRES was placed at the 5' end of the pCITE-1 substrate, or moved to internal sites in this and one other unrelated RNA (figures 4-1 to 4-6, and additional data not shown). Taken together, these observations indicate that the targeting phenomenon does not depend on the sequence context or location of the IRES within the transcript. The cleavages provoked by the inserted IRES occur at sites that are not preferentially used in substrates lacking the element (figures 4-3 to 4-6), demonstrating that the IRES behaves as a moveable element that targets cleavage to 3' flanking sequences. The unrelated poliovirus IRES displayed detectable but weaker activity, demonstrating that the EMCV IRES is not unique in this regard and raising the possibility that a variety of IRES elements are capable of similarly targeting vhs-dependent cleavage events.

How do the EMCV and poliovirus IRES elements target vhs-dependent cleavage events to 3' flanking sequences? One intriguing possibility is that this activity reflects the translational initiation function of these elements. As reviewed in the introduction, picornavirus IRES elements bind translational initiation factors that recruit the 40S ribosomal subunit to the RNA, thereby promoting cap-independent initiation of translation. Although our data demonstrate that ribosomes are not required for IRES-directed cleavage, it is possible that the vhs-dependent endoribonuclease activity is delivered to the RNA substrate through interactions with one or more of the translation initiation factors that act upstream of ribosome loading (e.g., eIF3, eIF4A, eIF4B, or eIF4G). This is an attractive hypothesis, as it could provide a functional link between vhs and the translational apparatus, and thus potentially explain how mRNAs (including those that lack an IRES) are selectively targeted for degradation *in vivo*. Moreover, this mechanism would preferentially target those mRNAs that are translated at the highest rate. Two additional features of our data are consistent with this model. First, the cluster of vhs-dependent cleavages provoked by the IRES in the pCITE-1 RNA is located around the site where the 40S ribosomal subunit loads (i.e., the initiation codon). Second, the EMCV IRES is substantially more active than the poliovirus IRES in targeting vhs-dependent cleavage, particularly when it is placed at an internal site in the RNA substrate (figure 4-7). This difference correlates with the relative translational initiation efficiency of these elements, as the EMCV IRES is significantly more active in promoting translation than the poliovirus IRES in RRL *in vitro* and in some cell lines *in vivo* (1, 2). The hypothesis that translation initiation factors serve to selectively target vhs activity to mRNAs is also generally consistent with our previous observation that vhs-induced

degradation of SRPα mRNA (lacking an IRES) appears to initiate by endoribonucleolytic cleavage events clustered over the 5' quadrant of the RNA (7).

Several lines of evidence could be interpreted to argue against the foregoing hypothesis. First, *in vivo* experiments using translational inhibitors have demonstrated that mRNAs need not be engaged in ongoing translation in order to be targeted by vhs (59, 67). However, all of the drugs used in these studies act at, or downstream of, ribosome loading onto the mRNA. Therefore, these data do not necessarily exclude a role for initiation factors that function to recruit ribosomes to the mRNA. Second, the hypothesis that vhs is recruited to mRNAs through interactions with translation initiation factors predicts that the 5' cap should markedly stimulate degradation of mRNAs that lack an IRES. However, we have previously shown that the presence of a 5' cap does not detectably alter the rate or mode of degradation of SRPα mRNA in the rabbit reticulocyte *in vitro* system (7). However, it is worth noting that translation initiation in rabbit reticulocyte lysates is relatively cap-independent (44). Therefore, this line of evidence does not definitively exclude the hypothesis. Third, Zelus et al. (75) have shown that extracts of partially purified HSV-1 virions contain vhs-dependent ribonuclease activity. These data suggest, but do not prove, that vhs displays detectable activity in the absence of translation initiation factors. If so, then it would be highly informative to determine if IRES elements retain their ability to target vhs-induced cleavage in such virion extracts.

Alternatively, it is possible that targeting is mechanistically unrelated to translation, and instead directly depends on one or more structural features of the IRES

element. We can envision at least three distinct structure-based mechanisms that could give rise to the observed cleavage pattern:

1. The IRES might serve as a preferred loading site for the vhs-dependent endoribonuclease. In this scenario, the nuclease directly recognizes and binds to the IRES (likely through one or more features of the extensive secondary structure adopted by the element), then tracks along the RNA into flanking sequences until it encounters relatively unstructured regions that are susceptible to cleavage. Our observation that cleavage occurs only in 3' flanking sequences could be readily explained under this scenario if vhs tracks exclusively in a 5' to 3' direction. In this context it is interesting to note that vhs displays weak but significant amino acid sequence similarity to the fen-1 family of nucleases that are involved in DNA replication and repair (6). Fen-1 loads onto the 5' end of DNA substrates, then tracks in a 3' direction until it encounters structural features that trigger cleavage (reviewed in reference (42)). Moreover, recent evidence suggests that fen-1 interacts with RNA substrates in a similar fashion (65).

The hypothesis that vhs tracks along the RNA in the 5' to 3' direction in search of cleavage sites is interesting, as it could also potentially explain our observation that two transcripts lacking an IRES are initially cleaved at multiple sites located over the 5' quadrant of the RNA ((7) and figure 4-3). Specifically, if one assumes that the 5' end of the RNA also serves as a preferred loading site for the vhs-dependent nuclease (as is the case for fen-1), then 5' loading and tracking would give rise to the observed pattern.

2. The highly structured IRES might serve as a barrier to the movement of the nuclease along the RNA, resulting in accumulation of the enzyme and clustering of

cleavage events at the boundary of the IRES. In order to accommodate the observation that only 3' flanking sequences are targeted, one would likely have to propose that the nuclease tracks in a 3' to 5' direction. This requirement is not obviously consistent with our observation that transcripts containing an internal IRES are also cleaved over their 5' quadrant, and that these 5' cleavage events appear to occur independently of those provoked by the IRES. Indeed, these observations are more compatible with scenario 1 as described above. Moreover, our preliminary results indicate that the highly structured HIV TAR element does not serve to target vhs-dependent cleavage, arguing that targeting requires specific structural features rather than secondary structure *per se*. (J. Perez-Parada, M. Elgadi, and J.R. Smiley, unpublished data).

3. The vhs-dependent nuclease might directly recognize and cleave the 3' junction between highly structured and relatively unstructured regions. Although we cannot exclude this possibility, we note that it does not directly predict that the IRES-induced cleavages would be distributed over a fairly broad zone (that in the case of the pSexA1 IRES transcript extends over ca. 300 nt, figure 4-3). This observation seems more compatible with scenarios 1 and 2.

The possibility that the vhs-dependent nuclease directly recognizes a structural feature of the IRES raises questions about the possible biological significance of this activity. Perhaps vhs is designed to selectively target cellular or viral transcripts that contain IRES elements or other highly structured regions. Alternatively, the activity might mirror another function of vhs. As noted above, vhs displays limited but significant amino acid sequence similarity to the fen-1 family of nucleases (6). These enzymes are

structure-specific endonucleases involved in DNA replication and repair (reviewed in reference (42)). Fen-1 specifically recognizes and cleaves 5' flap structures in DNA substrates, by loading onto the 5' end of the unpaired strand and tracking in 3' direction until it reaches the base of the flap. Fen-1 also performs structure-specific cleavage of RNA substrates, at the 5' base of stem-loops (65). The RNAse activity of fen-1 appears to mirror its DNAse activity in that fen-1 likely loads at the 5' end of the RNA and then tracks 5' to 3' until it encounters the secondary structure elements that trigger cleavage. By analogy with fen-1, it is conceivable that vhs is capable of structure-specific cleavage of both DNA and RNA substrates.

We have previously reported that vhs-dependent cleavage events at the extreme 5' end of SRPα RNA tend to occur between purine residues (7). In this report, we have mapped 26 additional cleavage sites at high resolution (figures 4-2, 4-4, and 4-6) . In addition, Zelus and colleagues mapped 6 cleavage sites in β-globin RNA (75). The combined data from these three studies indicate that of 37 sites analyzed, 19 occur between purine residues. The others are: GC (6), UG (5), AC (2), GU, CU, UU, UC, and CG (1 each). In combination, these data suggest that the vhs-induced endoribonuclease displays relaxed sequence specificity.

The experiments outlined in this report describe a novel and unanticipated effect of picornavirus IRES elements in targeting vhs-dependent endoribonucleolytic cleavage events. We suspect that further analysis of this activity may lead to increased understanding of how vhs targets mRNAs *in vivo*.

4.6. Acknowledgements.

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4.7. References.

1. **Borman, A. M., J. L. Bailly, M. Girard, and K. M. Kean.** 1995. Picornavirus internal ribosome entry segments: comparison of translation efficiency and the requirements for optimal internal initiation of translation in vitro. Nucleic Acids Res. **23:**3656-3663.

2. **Borman, A. M., P. Le Mercier, M. Girard, and K. M. Kean.** 1997. Comparison of picornaviral IRES-driven internal initiation of translation in cultured cells of different origins. Nucleic Acids Res. **25:**925-932.

3. **Buckley, B., and E. Ehrenfeld.** 1987. The cap-binding protein complex in uninfected and poliovirus-infected HeLa cells. J Biol. Chem. **262:**13599-13606.

4. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA. **81:**1991-1995.

5. **Devaney, M. A., V. N. Vakharia, R. E. Lloyd, E. Ehrenfeld, and M. J. Grubman.** 1988. Leader protein of foot-and-mouth disease virus is required for cleavage of the p220 component of the cap-binding protein complex. J. Virol. **62:**4407-4409.

6. **Doherty, A. J., L. C. Serpell, and C. P. Ponting.** 1996. The helix-hairpin-helix DNA-binding motif: a structural basis for non-sequence-specific recognition of DNA. Nucleic Acids Res. **24:**2488-2497.

7. **Elgadi, M. M., C. E. Hayes, and J. R. Smiley.** 1999. The Herpes simplex virus vhs protein induces endoribonucleolytic cleavage of target RNAs in cell-free extracts. J Virol. **In press.**

8. **Etchison, D., and S. Fout.** 1985. Human rhinovirus 14 infection of HeLa cells results in the proteolytic cleavage of the p220 cap-binding complex subunit and inactivates globin mRNA translation in vitro. J. Virol. **54:**634-638.

9. **Falcone, D., and D. W. Andrews.** 1991. Both the 5' untranslated region and the sequence surrounding the start site contribute to efficient initiation of translation in vitro. Mol. Cell. Biol. **11:**2656-2664.

10. **Feigenblum, D., and R. J. Schneider.** 1993. Modification of eukaryotic initiation factor 4F during infection by influenza virus. J. Virol. **67:**3027-3035.

11. **Fenwick, M. L.** 1984. The effect of herpesviruses on cellular macromolecule synthesis., p. 359-390. *In* H. Fraenkel-Conrat and R. K. Wagner (ed.), Comprehensive Virology, vol. 19. Plenum Press Corp., New York.

12. **Fenwick, M. L., and J. Clark.** 1982. Early and delayed shut-off of host protein synthesis in cells infected with herpes simplex virus. J. Gen. Virol. **61:**121-125.

13. **Fenwick, M. L., and M. M. McMenamin.** 1984. Early virion-associated suppression of cellular protein synthesis by herpes simplex virus is accompanied by inactivation of mRNA. J. Gen. Virol. **65:**1225-1228.

14. **Fenwick, M. L., and S. A. Owen.** 1988. On the control of immediate early (alpha) mRNA survival in cells infected with herpes simplex virus. J. Gen. Virol. **69:**2869-2877.

15. **Fenwick, M. L., and M. J. Walker.** 1978. Suppression of synthesis of cellular macromolecules by herpes simplex virus. J. Gen. Virol. **41:**37-51.

16. **Gallie, D. R.** 1998. A tale of two termini: a functional interaction between the termini of an mRNA is a prerequisite for efficient translation initiation. Gene. **216:**1-11.

17. **Garfinkel, M. S., and M. G. Katze.** 1993. Translational control by influenza virus. Selective translation is mediated by sequences within the viral mRNA 5'-untranslated region. J. Biol. Chem. **268:**22223-22226.

18. **Gingras, A. C., and N. Sonenberg.** 1997. Adenovirus infection inactivates the translational inhibitors 4E-BP1 and 4E-BP2. Virology. **237:**182-186.

19. **Gingras, A. C., Y. Svitkin, G. J. Belsham, A. Pause, and N. Sonenberg.** 1996. Activation of the translational suppressor 4E-BP1 following infection with encephalomyocarditis virus and poliovirus. Proc. Natl. Acad. Sci. U S A. **93:**5578-5583.

20. **Gradi, A., H. Imataka, Y. V. Svitkin, E. Rom, B. Raught, S. Morino, and N. Sonenberg.** 1998. A novel functional human eukaryotic translation initiation factor 4G. Mol. Cell Biol. **18:**334-342.

21. **Gradi, A., Y. V. Svitkin, H. Imataka, and N. Sonenberg.** 1998. Proteolysis of human eukaryotic translation initiation factor eIF4GII, but not eIF4GI, coincides with the shutoff of host protein synthesis after poliovirus infection. Proc. Natl. Acad. Sci. U S A. **95:**11089-11094.

22. **Haghighat, A., S. Mader, A. Pause, and N. Sonenberg.** 1995. Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. Embo J. **14:**5701-5709.

23. **Haghighat, A., Y. Svitkin, I. Novoa, E. Kuechler, T. Skern, and N. Sonenberg.** 1996. The eIF4G-eIF4E complex is the target for direct cleavage by the rhinovirus 2A proteinase. J. Virol. **70:**8444-8450.

24. **Hentze, M. W.** 1997. eIF4G: a multipurpose ribosome adapter? [published erratum appears in Science 1997 Mar 14;275(5306):1553]. Science. **275:**500-501.

25. **Huang, J. T., and R. J. Schneider.** 1991. Adenovirus inhibition of cellular protein synthesis involves inactivation of cap-binding protein. Cell. **65:**271-280.

26. **Imataka, H., A. Gradi, and N. Sonenberg.** 1998. A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. Embo J. **17:**7480-7489.

27. **Imataka, H., and N. Sonenberg.** 1997. Human eukaryotic translation initiation factor 4G (eIF4G) possesses two separate and independent binding sites for eIF4A. Mol. Cell. Biol. **17:**6940-6947.

28. **Jang, S. K., M. V. Davies, R. J. Kaufman, and E. Wimmer.** 1989. Initiation of protein synthesis by internal entry of ribosomes into the 5' nontranslated region of encephalomyocarditis virus RNA in vivo. J. Virol. **63:**1651-1660.

29. **Jang, S. K., H. G. Krausslich, M. J. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer.** 1988. A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J. Virol. **62:**2636-2643.

30. **Joachims, M., P. C. Van Breugel, and R. E. Lloyd.** 1999. Cleavage of poly(A)-binding protein by enterovirus proteases concurrent with inhibition of translation in vitro. J. Virol. **73:**718-727.

31. **Jones, F. E., C. A. Smibert, and J. R. Smiley.** 1995. Mutational analysis of the herpes simplex virus virion host shutoff protein: evidence that vhs functions in the absence of other viral proteins. J. Virol. **69:**4863-4871.

32. **Kerekatte, V., B. D. Keiper, C. Badorff, A. Cai, K. U. Knowlton, and R. E. Rhoads.** 1999. Cleavage of Poly(A)-binding protein by coxsackievirus 2A protease in vitro and in vivo: another mechanism for host protein synthesis shutoff? J. Virol. **73:**709-717.

33. **Kleijn, M., C. L. Vrins, H. O. Voorma, and A. A. Thomas.** 1996. Phosphorylation state of the cap-binding protein eIF4E during viral infection. Virology. **217:**486-494.

34. **Krikorian, C. R., and G. S. Read.** 1991. In Vitro mRNA degradation system to study the virion host shutoff function of herpes simplex virus. J. Virol. **65:**112-122.

35. **Kwong, A. D., and N. Frenkel.** 1989. The herpes simplex virus virion host shutoff function. J. Virol. **63:**4834-4839.

36. **Kwong, A. D., and N. Frenkel.** 1987. Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs. Proc. Natl. Acad. Sci. USA. **84:**1926-1930.

37. **Kwong, A. D., J. A. Kruper, and N. Frenkel.** 1988. Herpes simplex virus virion host shutoff function. J. Virol. **62:**912-921.

38. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **227:**680-685.

39. **Lam, Q., C. A. Smibert, K. E. Koop, C. Lavery, J. P. Capone, S. P. Weinheimer, and J. R. Smiley.** 1996. Herpes simplex virus VP16 rescues viral mRNA from destruction by the virion host shutoff function. Embo J. **15:**2575-2581.

40. **Lamphear, B. J., R. Kirchweger, T. Skern, and R. E. Rhoads.** 1995. Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. Implications for cap-dependent and cap-independent translational initiation. J. Biol. Chem. **270:**21975-21983.

41. **Lamphear, B. J., R. Yan, F. Yang, D. Waters, H. D. Liebig, H. Klump, E. Kuechler, T. Skern, and R. E. Rhoads.** 1993. Mapping the cleavage site in protein synthesis initiation factor eIF-4 gamma of the 2A proteases from human Coxsackievirus and rhinovirus. J. Biol. Chem. **268:**19200-19203.

42. **Lieber, M. R.** 1997. The FEN-1 family of structure-specific nucleases in eukaryotic DNA replication, recombination and repair. Bioessays. **19:**233-240.

43. **Lin, T. A., X. Kong, T. A. Haystead, A. Pause, G. Belsham, N. Sonenberg, and J. C. Lawrence, Jr.** 1994. PHAS-I as a link between mitogen-activated protein kinase and translation initiation [see comments]. Science. **266:**653-656.

44. **Lodish, H. F., and J. K. Rose.** 1977. Relative importance of 7-methylguanosine in ribosome binding and translation of vesicular stomatitis virus mRNA in wheat germ and reticulocyte cell-free systems. J. Biol. Chem. **252:**1181-1188.

45. **Mader, S., H. Lee, A. Pause, and N. Sonenberg.** 1995. The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins. Mol. Cell. Biol. **15:**4990-4997.

46. **Nishioka, Y., and S. Silverstein.** 1977. Degradation of cellular mRNAs during infection by herpes simplex virus. Proc. Natl. Acad. Sci. USA. **74:**2370-2374.

47. **Nishioka, Y., and S. Silverstein.** 1978. Requirement of protein synthesis for the degradation of host mRNA in friend erythroleukemia cells infected with herpes simplex virus type 1. J. Virol. **27:**619-627.

48. **Ohlmann, T., M. Rau, V. M. Pain, and S. J. Morley.** 1996. The C-terminal domain of eukaryotic protein synthesis initiation factor (eIF) 4G is sufficient to support cap-independent translation in the absence of eIF4E. Embo J. **15:**1371-1382.

49. **Oroskar, A. A., and G. S. Read.** 1989. Control of mRNA stability by the virion host shutoff function of herpes simplex virus. J. Virol. **63:**1897-1906.

50. **Oroskar, A. A., and G. S. Read.** 1987. A mutant of herpes simplex virus type 1 exhibits increased stability of immediate-early (alpha) mRNAs. J. Virol. **61:**604-606.

51. **Pak, A. S., D. N. Everly, K. Knight, and G. S. Read.** 1995. The virion host shutoff protein of herpes simplex virus inhibits reporter gene expression in the absence of other viral gene products. Virology. **211:**491-506.

52. **Park, Y. W., and M. G. Katze.** 1995. Translational control by influenza virus. Identification of cis-acting sequences and trans-acting factors which may regulate selective viral mRNA translation. J. Biol. Chem. **270:**28433-28439.

53. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature. **334:**320-325.

54. **Pestova, T. V., S. V. Maslova, V. K. Potapov, and V. I. Agol.** 1989. Distinct modes of poliovirus polyprotein initiation in vitro. Virus Res. **14:**107-118.

55. **Pestova, T. V., I. N. Shatsky, and C. U. Hellen.** 1996. Functional dissection of eukaryotic initiation factor 4F: the 4A subunit and the central domain of the 4G subunit are sufficient to mediate internal entry of 43S preinitiation complexes. Mol. Cell. Biol. **16:**6870-6878.

56. **Preiss, T., and M. W. Hentze.** 1998. Dual function of the messenger RNA cap structure in poly(A)-tail- promoted translation in yeast. Nature. **392:**516-520.

57. **Read, G. S., and N. Frenkel.** 1983. Herpes simplex virus mutants defective in the virion-associated shutoff of host polypeptide synthesis and exhibiting abnormal synthesis of a (immediate early) viral polypeptides. J. Virol. **46:**498-512.

58. **Read, G. S., B. M. Karr, and K. Knight.** 1993. Isolation of a herpes simplex virus type 1 mutant with a deletion in the virion host shutoff gene and identification of multiple forms of the vhs (UL41) polypeptide. J. Virol. **67:**7149-7160.

59. **Schek, N., and S. L. Bachenheimer.** 1985. Degradation of cellular mRNAs induced by a virion-associated factor during herpes simplex virus infection of Vero cells. J. Virol. **55:**601-610.

60. **Smibert, C. A., D. C. Johnson, and J. R. Smiley.** 1992. Identification and characterization of the virion-induced host shutoff product of herpes simplex virus gene UL41. J. Gen. Virol. **73:**467-470.

61. **Smibert, C. A., B. Popova, P. Xiao, J. P. Capone, and J. R. Smiley.** 1994. Herpes simplex virus VP16 forms a complex with the virion host shutoff protein vhs. J. Virol. **68:**2339-2346.

62. **Smibert, C. A., and J. R. Smiley.** 1990. Differential regulation of endogenous and transduced beta-globin genes during infection of erythroid cells with a herpes simplex virus type 1 recombinant. J.Virol. **64:**3882-3894.

63. **Sonenberg, N.** 1994. Regulation of translation and cell growth by eIF4E. Biochimie. **76:**839-846.

64. **Sorenson, C. M., P. A. Hart, and J. Ross.** 1991. Analysis of herpes simplex virus-induced mRNA destabilizing activity using an in vitro mRNA decay system. Nucleic Acids Res. **19:**4459-4465.

65. **Stevens, A.** 1998. Endonucleolytic cleavage of RNA at 5' endogenous stem structures by human flap endonuclease 1. Biochem. Biophys. Res. Com. **251:**501-508.

66. **Strelow, L. I., and D. A. Leib.** 1995. Role of the virion host shutoff (vhs) of herpes simplex virus type 1 in latency and pathogenesis. J. Virol. **69:**6779-6786.

67. **Strom, T., and N. Frenkel.** 1987. Effect of herpes simplex virus on mRNA stability. J. Virol. **61:**2198-2207.

68. **Svitkin, Y. V., A. Gradi, H. Imataka, S. Morino, and N. Sonenberg.** 1999. Eukaryotic initiation factor 4GII (eIF4GII), but not eIF4GI, cleavage correlates with inhibition of host cell protein synthesis after human rhinovirus infection. J. Virol. **73:**3467-3472.

69. **Sydiskis, R. J., and B. Roizman.** 1967. The disaggregation of host polyribosomes in productive and abortive infection with herpes simplex virus. Virology. **32:**678-686.

70. **Sydiskis, R. J., and B. Roizman.** 1966. Polysomes and protein synthesis in cells infected with a DNA virus. Science. **153:**76-78.

71. **Tarun, S. Z., Jr., S. E. Wells, J. A. Deardorff, and A. B. Sachs.** 1997. Translation initiation factor eIF4G mediates in vitro poly(A) tail- dependent translation. Proc. Natl. Acad. Sci. U S A. **94:**9046-9051.

72. **Witherell, G. W., and E. Wimmer.** 1994. Encephalomyocarditis virus internal ribosomal entry site RNA-protein interaction. J. Virol. **68:**3183-3192.

73. **Young, J. C., J. Ursini, K. R. Legate, J. D. Miller, P. Walter, and D. W. Andrews.** 1995. An amino terminal domain containing hydrophobic and hydrophilic sequences binds the signal recognition particle receptor alpha subunit to the beta subunit on the endoplasmic reticulum membrane. J. Biol. Chem. **270:**15650-15657.

74. **Yueh, A., and R. J. Schneider.** 1996. Selective translation initiation by ribosome jumping in adenovirus- infected and heat-shocked cells. Genes Dev. **10:**1557-1567.

75. **Zelus, B. D., R. S. Stewart, and J. Ross.** 1996. The virion host shutoff protein of herpes simplex virus type1: messenger ribonucleolytic activity in vitro. J. Virol. **70:**2411-2419.

76. **Zhang, Y., D. Feigenblum, and R. J. Schneider.** 1994. A late adenovirus factor induces eIF-4E dephosphorylation and inhibition of cell protein synthesis. J. Virol. **68:**7040-7050.

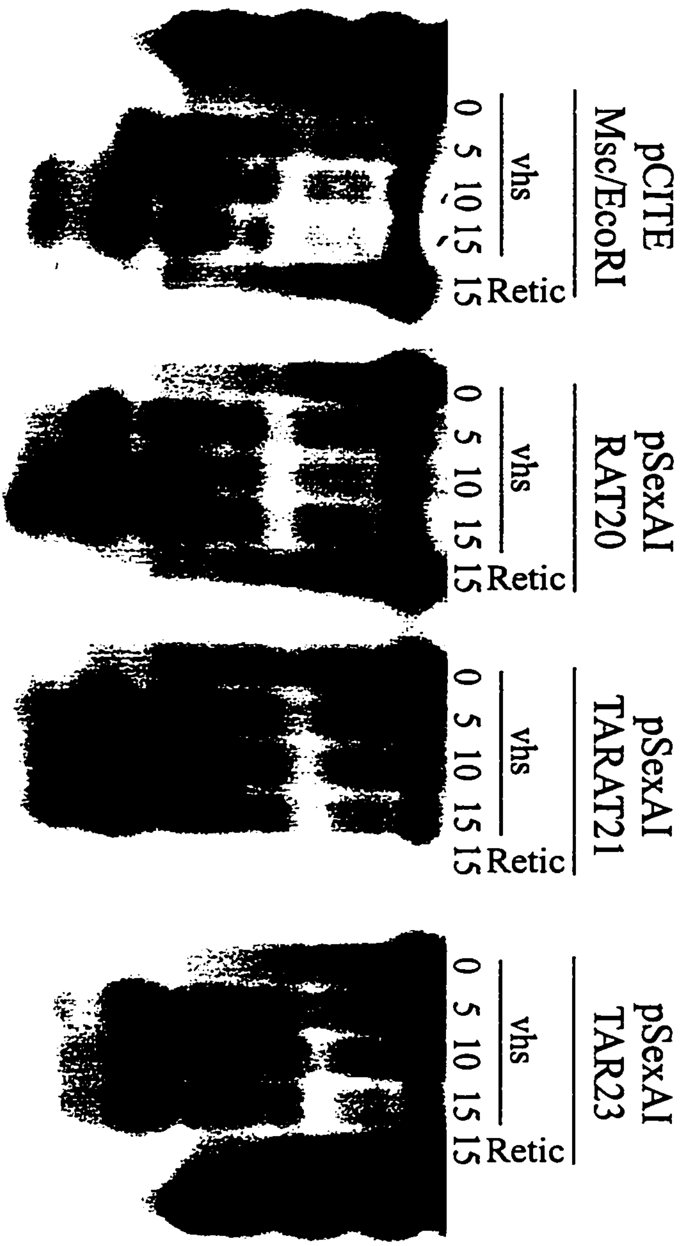
4.8. Addendum.

4.8.1. Data not shown in chapter 4.

The data described in chapter 4 show that the IRES elements of the EMCV and poliovirus target vhs-induced endoribonucleolytic RNA cleavage to 3' flanking sequences. These data raise the question of how these elements target vhs-dependent RNA cleavage. IRES elements are highly structured RNA sequences that mediate translation initiation by a cap- and 5' end-independent mechanism (reviewed by Hentze 1997). Two possible mechanisms of IRES-mediated vhs targeting were proposed in chapter 4. One is that vhs gains access to mRNAs by interacting with one or more components of the translation apparatus that interact with IRES elements. Alternatively, secondary structure features of the IRES may function to target vhs to mRNAs. The conclusion that secondary structure does not suffice to target vhs-induced RNA cleavage was based on the observation that the degradation profile of RNA substrates containing the highly structured HIV TAR sequence does not differ from the parental substrate lacking these sequences. However, the data was not presented. The purpose of this section is to show these data.

An oligonucleotide duplex containing the TAR sequence of HIV was inserted at the SexAI site of pCITE Msc/RI (lacking the IRES element). Three plasmids containing TAR sequences were generated: pSexAI RAT20 contains the TAR sequence in the antisense orientation, pSexA1 TARAT21 contains a sense-antisense concatemer of the

Figure 4-9. The presence of highly structured elements within the RNA is not sufficient to target vhs activity. Cap-labeled RNA substrates containing the HIV TAR elements (antisense orientation, RAT20; sense-antisense concatemer, TARAT21; sense orientation, TAR23) were reacted with control and vhs-containing RRLs. RNAs recovered from samples collected at indicated times (numbers above lanes, minutes) were resolved on a 1% agarose/6% formaldehyde gel as outlined in the material and methods. The cap label was detected by autoradiography using Kodak X-OMAT AR film.


pCITE
Msc/EcoRI
vhs
0 5 10 15 15 Retic
pSexAI
RAT20
vhs
0 5 10 15 15 Retic
pSexAI
TARAT21
vhs
0 5 10 15 15 Retic
pSexAI
TAR23
vhs
0 5 10 15 15 Retic

TAR sequence, and pSexAI TAR23 contains the TAR sequence in the sense orientation. Run-off transcripts generated by T7 RNA polymerase transcription using EcoNI-linearized plasmid DNAs as templates were cap labeled and used as substrate in RRL containing vhs. The results shown in figure 4-9, demonstrate that the presence of highly structured elements within the RNA is not sufficient to target vhs-dependent cleavage, inasmuch as the overall degradation profile of these RNAs does not differ from that of the parental pCITE Msc/RI transcript. These data do not, however, exclude the possibility that specific secondary structure features of the IRES are required to mediate vhs targeting. That is, a specific stem-loop or stem-loop tertiary arrangement within the context of other structures may be responsible for targeting vhs activity to RNA.

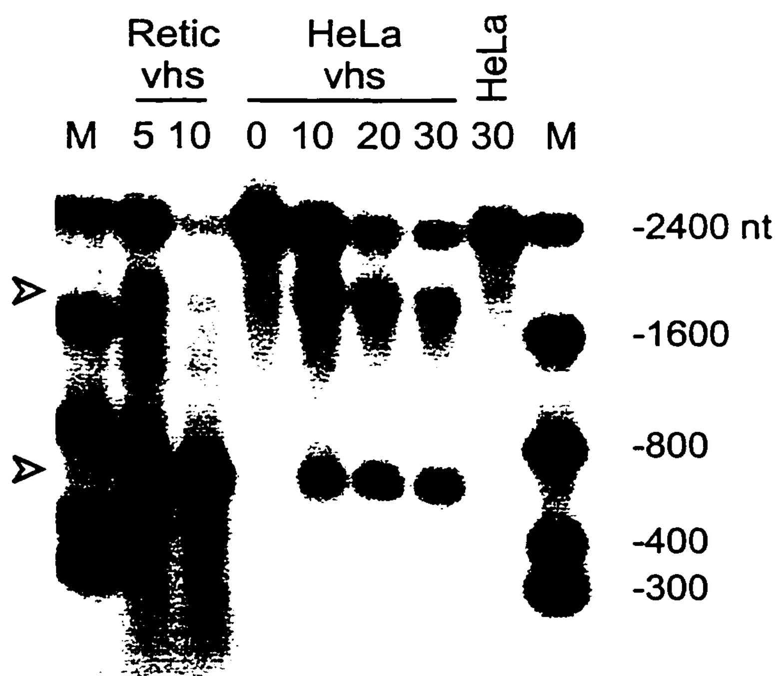
4.8.2. Unpublished data relating to chapter 4.

i) The EMCV IRES targets vhs-induced cleavage in extracts derived from HeLa cells.

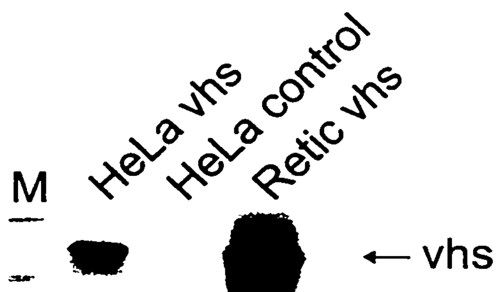
The data presented in chapter 4 clearly demonstrate that IRES elements target vhs-dependent RNA degradation activity to RNA in RRL. Although unlikely, it was possible that IRES-mediated vhs targeting is restricted to the RRL *in vitro* system. To test for generality of this targeting function, I compared the degradation profiles of uncapped, internally labeled pCITE mRNA in RRL and HeLa cell extracts containing vhs. The results clearly show that the EMCV IRES element functions as a vhs targeting element in both systems (figure 4-10A). Moreover, the degradation intermediates exhibited similar (if not identical) electrophoretic mobilities in both systems,

Figure 4-10. The EMCV IRES functions as a vhs targeting element in extracts derived from HeLa cell. (A). Uncapped internally labeled pCITE RNA was reacted with RRL and HeLa cell extracts containing pretranslated vhs. RNA recovered at the indicated times (numbers above lanes, minutes) were analyzed by 1% agarose/6% formaldehyde gel electrophoresis and radioactive signals were detected by autoradiography. Numbers to the right of panel A indicate the size of RNA markers in nucleotides. (B) Samples of the vhs-containing and control lysates used in panel A were analyzed by SDS-PAGE to determine the amount of 35S-labeled vhs protein present. Note that panel B is the same as that shown in figure 3-5B in chapter 3. This is because the same lysates were used to analyze the degradation profile of both SRPα (chapter 3, figure 3-5B) and pCITE (here) substrates.

A.



B.



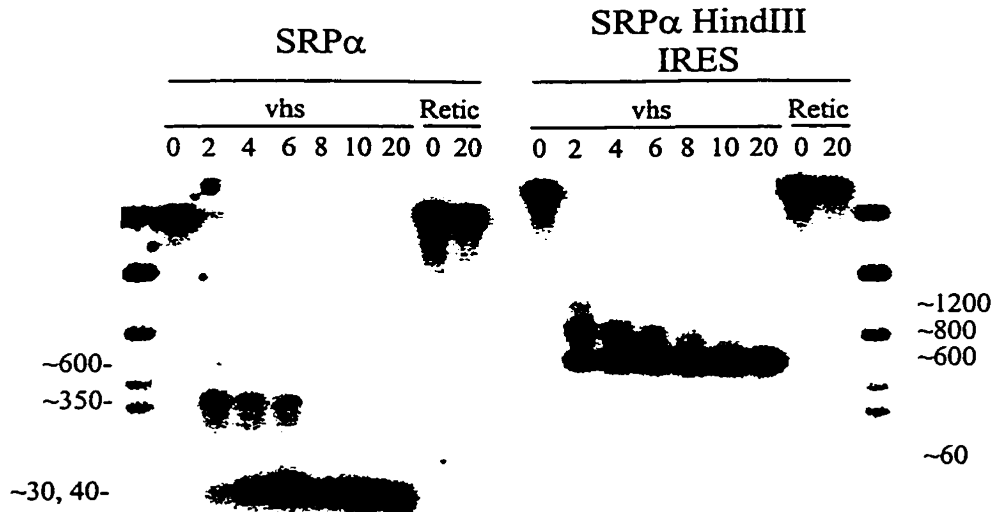
demonstrating that degradation occurs through a similar (if not the same) mechanism in the two systems. The low level of RNA degradation activity in HeLa cell extracts is most likely due to the fact that this extract contains much less vhs protein than RRL (figure 4-10B). These data argue for the biological relevance of our data.

ii) Specificity of vhs-induced cleavage of SRPα RNA.

Detailed analysis of the vhs-induced SRPα RNA degradation revealed that the RNA was initially cleaved at a number of sites non-randomly distributed over the 5' quadrant of the RNA (chapter 3). I also showed that the EMCV IRES functions as a sequence context- and position-independent vhs targeting element *in vitro* (chapter 4). This conclusion was reached following the detailed analysis of two RNA substrates that contain the IRES at internal position. An additional plasmid containing the IRES element inserted at the HindIII site (7 bp downstream of the SP6 transcriptional start site) of the SRPα RNA was also constructed. Comparison of the degradation profile of this RNA to that of SRPα RNA is shown in figure 4-11. As in the case of pCITE RNA, reaction of cap-labeled SRPα HindIII IRES with RRL-vhs yields a prominent and stable cap-labeled product of ca. 600 nt corresponding to cleavage at sites that map just downstream of the IRES (figure 4-11A and D). In addition to the 600 nt IRES fragment, cap-labeled fragments in the range of 800 to 1200 nt were detected (figure 4-11A). These fragments were unstable and degrade further as the reaction proceeds in favor of the 600 nt IRES band. These products result from cleavage at sites corresponding to the 200-600 nt sites detected on SRPα RNA displaced in the 3' direction by 600 nt (size of the inserted IRES)

Figure 4-11. The cleavage sites clustered over the 5' end of SRPα are 5' end-independent. Cap-labeled (A) and internally labeled (B) SRPα and SRPα HindIII IRES RNAs were added to RRL containing vhs and control RRL. RNA samples recovered at various time points (numbers above lanes, minutes) were analyzed by 1% agarose/6% formaldehyde gel electrophoresis. (C) Following the decay of the ^{32}P signal from the cap label for 3 months (more than 6 half-lives) the membrane in panel A was hybridized to a random primer ^{32}P-labeled DNA probe corresponding to the 3'-most 400 nt of SRPα RNA. (D) Schematic representation of the RNA substrates indicating the approximate position of vhs-dependent cleavage sites (arrows and accompanying numbers in nucleotides). Numbers to the sides of panels A, B, and C represent the estimated size of vhs-dependent fragments in nucleotides.

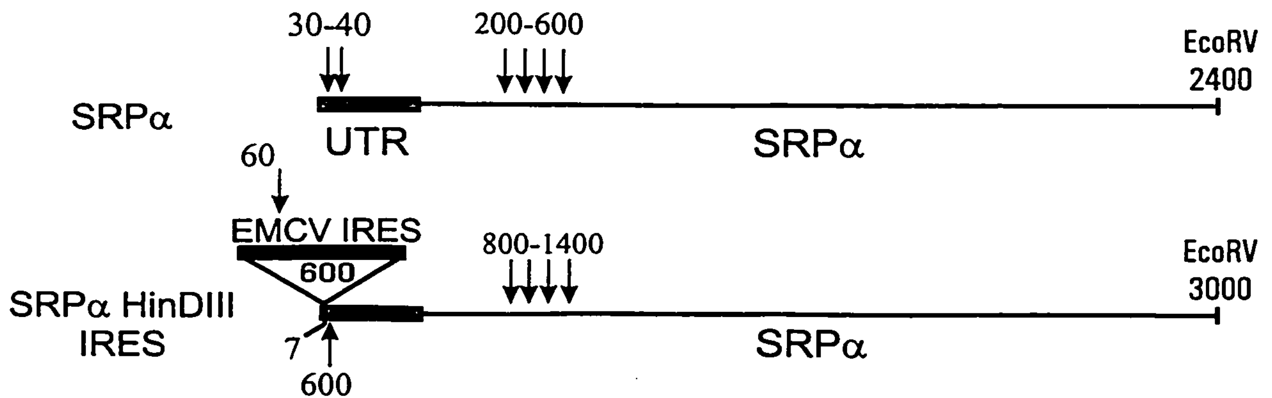
A.



B.

C.

D.



(illustrated schematically in figure 4-11D). Internally labeled SRPα HindIII IRES yield a stable fragment of ca. 600 nt (not detected on the parental SRPα RNA) corresponding to the IRES element (figure 4-11B). In addition, high molecular weight fragments that co-migrate with SRPα 3' cleavage products were detected. That these fragments represent 3' cleavage products is confirmed by probing the blot in panel A (after the cap signal was allowed to decay for more than six half-lives) with a DNA probe corresponding to the SRPα 3' most 400 nt. As expected, the profile of 3' products generated from SRPα and SRPα HindIII IRES were indistinguishable (figure 4-11C).

This degradation profile lends further support to the conclusion that the IRES element serves as a movable vhs targeting element. The data also support the conclusion that the vhs-dependent degradation of SRPα RNA initiates at prominent sites ca. 200-600 nt from the 5' end, since cleavages at sites further downstream (800-1200 nt) are easily detectable using SRPα HindIII IRES RNA. More importantly, these data show that the preferential cleavage of the 200-600 nt sites in SRPα RNA is independent of their location relative to the 5' end of the RNA. Instead, the data seem to suggest that specific features at these locations (such as sequence or secondary structure) render them preferred sites for vhs-dependent cleavage. It will be interesting to determine the degradation profile of SRPα RNAs in which these sites have been deleted or translocated closer to the 3' end.

CHAPTER 5: Discussion and Future Directions.

The limited coding capacity of viruses necessitates that they rely on the host cell's synthetic machinery for replication and production of progeny. In most, if not all, cases this occurs at the expense of the host cell in that the cellular transcription, replication, and translation apparatus are hijacked for the synthesis of viral components leading to cell death and release of infectious progeny virions. HSV is no exception. HSV lytic infection of permissive cells leads to efficient synthesis of viral DNA, mRNA, and protein and dramatic inhibition of the cellular counterparts. The selective inhibition of these cellular processes insures the availability of the synthetic apparatus and precursor molecules for the metabolism of viral components.

The HSV virions contain a number of regulatory functions that are delivered into cells upon entry and disassembly of the infecting virions. As such, these molecules are strategically poised to affect some of the earliest stages of infection, prior to the onset of viral gene expression. The best understood of these regulators is the potent transcriptional transactivator VP16, which stimulates the expression of the viral immediate early genes (Campbell *et al.*, 1984, Post *et al.*, 1981). Another, less understood, virion-associated regulatory activity is the virion-induced shutoff of host cell protein synthesis. Upon infection, HSV virions induce inhibition of host cell translation,

disaggregation of pre-existing cellular polysomes, and degradation of pre-existing cellular mRNAs. These effects (termed virion-induced host shutoff, vhs) occur in the absence of *de novo* viral gene expression and are dependent on a functional product of the HSV UL41 gene (Fenwick & Clark, 1982, Fenwick & McMenamin, 1984, Fenwick & Walker, 1978, Kwong *et al.*, 1988, Nishioka & Silverstein, 1977, Nishioka & Silverstein, 1978, Oroskar & Read, 1987, Oroskar & Read, 1989, Read & Frenkel, 1983, Roizman *et al.*, 1965, Smibert & Smiley, 1990, Sydiskis & Roizman, 1966, Sydiskis & Roizman, 1967, Sydiskis & Roizman, 1968). The UL41 gene encodes a 58-kDa phosphoprotein that is synthesized late during infection and packaged into the tegument of the virion (Read *et al.*, 1993, Smibert *et al.*, 1992).

At the time I initiated the investigation outlined in this thesis, little was known about the mechanism of action of the vhs protein. The data described here show that the HSV vhs protein induces translation arrest and RNA degradation when produced as the only HSV protein in an *in vitro* system derived from rabbit reticulocyte lysate. Detailed analysis of the vhs-induced RNA degradation *in vitro* revealed that degradation initiates via endoribonucleolytic cleavage events non-randomly distributed over the 5' quadrant of two unrelated transcripts. Moreover, placing the IRES element from EMCV or poliovirus within the RNA leads to novel vhs-dependent endoribonucleolytic cleavage at sites immediately downstream of the element, leading to the conclusion that these elements target vhs-induced cleavage to RNA. This targeting function appears to be sequence context- and position-independent since placing the IRES element at different internal locations in two unrelated RNAs does not alter the ability of these elements to

target vhs to the 3' flanking sequences. The 5' cap structure and 3' poly(A) tail of RNA do not detectably alter the vhs-dependent RNA degradation activity *in vitro*. The activity displays a marked dependence on divalent Mg^{++} ions but not ATP and ribosomes. Detailed description and discussion of these data are presented in chapters 3 and 4. Therefore, this section is intended to draw the overall conclusions emerging from the presented data, outline the significance of these findings, and suggest possible approaches to address some of the outstanding questions.

Several observations argue for the biological relevance of the data presented in this thesis. First, the Mg^{++} ion and ATP requirements of the vhs-induced RNA degradation activity *in vitro* are consistent with those reported in *in vitro* systems derived from infected cells (Krikorian & Read, 1991). Second, the ribosome- and translation-independence of the vhs activity *in vitro* mirror that reported during infection and in infected cell extracts *in vitro* (Fenwick & Walker, 1978, Schek & Bachenheimer, 1985, Sorenson *et al.*, 1991). Third, the degradation of RNAs lacking a poly(A) tail is consistent with the observation that histone H3 and H4 mRNAs (which are non-polyadenylated) are targeted for degradation in HSV infected cells (Schek & Bachenheimer, 1985). Fourth, vhs induces RNA degradation in *in vitro* systems derived from mammalian cells (related to its natural host) but not from wheat germ. Fourth, the tendency of the vhs activity to initiate RNA cleavage near the 5' end *in vitro* is consistent with the overall 5' to 3' polarity of the vhs activity in HSV infected cells (Karr and Read, 1999).

An outstanding question regarding vhs activity is whether vhs itself is a nuclease? Existing data showing that vhs displays a weak but significant homology to the fen-1 family of endonucleases (Doherty *et al.*, 1996) suggest that vhs is a nuclease. This hypothesis is supported by the recent demonstration that mutations in the vhs protein that alter residues conserved with and critical for fen-1 function render vhs inactive (Everly & Read, 1999). Moreover, the observation that vhs protein isolated from partially purified virions displays an RNase activity that is inhibited by vhs-specific antibodies *in vitro* (discussed in more detail below; (Zelus *et al.*, 1996)) is consistent with vhs being a nuclease or an essential component of a nuclease complex. Although my data do not directly address this question, the observation that some of the sites of cleavage on SRPα induced by the pseudorabies virus vhs protein are distinct from those induced by the HSV-1 vhs protein demonstrates the vhs protein is involved in cleavage site selection. This is consistent with the hypothesis that vhs is a nuclease or a component of a nuclease complex. Purified vhs protein is required to directly address this issue.

Although the data presented here clearly show that vhs-induced RNA decay *in vitro* is independent of the 5' cap structure and the 3' poly(A) tail, they do not directly address the issue of what (if any) other features of mRNAs selectively render them targets for vhs-dependent degradation. I have put forth two possible mechanisms to account for the selective decay of mRNA induced by vhs *in vitro* and *in vivo*. One mechanism is centered around the recruitment of vhs to mRNA through an interaction with one or more components of the translational apparatus. The finding that vhs produced in wheat germ (my data) or yeast extracts (P. Lu and J. Smiley, unpublished

data) does not induce RNA degradation unless supplemented with naive reticulocyte lysates is consistent with this mechanism and suggests that reticulocyte lysates may contain a vhs co-factor that is lacking in wheat germ and yeast extracts. Moreover, the observation that the poliovirus IRES targets vhs to RNA with much lower efficiency than the EMCV IRES provides a possible connection between translation initiation and vhs activity, inasmuch as the poliovirus IRES is also much less efficient in translation initiation than the EMCV IRES both *in vitro* and *in vivo* (Borman *et al.*, 1995, Borman *et al.*, 1997). This mechanism could provide a link between the vhs-dependent cleavage events near the 5' end and those downstream of an IRES element, since both of these sequences (5' end and IRES) interact with overlapping components of the translation machinery.

The possible involvement of components of the translation apparatus is even more intriguing in light of the very recent data showing that the HSV-1 vhs, but not vhs1, protein specifically interacts with the newly identified eukaryotic translation initiation factor eIF4H in the yeast two-hybrid system (GS Read 1999, presented at the 24th International Herpesvirus Workshop). Although its role in translation initiation remains to be clearly established, eIF4H has been shown to contain RNA binding motifs and stimulate the activity of the eIF4A/eIF4B helicase and eIF4F in globin mRNA translation assays (Richter-Cook *et al.*, 1998, Rogers *et al.*, 1999). Whether or not this interaction occurs in mammalian cells remains to be demonstrated. Co-immunoprecipitation analysis may help in determining if this interaction occurs in rabbit reticulocyte lysates. If it does occur, then one approach to test the significance of this interaction is to ask if

immunodepletion of eIF4H from rabbit reticulocyte lysates containing vhs has any effect on vhs activity using IRES and non-IRES RNAs.

During the course of my investigation, Zelus and colleagues reported that vhs protein extracted from partially purified virions is associated with an RNase activity in the absence of cellular and viral proteins *in vitro* (Zelus *et al.*, 1996). These data appear to contradict the possible involvement of cellular proteins in vhs activity. However, the method used by these authors does not exclude cellular proteins from the preparations. The virion preparations used in their studies were partially purified by pelleting the extracellular medium after cytopathic effect was observed (2-3 days following infection) and, thus are likely to contain cellular proteins. This is evident by the observation that preparations from cells infected with the HSV-1 mutant vhs-ΔSma (contains a 588 bp deletion in the UL41 gene (Read *et al.*, 1993)) contain the mutant vhs protein. Read and colleagues (1993) demonstrated that, although synthesized in infected cells, this mutant protein is excluded from virions purified by density gradient centrifugation. Therefore, the vhs-ΔSma protein detected in the extracts prepared by Zelus and co-workers likely originated from contaminating cell lysate rather than virions. The data provided by Zelus and colleagues also do not exclude the possibility that vhs is associated with cellular proteins in the tegument of virions. Indeed, overexpression of the major tegument protein VP22 in infected cells results in a corresponding increase in incorporation of the protein into the tegument (Leslie *et al.*, 1996), demonstrating that the tegument is flexible enough to accommodate excess viral and perhaps cellular proteins.

One approach to further investigate the possible link between vhs activity and the translational apparatus is to determine the effect of the yeast inhibitory RNA (I-RNA) on the cleavage profile of the pSexAI P2 substrate. The I-RNA is a 60 nt long *S. cerevisiae* RNA that inhibits poliovirus IRES-mediated but not cap-dependent translation initiation (Das *et al.*, 1994, Das *et al.*, 1996, Isoyama *et al.*, 1999). The I-RNA, as well as 16 and 25 nt derivatives, effectively competes with the poliovirus IRES for cellular factors required for cap-independent translation initiation including the La autoantigen. Indeed addition of exogenous La protein reverses the inhibitory effect of I-RNA (Das *et al.*, 1994, Das *et al.*, 1996). Thus, if translation initiation and vhs targeting are linked, I-RNA, or the smaller derivatives, may inhibit poliovirus IRES-targeted vhs cleavage without affecting cleavage near the 5'-end of the transcript. If so, then addition of exogenous La protein to the reaction would be expected to reverse this inhibition. A negative result (i.e. I-RNA has no effect on IRES-mediated vhs targeting) will not rule out the hypotheses that vhs targets mRNA through a component(s) of the translational machinery, inasmuch as vhs may be recruited to RNA by factors that load onto the IRES independent of La and translation initiation (for example, factors that load prior to La binding). An alternative approach to determine if cellular factors are involved in vhs activity is to supplement wheat germ or yeast extracts containing vhs with chromatographic fractions of naïve reticulocyte lysates and attempt to identify potential cellular vhs co-factors (if any) from fractions that restore vhs activity. Ultimately, definitive determination of whether vhs activity requires a cellular co-factor requires pure preparations of vhs protein.

The other possible mechanism of vhs-dependent selective mRNA degradation involves elements of mRNA secondary structure. In chapter 4, I proposed three different possibilities by which secondary structure may serve as vhs target or targeting element. None of these possibilities exclude the involvement of cellular cofactors in vhs activity. That the IRES may serve to block the movement of vhs along the RNA leading to cleavage is an unlikely scenario since the highly structured HIV TAR sequence does not induce novel vhs-dependent cleavages when placed at an internal site of the pCITE Msc/RI transcript.

As discussed in chapter 4, one possibility is that vhs may independently load at IRES (or IRES-like) elements and 5' end of RNAs and migrate in the 5' to 3' direction until it encounters specific determinants where it cleaves. This possibility is compatible with the degradation profiles of the SRPα, SRPα StuI IRES, pCITE Msc/RI, and SexAI IRES (figures 3-2, 4-1, 4-3, and 4-5) transcripts but may seem inconsistent with that of SRPα HindIII IRES (figure 4-11). That is, loading of vhs at the IRES in SRPα HindIII IRES is predicted to induce cleavages in the 3' flanking sequences but not at discrete sites located 300 to 700 nt downstream, unless a subset of the nuclease molecules that load at the IRES track past the cleavage sites located just downstream of the IRES until they encounter strong cleavage sites further downstream. Recall that the RNA fragments resulting from cleavage downstream of the EMCV IRES (the pSexAI IRES RNA) migrate as a broad band of ca 1400-1700 nt at early times and that these fragments collapse into a more discrete band of ca. 1400 nt as the reaction proceeds (figures 4-3 and 4-7). This pattern demonstrates that some RNA molecules were initially cleaved at sites

ca. 300 nt downstream of the IRES and subsequently cleaved at sites closer to the IRES. If one assumes that the nuclease loads at the IRES and then tracks 3', then the data strongly suggest that some of the nuclease molecules may in fact transverse potential cleavage sites without cleaving.

Loading and 3' tracking is a mechanism employed by fen-1 and the prokaryotic RNase E endonucleases (Mackie, 1998, Stevens, 1998). The activity of both of these enzymes occurs in two independent recognition steps, a 5'-end-dependent recognition of RNA and recognition of cleavage sites downstream. In both cases, cleavages seem to be non-randomly distributed over the 5' portion of the RNA, even though other strong potential cleavage sites can be found in other parts of the RNA. Like these nucleases, vhs-induced cleavage of two unrelated non-IRES RNAs (SRPα and pCITE Msc/RI) initiates at sites non-randomly distributed over the 5' quadrants of the transcripts. Together with the observation that mutations in the vhs protein that alter amino acid residues critical for fen-1 activity and conserved between vhs and fen-1 render vhs inactive (Everly & Read, 1999), these data are consistent with the hypothesis that vhs utilizes a loading/3' tracking mode of action. Analysis of the degradation profile of a variety of non-IRES RNA is required to definitively determine if 5' clustering of cleavage events is a general feature of the vhs activity.

Alternatively, the cleavage profile of SRPα HindIII IRES can be generated if the vhs-dependent nuclease directly loads at, or close to, potential cleavage sites. In this case, cleavage of SRPα in the 200-600 nt range (and SRPα HindIII IRES in the 800-1400 nt range) may be the consequence of direct recognition of these sites independently of the

IRES (figures 3-2 and 4-11). It is interesting to note that recent preliminary data from Dr. Smiley's laboratory (University of Alberta) shows that the vhs1 mutant protein (Thr 214 to Ile) cleaves pCITE RNA as efficiently as wild type vhs (P. Lu and J. Smiley, unpublished data). Given my finding that the SRPα RNA is essentially immune to cleavage by the vhs1 mutant protein, these data imply that 5'-end and IRES mediated substrate recognition are indeed independent events. In addition, the detection of the 800-1400 nt products from cap labeled SRPα HindIII IRES strongly suggests that cleavage at these sites (same site at 200-600 nt from the 5' end of SRPα but displaced 3'by 600 nt [inserted IRES]) is independent of the 5' end of the transcripts. This is consistent with a mechanism in which the vhs-dependent nuclease directly recognizes specific RNA features at these sites. Such features are likely to be secondary structure since the vhs-dependent RNA cleavage displays very weak (if any) sequence specificity. Characterization of the susceptibility of covalently closed circular RNAs to vhs-dependent cleavage will definitively address the requirement for a free 5' end in vhs activity.

The data presented here do not answer the question of how IRES elements target vhs activity to RNA. The IRES elements could target vhs cleavage through their function (translation initiation) or structure. As mentioned above, one approach that might help distinguish between these possibilities is analysis of the effect of the yeast I-RNA on the vhs targeting activity of the poliovirus IRES. A complementary approach involves analysis of the ability of the IRES element of rhinovirus type 2 to target vhs activity to RNA. This element supports cap-independent translation initiation in HeLa cell extracts

but not RRL, because it requires cellular factors that are lacking from RRL (Hunt *et al.*, 1999, Hunt & Jackson, 1999). Therefore, the rhinovirus type 2 IRES is predicted to target vhs activity to RNA in HeLa extracts but not RRL if the IRES vhs targeting activity is mediated through its role in translation initiation. Alternatively, if the vhs targeting activity is mediated through structural features of the IRES, then the rhinovirus type 2 IRES would serve as a vhs-targeting element in both systems. Characterization of the ability of EMCV IRES mutants, that alter the function (reduced translation initiation activity) but not the structure of the element, to target vhs activity of RNA may also help in the determining how IRES's function to target vhs activity to RNA.

If secondary structure is involved in targeting vhs activity to RNA, then transfer of segments of the IRES (defined stem-loop structures) to internal positions of RNA may address the issue of whether specific structures of the IRES serves as vhs recognition /recruitment elements. Furthermore, deletion and translocation analysis of the 200-700 nt region of the SRPα RNA may help distinguish between the 5' loading/3' tracking and direct recognition models of vhs action. If sequences within the 200-700 nt region of SRPα prove to be vhs recognition sites, then secondary structure prediction at these sequences and comparison to the structure of IRES elements may lead to identification of vhs recognition motifs.

The results of my experiments demonstrate that neither a 5' cap structure nor a 3' poly(A) tail is required for vhs activity in the rabbit reticulocyte lysate *in vitro* system. Although these findings are consistent with previously published data (Schek & Bachenheimer, 1985, Zelus *et al.*, 1996), they do not exclude a role for translation

initiation factors in targeting vhs activity. This is due to the fact that translation in rabbit reticulocyte lysates is inherently cap-independent unless the lysate is supplemented with general RNA binding proteins such as the La autoantigen and the polypyrimidine tract binding protein, PTB (Lodish & Rose, 1977, Svitkin *et al.*, 1996, Svitkin *et al.*, 1994). Analysis of the vhs-dependent RNA degradation using capped and uncapped RNA substrates in a cap-dependent system such as HeLa cell extract or in rabbit reticulocyte lysates supplemented with La or PTB will be necessary to resolve this issue.

Comparison of the activity of the HSV-1 and HSV-2 vhs proteins in RRL revealed that these molecules induce SRPα RNA cleavage with very similar rates. This is surprising since the type 2 vhs displays a much stronger shutoff phenotype than type 1 vhs in infected cells and when expressed as the only HSV protein in transiently transfected cells (Everly & Read, 1997, Fenwick & Everett, 1990, Powell & Courtney, 1975, Shivack and Smiley, unpublished data). The basis for the difference between Type 1 and type 2 vhs proteins are not clear. One possibility is that vhs localizes to defined cellular compartments which are more efficiently targeted by the type 2 proteins. That these compartments do not exist in cell free systems may explain the lack of difference between these molecules in RRL. Further analysis of the activity of these molecules in different *in vitro* systems and using a variety of substrates (including RNAs bearing IRES elements) may help in understanding this observation.

In conclusion, the data described in this thesis provide insight into the mechanism of action of the HSV virion host shutoff protein and begin to define some of the requirements of the vhs-induced RNA degradation reaction. The *in vitro* vhs activity

system I developed is rapid, economical, and most importantly highly sensitive and reproducible. Further characterization of the vhs activity in this system will lead to a better understanding of the mechanism of vhs activity and, perhaps, analogous cellular mRNA turnover pathways.

CHAPTER 6: References.

Ablashi, D. V., Lapps, W., Kaplan, M., Whitman, J. E., Richert, J. R. & Pearson, G. R. (1998). Human Herpesvirus-6 (HHV-6) infection in multiple sclerosis: a preliminary report. *Mult Scler* **4**, 490-6.

Ace, C. I., Dalrymple, M. A., Ramsay, F. H., Preston, V. G. & Preston, C. M. (1988). Mutational analysis of the herpes simplex virus type 1 trans-inducing factor Vmw65. *J Gen Virol* **69**, 2595-605.

Ace, C. I., McKee, T. A., Ryan, J. M., Cameron, J. M. & Preston, C. M. (1989). Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. *J Virol* **63**, 2260-9.

Addison, C., Rixon, F. J. & Preston, V. G. (1990). Herpes simplex virus type 1 UL28 gene product is important for the formation of mature capsids. *J Gen Virol* **71**, 2377-84.

Ahn, K., Meyer, T. H., Uebel, S., Sempe, P., Djaballah, H., Yang, Y., Peterson, P. A., Fruh, K. & Tampe, R. (1996). Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus ICP47. *Embo J* **15**, 3247-55.

Anderson, K. P., Frink, R. J., Devi, G. B., Gaylord, B. H., Costa, R. H. & Wagner, E. K. (1981). Detailed characterization of the mRNA mapping in the HindIII fragment K region of the herpes simplex virus type 1 genome. *J Virol* **37**, 1011-27.

Arvin, A. M. (1996). Varicella-Zoster Virus. In *Fields Virology*, 3rd edn, pp. 2547-2586. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott-Raven.

Aurelian, L. & Roizman, B. (1965). Abortive infection of canine cells by HSV. II. The alternative suppression of synthesis of interferon and viral constituents. *J Mol Biol* **11**, 539-.

Baines, J. D. & Roizman, B. (1992). The UL11 gene of herpes simplex virus 1 encodes a function that facilitates nucleocapsid envelopment and egress from cells. *J Virol* **66**, 5168-74.

Baines, J. D., Ward, P. L., Campadelli-Fiume, G. & Roizman, B. (1991). The UL20 gene of herpes simplex virus 1 encodes a function necessary for viral egress. *J Virol* **65**, 6414-24.

Banfield, B. W., Leduc, Y., Esford, L., Visalli, R. J., Brandt, C. R. & Tufaro, F. (1995). Evidence for an interaction of herpes simplex virus with chondroitin sulfate proteoglycans during infection. *Virology* **208**, 531-9.

Batterson, W., Furlong, D. & Roizman, B. (1983). Molecular genetics of herpes simplex virus. VIII. further characterization of a temperature-sensitive mutant defective in release of viral DNA and in other stages of the viral reproductive cycle. *J Virol* **45**, 397-407.

Batterson, W. & Roizman, B. (1983). Characterization of the herpes simplex virion-associated factor responsible for the induction of alpha genes. *J Virol* **46**, 371-7.

Beck, T. W. & Millette, R. L. (1982). In vitro transcription of herpes simplex virus genes. Partial purification and properties of RNA polymerase II from uninfected and infected Hep-2 cells. *J Biol Chem* **257**, 12780-8.

Becker, Y., Asher, Y., Friedmann, A. & Kessler, E. (1978a). Circular, circular-linear and branched herpes simplex virus DNA molecules from arginine deprived cells. *J Gen Virol* **41**, 629-33.

Becker, Y., Asher, Y., Weinberg-Zahlering, E., Rabkin, S., Friedmann, A. & Kessler, E. (1978b). Defective herpes simplex virus DNA: circular and circular-linear molecules resembling rolling circles. *J Gen Virol* **40**, 319-35.

Becker, Y., Dym, H. & Sarov, I. (1968). Herpes simplex virus DNA. *Virology* **36**, 184-92.

Berthomme, H., Jacquemont, B. & Epstein, A. (1993). The pseudorabies virus host-shutoff homolog gene: nucleotide sequence and comparison with alphaherpesvirus protein counterparts. *Virology* **193**, 1028-32.

Binder, P. S. (1977). Herpes simplex keratitis. *Surv Ophthalmol* **21**, 313-31.

Blaho, J. A., Mitchell, C. & Roizman, B. (1993). Guanylylation and adenylylation of the alpha regulatory proteins of herpes simplex virus require a viral beta or gamma function. *J Virol* **67**, 3891-900.

Boehmer, P. E. & Lehman, I. R. (1997). Herpes simplex virus DNA replication. *Annu Rev Biochem* **66**, 347-84.

Borman, A. M., Bailly, J. L., Girard, M. & Kean, K. M. (1995). Picornavirus internal ribosome entry segments: comparison of translation efficiency and the

requirements for optimal internal initiation of translation in vitro. *Nucleic Acids Res* **23**, 3656-63.

Borman, A. M., Le Mercier, P., Girard, M. & Kean, K. M. (1997). Comparison of picornaviral IRES-driven internal initiation of translation in cultured cells of different origins. *Nucleic Acids Res* **25**, 925-32.

Britt, W. J. & Alford, C. A. (1996). Cytomegalovirus. In *Fields Virology*, 3rd edn, pp. 2493-2524. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott-Raven Press.

Brown, C. R., Nakamura, M. S., Mosca, J. D., Hayward, G. S., Straus, S. E. & Perera, L. P. (1995). Herpes simplex virus trans-regulatory protein ICP27 stabilizes and binds to 3' ends of labile mRNA. *J Virol* **69**, 7187-95.

Browne, H., Bell, S., Minson, T. & Wilson, D. W. (1996). An endoplasmic reticulum-retained herpes simplex virus glycoprotein H is absent from secreted virions: evidence for reenvelopment during egress. *J Virol* **70**, 4311-6.

Brugha, R., Keersmaekers, K., Renton, A. & Meheus, A. (1997). Genital herpes infection: a review. *Int J Epidemiol* **26**, 698-709.

Cai, W. & Schaffer, P. A. (1991). A cellular function can enhance gene expression and plating efficiency of a mutant defective in the gene for ICP0, a transactivating protein of herpes simplex virus type 1. *J Virol* **65**, 4078-90.

Cai, W. & Schaffer, P. A. (1992). Herpes simplex virus type 1 ICP0 regulates expression of immediate- early, early, and late genes in productively infected cells. *J Virol* **66**, 2904-15.

Cai, W. H., Gu, B. & Person, S. (1988). Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion [published erratum appears in J Virol 1988 Nov;62(11):4438]. *J Virol* **62**, 2596-604.

Campadelli-Fiume, G., Arsenakis, M., Farabegoli, F. & Roizman, B. (1988). Entry of herpes simplex virus 1 in BJ cells that constitutively express viral glycoprotein D is by endocytosis and results in degradation of the virus. *J Virol* **62**, 159-67.

Campadelli-Fiume, G., Farabegoli, F., Di Gaeta, S. & Roizman, B. (1991). Origin of unenveloped capsids in the cytoplasm of cells infected with herpes simplex virus 1. *J Virol* **65**, 1589-95.

Campadelli-Fiume, G., Mirandola, P. & Menotti, L. (1999). Human herpesvirus 6: An emerging pathogen [In Process Citation]. *Emerg Infect Dis* **5**, 353-66.

Campbell, M. E., Palfreyman, J. W. & Preston, C. M. (1984). Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription. *J Mol Biol* **180**, 1-19.

Carroll, R. & Lucas-Lenard, J. (1993). Preparation of a cell-free translation system with minimal loss of initiation factor eIF-2/eIF-2B activity. *Anal Biochem* **212**, 17-23.

Carrozza, M. J. & DeLuca, N. A. (1996). Interaction of the viral activator protein ICP4 with TFIID through TAF250. *Mol Cell Biol* **16**, 3085-93.

Challberg, M. D. (1986). A method for identifying the viral genes required for herpesvirus DNA replication. *Proc Natl Acad Sci U S A* **83**, 9094-8.

Challberg, M. D. & Kelly, T. J. (1989). Animal virus DNA replication. *Annu Rev Biochem* **58**, 671-717.

Chapman, A. L. & Rickinson, A. B. (1998). Epstein-Barr virus in Hodgkin's disease. *Ann Oncol* **9**, S5-16.

Chelbi-Alix, M. K. & de The, H. (1999). Herpes virus induced proteasome-dependent degradation of the nuclear bodies-associated PML and Sp100 proteins. *Oncogene* **18**, 935-41.

Chen, J. & Silverstein, S. (1992). Herpes simplex viruses with mutations in the gene encoding ICP0 are defective in gene expression. *J Virol* **66**, 2916-27.

Cheung, P., Banfield, B. W. & Tufaro, F. (1991). Brefeldin A arrests the maturation and egress of herpes simplex virus particles during infection. *J Virol* **65**, 1893-904.

Cheung, P., Panning, B. & Smiley, J. R. (1997). Herpes simplex virus immediate-early proteins ICP0 and ICP4 activate the endogenous human alpha-globin gene in nonerythroid cells. *J Virol* **71**, 1784-93.

Church, G. A. & Wilson, D. W. (1997). Study of herpes simplex virus maturation during a synchronous wave of assembly. *J Virol* **71**, 3603-12.

Church, G. M. & Gilbert, W. (1984). Genomic sequencing. *Proc Natl Acad Sci USA* **81**, 1991-1995.

Cocchi, F., Lopez, M., Menotti, L., Aoubala, M., Dubreuil, P. & Campadelli-Fiume, G. (1998a). The V domain of herpesvirus Ig-like receptor (HIgR) contains a major functional region in herpes simplex virus-1 entry into cells and interacts physically with the viral glycoprotein D. *Proc Natl Acad Sci U S A* **95**, 15700-5.

Cocchi, F., Menotti, L., Mirandola, P., Lopez, M. & Campadelli-Fiume, G. (1998b). The ectodomain of a novel member of the immunoglobulin subfamily related to the

poliovirus receptor has the attributes of a bona fide receptor for herpes simplex virus types 1 and 2 in human cells. *J Virol* **72**, 9992-10002.

Compton, T. & Courtney, R. J. (1984a). Evidence for post-translational glycosylation of a nonglycosylated precursor protein of herpes simplex virus type 1. *J Virol* **52**, 630-7.

Compton, T. & Courtney, R. J. (1984b). Virus-specific glycoproteins associated with the nuclear fraction of herpes simplex virus type 1-infected cells. *J Virol* **49**, 594-7.

Corey, L., Adams, H. G., Brown, Z. A. & Holmes, K. K. (1983). Genital herpes simplex virus infections: clinical manifestations, course, and complications. *Ann Intern Med* **98**, 958-972.

Dabrowski, C. E., Carmillo, P. J. & Schaffer, P. A. (1994). Cellular protein interactions with herpes simplex virus type 1 oriS. *Mol Cell Biol* **14**, 2545-55.

Darlington, R. W. & Moss, L. H. d. (1968). Herpesvirus envelopment. *J Virol* **2**, 48-55.

Das, S., Coward, P. & Dasgupta, A. (1994). A small yeast RNA selectively inhibits internal initiation of translation programmed by poliovirus RNA: specific interaction with cellular proteins that bind to the viral 5'-untranslated region. *J Virol* **68**, 7200-11.

Das, S., Kenan, D. J., Bocskai, D., Keene, J. D. & Dasgupta, A. (1996). Sequences within a small yeast RNA required for inhibition of internal initiation of translation: interaction with La and other cellular proteins influences its inhibitory activity. *J Virol* **70**, 1624-32.

Davison, A. J. & Wilkie, N. M. (1983). Inversion of the two segments of the herpes simplex virus genome in intertypic recombinants. *J Gen Virol* **64**, 1-18.

de Bruyn Kops, A. & Knipe, D. M. (1988). Formation of DNA replication structures in herpes virus-infected cells requires a viral DNA binding protein. *Cell* **55**, 857-68.

de Bruyn Kops, A. & Knipe, D. M. (1994). Preexisting nuclear architecture defines the intranuclear location of herpesvirus DNA replication structures. *J Virol* **68**, 3512-26.

Dean, H. J., Terhune, S. S., Shieh, M. T., Susmarski, N. & Spear, P. G. (1994). Single amino acid substitutions in gD of herpes simplex virus 1 confer resistance to gD-mediated interference and cause cell-type-dependent alterations in infectivity. *Virology* **199**, 67-80.

Deiss, L. P., Chou, J. & Frenkel, N. (1986). Functional domains within the a sequence involved in the cleavage- packaging of herpes simplex virus DNA. *J Virol* **59**, 605-18.

Deiss, L. P. & Frenkel, N. (1986). Herpes simplex virus amplicon: cleavage of concatemeric DNA is linked to packaging and involves amplification of the terminally reiterated a sequence. *J Virol* **57**, 933-41.

Delius, H. & Clements, J. B. (1976). A partial denaturation map of herpes simplex virus type 1 DNA: evidence for inversions of the unique DNA regions. *J Gen Virol* **33**, 125-33.

DeLuca, N. A., McCarthy, A. M. & Schaffer, P. A. (1985). Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. *J Virol* **56**, 558-70.

DeLuca, N. A. & Schaffer, P. A. (1985). Activation of immediate-early, early, and late promoters by temperature- sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. *Mol Cell Biol* **5**, 1997-208.

Desai, P. & Person, S. (1998). Incorporation of the green fluorescent protein into the herpes simplex virus type 1 capsid. *J Virol* **72**, 7563-8.

Dixon, R. A. & Schaffer, P. A. (1980). Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175. *J Virol* **36**, 189-203.

Doherty, A. J., Serpell, L. C. & Ponting, C. P. (1996). The helix-hairpin-helix DNA-binding motif: a structural basis for non-sequence-specific recognition of DNA. *Nucleic Acids Res* **24**, 2488-2497.

Dupin, N., Fisher, C., Kellam, P., Ariad, S., Tulliez, M., Franck, N., van Marck, E., Salmon, D., Gorin, I., Escande, J. P., Weiss, R. A., Alitalo, K. & Boshoff, C. (1999). Distribution of human herpesvirus-8 latently infected cells in Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma. *Proc Natl Acad Sci U S A* **96**, 4546-51.

Dyer, A. P., Banfield, B. W., Martindale, D., Spannier, D. M. & Tufaro, F. (1997). Dextran sulfate can act as an artificial receptor to mediate a type- specific herpes simplex virus infection via glycoprotein B. *J Virol* **71**, 191-8.

Elliott, G. & O'Hare, P. (1998). Herpes simplex virus type 1 tegument protein VP22 induces the stabilization and hyperacetylation of microtubules. *J Virol* **72**, 6448-55.

Elliott, G. & O'Hare, P. (1999). Live-cell analysis of a green fluorescent protein-tagged herpes simplex virus infection. *J Virol* **73**, 4110-9.

Elliott, G. G., Mouzakitis, G. & O'Hare, P. (1995). VP16 interacts via its activation domain with VP22, a tegument protein of herpes simplex virus, and is relocated to novel macromolecular assembly in coexressing cells. *J Virol* **69**, 7932-7941.

Everett, R. D. (1984). Trans activation of transcription by herpes virus products: requirement for two HSV-1 immediate-early polypeptides for maximum activity. *Embo J* **3**, 3135-41.

Everett, R. D. (1985). Activation of cellular promoters during herpes virus infection of biochemically transformed cells. *Embo J* **4**, 1973-80.

Everett, R. D. (1986). The products of herpes simplex virus type 1 (HSV-1) immediate early genes 1, 2 and 3 can activate HSV-1 gene expression in trans. *J Gen Virol* **67**, 2507-13.

Everett, R. D. (1988). Promoter sequence and cell type can dramatically affect the efficiency of transcriptional activation induced by herpes simplex virus type 1 and its immediate-early gene products Vmw175 and Vmw110. *J Mol Biol* **203**, 739-51.

Everett, R. D. (1989). Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1. *J Gen Virol* **70**, 1185-202.

Everett, R. D. & Fenwick, M. L. (1990). Comparative DNA sequence analysis of the host shutoff genes of different strains of herpes simplex virus: type 2 strain HG52 encodes a truncated UL41 product. *J Gen Virol* **71**, 1387-90.

Everett, R. D., Freemont, P., Saitoh, H., Dasso, M., Orr, A., Kathoria, M. & Parkinson, J. (1998a). The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. *J Virol* **72**, 6581-91.

Everett, R. D. & Maul, G. G. (1994). HSV-1 IE protein Vmw110 causes redistribution of PML. *Embo J* **13**, 5062-9.

Everett, R. D., Meredith, M., Orr, A., Cross, A., Kathoria, M. & Parkinson, J. (1997). A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein [corrected and republished article originally printed in EMBO J 1997 Feb 3;16(3):566-77]. *Embo J* **16**, 1519-30.

Everett, R. D., Orr, A. & Preston, C. M. (1998b). A viral activator of gene expression functions via the ubiquitin- proteasome pathway. *Embo J* **17**, 7161-9.

Everly, D. N. & Read, G. S. (1999). Mutational analysis of the HSV virion host shutoff protein (UL41): mutation of residues conserved in vha and cellular nuclease. In *24th International Herpesvirus Workshop*, pp. Abstract No. 15.006. York, UK.

Faber, S. W. & Wilcox, K. W. (1986). Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA. *Nucleic Acids Res* **14**, 6067-83.

Faber, S. W. & Wilcox, K. W. (1988). Association of herpes simplex virus regulatory protein ICP4 with sequences spanning the ICP4 gene transcription initiation site. *Nucleic Acids Res* **16**, 555-70.

Falcone, D. & Andrews, D. W. (1991). Both the 5' untranslated region and the sequence surrounding the start site contribute to efficient initiation of translation in vitro. *Mol Cell Biol* **11**, 2656-2664.

Fenwick, M. L. & Clark, J. (1982). Early and delayed shut-off of host protein synthesis in cells infected with herpes simplex virus. *J Gen Virol* **61**, 121-5.

Fenwick, M. L. & Clark, J. (1983). The effect of cycloheximide on the accumulation and stability of functional alpha-mRNA in cells infected with herpes simplex virus. *J Gen Virol* **64**, 1955-63.

Fenwick, M. L. & Everett, R. D. (1990a). Inactivation of the shutoff gene (UL41) of herpes simplex virus types 1 and 2. *J Gen Virol* **71**, 2961-7.

Fenwick, M. L. & Everett, R. D. (1990b). Transfer of UL41, the gene controlling virion-associated host cell shutoff, between different strains of herpes simplex virus. *J Gen Virol* **71**, 411-8.

Fenwick, M. L. & McMenamin, M. M. (1984). Early virion-associated suppression of cellular protein synthesis by herpes simplex virus is accompanied by inactivation of mRNA. *J Gen Virol* **65**, 1225-8.

Fenwick, M. L. & Owen, S. A. (1988). On the control of immediate early (alpha) mRNA survival in cells infected with herpes simplex virus. *J Gen Virol* **69**, 2869-77.

Fenwick, M. L. & Walker, M. J. (1978). Suppression of the synthesis of cellular macromolecules by herpes simplex virus. *J Gen Virol* **41**, 37-51.

Flaitz, C. M. & Hicks, M. J. (1998). Molecular Piracy: the viral link to carcinogenesis. *Oral Oncol* **34**, 448-453.

Forrester, A., Farrell, H., Wilkinson, G., Kaye, J., Davis-Poynter, N. & Minson, T. (1992). Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. *J Virol* **66**, 341-8.

Freiman, R. N. & Herr, W. (1997). Viral mimicry: common mode of association with HCF by VP16 and the cellular protein LZIP. *Genes Dev* **11**, 3122-7.

Frenkel, N. & Roffman, E. (1996). Human herpesvirus 7. In *Fields Virology*, 3rd edn, pp. 2609-2623. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Fields Virology: Lippincott-Raven.

Friedmann, A., Shlomai, J. & Becker, Y. (1977). Electron microscopy of herpes simplex virus DNA molecules isolated from infected cells by centrifugation in CsCl density gradients. *J Gen Virol* **34**, 507-22.

Fruh, K., Ahn, K., Djaballah, H., Sempe, P., van Endert, P. M., Tampe, R., Peterson, P. A. & Yang, Y. (1995). A viral inhibitor of peptide transporters for antigen presentation. *Nature* **375**, 415-8.

Fuller, A. O., Santos, R. E. & Spear, P. G. (1989). Neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit viral attachment to cells but prevent penetration. *J Virol* **63**, 3435-43.

Fuller, A. O. & Spear, P. G. (1985). Specificities of monoclonal and polyclonal antibodies that inhibit adsorption of herpes simplex virus to cells and lack of inhibition by potent neutralizing antibodies. *J Virol* **55**, 475-82.

Furlong, D., Swift, H. & Roizman, B. (1972). Arrangement of herpesvirus deoxyribonucleic acid in the core. *J Virol* **10**, 1071-4.

Gao, M., Matusick-Kumar, L., Hurlburt, W., DiTusa, S. F., Newcomb, W. W., Brown, J. C., McCann, P. J., 3rd, Deckman, I. & Colonno, R. J. (1994). The protease of herpes simplex virus type 1 is essential for functional capsid formation and viral growth. *J Virol* **68**, 3702-12.

Gelman, I. H. & Silverstein, S. (1985). Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. *Proc Natl Acad Sci U S A* **82**, 5265-9.

Gelman, I. H. & Silverstein, S. (1986). Co-ordinate regulation of herpes simplex virus gene expression is mediated by the functional interaction of two immediate early gene products. *J Mol Biol* **191**, 395-409.

Geraghty, R. J., Krummenacher, C., Cohen, G. H., Eisenberg, R. J. & Spear, P. G. (1998). Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science* **280**, 1618-20.

Gershon, A. A., Sherman, D. L., Zhu, Z., Gabel, C. A., Ambron, R. T. & Gershon, M. D. (1994). Intracellular transport of newly synthesized varicella-zoster virus: final envelopment in the trans-Golgi network. *J Virol* **68**, 6372-90.

Gerster, T. & Roeder, R. G. (1988). A herpesvirus trans-activating protein interacts with transcription factor OTF-1 and other cellular proteins. *Proc Natl Acad Sci U S A* **85**, 6347-51.

Goldstein, J. N. & Weller, S. K. (1998). In vitro processing of herpes simplex virus type 1 DNA replication intermediates by the viral alkaline nuclease, UL12. *J Virol* **72**, 8772-81.

Goodrich, J. A., Hoey, T., Thut, C. J., Admon, A. & Tjian, R. (1993). Drosophila TAFII40 interacts with both a VP16 activation domain and the basal transcription factor TFIIB. *Cell* **75**, 519-30.

Goto, H., Motomura, S., Wilson, A. C., Freiman, R. N., Nakabeppu, Y., Fukushima, K., Fujishima, M., Herr, W. & Nishimoto, T. (1997). A single-point mutation in HCF causes temperature-sensitive cell-cycle arrest and disrupts VP16 function. *Genes Dev* **11**, 726-37.

Graham, F. L., Veldhuisen, G. & Wilkie, N. M. (1973). Infectious herpesvirus DNA. *Nat New Biol* **245**, 265-6.

Greaves, R. & O'Hare, P. (1989). Separation of requirements for protein-DNA complex assembly from those for functional activity in the herpes simplex virus regulatory protein Vmw65. *J Virol* **63**, 1641-50.

Gruenheid, S., Gatzke, L., Meadows, H. & Tufaro, F. (1993). Herpes simplex virus infection and propagation in a mouse L cell mutant lacking heparan sulfate proteoglycans. *J Virol* **67**, 93-100.

Gu, B. & DeLuca, N. (1994). Requirements for activation of the herpes simplex virus glycoprotein C promoter in vitro by the viral regulatory protein ICP4. *J Virol* **68**, 7953-65.

Gu, B., Kuddus, R. & DeLuca, N. A. (1995). Repression of activator-mediated transcription by herpes simplex virus ICP4 via a mechanism involving interactions with the basal transcription factors TATA-binding protein and TFIIB. *Mol Cell Biol* **15**, 3618-26.

Guldner, H. H., Szostecki, C., Grotzinger, T. & Will, H. (1992). IFN enhance expression of Sp100, an autoantigen in primary biliary cirrhosis. *J Immunol* **149**, 4067-73.

Gupta, R., Emili, A., Pan, G., Xiao, H., Shales, M., Greenblatt, J. & Ingles, C. J. (1996). Characterization of the interaction between the acidic activation domain of VP16

and the RNA polymerase II initiation factor TFIIB. *Nucleic Acids Res* **24**, 2324-30.

Gutierrez, K. M., Falkovitz Halpern, M. S., Maldonado, Y. & Arvin, A. M. (1999). The Epidemiology of Neonatal Herpes Simplex Virus Infections in California from 1985 to 1995. *J Infect Dis* **180**, 199-202.

Haigh, A., Greaves, R. & O'Hare, P. (1990). Interference with the assembly of a virus-host transcription complex by peptide competition. *Nature* **344**, 257-9.

Hardwicke, M. A. & Sandri-Goldin, R. M. (1994). The herpes simplex virus regulatory protein ICP27 contributes to the decrease in cellular mRNA levels during infection. *J Virol* **68**, 4797-810.

Hardy, W. R. & Sandri-Goldin, R. M. (1994). Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect. *J Virol* **68**, 7790-9.

Harris, N. L. (1998). The many faces of Hodgkin's disease around the world: what have we learned from its pathology? *Ann Oncol* **9**, S45-56.

Harris, R. A., Everett, R. D., Zhu, X. X., Silverstein, S. & Preston, C. M. (1989). Herpes simplex virus type 1 immediate-early protein Vmw110 reactivates latent herpes simplex virus type 2 in an in vitro latency system. *J Virol* **63**, 3513-5.

Harris, R. A. & Preston, C. M. (1991). Establishment of latency in vitro by the herpes simplex virus type 1 mutant in1814. *J Gen Virol* **72**, 907-13.

Hayward, G. S., Jacob, R. J., Wadsworth, S. C. & Roizman, B. (1975). Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short components. *Proc Natl Acad Sci U S A* **72**, 4243-7.

Heine, J. W., Honess, R. W., Cassai, E. & Roizman, B. (1974). Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. *J Virol* **14**, 640-51.

Herold, B. C., Visalli, R. J., Susmarski, N., Brandt, C. R. & Spear, P. G. (1994). Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein B. *J Gen Virol* **75**, 1211-22.

Herold, B. C., WuDunn, D., Soltys, N. & Spear, P. G. (1991). Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. *J Virol* **65**, 1090-8.

Hill, A., Jugovic, P., York, I., Russ, G., Bennink, J., Yewdell, J., Ploegh, H. & Johnson, D. (1995). Herpes simplex virus turns off the TAP to evade host immunity. *Nature* **375**, 411-5.

Hill, T. M., Sadler, J. R. & Betz, J. L. (1985). Virion component of herpes simplex virus type 1 KOS interferes with early shutoff of host protein synthesis induced by herpes simplex virus type 2 186. *J Virol* **56**, 312-6.

Honess, R. W. & Roizman, B. (1974). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J Virol* **14**, 8-19.

Hong, Z., Beaudet-Miller, M., Durkin, J., Zhang, R. & Kwong, A. D. (1996). Identification of a minimal hydrophobic domain in the herpes simplex virus type 1 scaffolding protein which is required for interaction with the major capsid protein. *J Virol* **70**, 533-40.

Hunt, S. L., Hsuan, J. J., Totty, N. & Jackson, R. J. (1999). unr, a cellular cytoplasmic RNA-binding protein with five cold-shock domains, is required for internal initiation of translation of human rhinovirus RNA. *Genes Dev* **13**, 437-48.

Hunt, S. L. & Jackson, R. J. (1999). Polypyrimidine-tract binding protein (PTB) is necessary, but not sufficient, for efficient internal initiation of translation of human rhinovirus-2 RNA. *Rna* **5**, 344-59.

Hutchinson, L., Browne, H., Wargent, V., Davis-Poynter, N., Primorac, S., Goldsmith, K., Minson, A. C. & Johnson, D. C. (1992). A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH. *J Virol* **66**, 2240-50.

Imbalzano, A. N., Shepard, A. A. & DeLuca, N. A. (1990). Functional relevance of specific interactions between herpes simplex virus type 1 ICP4 and sequences from the promoter-regulatory domain of the viral thymidine kinase gene. *J Virol* **64**, 2620-31.

Ingles, C. J., Shales, M., Cress, W. D., Triezenberg, S. J. & Greenblatt, J. (1991). Reduced binding of TFIID to transcriptionally compromised mutants of VP16. *Nature* **351**, 588-90.

Ingram, A., Phelan, A., Dunlop, J. & Clements, J. B. (1996). Immediate early protein IE63 of herpes simplex virus type 1 binds RNA directly. *J Gen Virol* **77**, 1847-51.

Ishov, A. M. & Maul, G. G. (1996). The periphery of nuclear domain 10 (ND10) as site of DNA virus deposition. *J Cell Biol* **134**, 815-26.

Isoyama, T., Kamoshita, N., Yasui, K., Iwai, A., Shiroki, K., Toyoda, H., Yamada, A., Takasaki, Y. & Nomoto, A. (1999). Lower concentration of La protein required for internal ribosome entry on hepatitis C virus than on poliovirus RNA. *J Gen Virol* **80**, 2319-2327.

Jacob, R. J., Morse, L. S. & Roizman, B. (1979). Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. *J Virol* **29**, 448-57.

Jayachandra, S., Baghian, A. & Kousoulas, K. G. (1997). Herpes simplex virus type 1 glycoprotein K is not essential for infectious virus production in actively replicating cells but is required for efficient envelopment and translocation of infectious virions from the cytoplasm to the extracellular space. *J Virol* **71**, 5012-24.

Johnson, D. C., Burke, R. L. & Gregory, T. (1990). Soluble forms of herpes simplex virus glycoprotein D bind to a limited number of cell surface receptors and inhibit virus entry into cells. *J Virol* **64**, 2569-76.

Johnson, D. C. & Ligas, M. W. (1988). Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. *J Virol* **62**, 4605-12.

Johnson, D. C. & Spear, P. G. (1982). Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. *J Virol* **43**, 1102-12.

Johnson, K. M., Mahajan, S. S. & Wilson, A. C. (1999). Herpes simplex virus transactivator VP16 discriminates between HCF-1 and a novel family member, HCF-2. *J Virol* **73**, 3930-40.

Johnson, R. M. & Spear, P. G. (1989). Herpes simplex virus glycoprotein D mediates interference with herpes simplex virus infection. *J Virol* **63**, 819-27.

Jones, F. & Grose, C. (1988). Role of cytoplasmic vacuoles in varicella-zoster virus glycoprotein trafficking and virion envelopment. *J Virol* **62**, 2701-11.

Jones, F. E., Smibert, C. A. & Smiley, J. R. (1995). Mutational analysis of the herpes simplex virus virion host shutoff protein: evidence that vhs functions in the absence of other viral proteins. *J Virol* **69**, 4863-71.

Jordan, R. & Schaffer, P. A. (1997). Activation of gene expression by herpes simplex virus type 1 ICP0 occurs at the level of mRNA synthesis. *J Virol* **71**, 6850-62.

Karr, B. M. & Read, G. S. (1999). The virion host shutoff function of herpes simplex virus degrades the 5' end of target mRNA before the 3' end. *Virology* **10**, 195-204.

Katan, M., Haigh, A., Verrijzer, C. P., van der Vliet, P. C. & O'Hare, P. (1990). Characterization of a cellular factor which interacts functionally with Oct-1 in the assembly of a multicomponent transcription complex. *Nucleic Acids Res* **18**, 6871-80.

Kieff, E. D., Bachenheimer, S. L. & Roizman, B. (1971). Size, composition, and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. *J Virol* **8**, 125-32.

Kimberlin, D. W. & Whitley, R. J. (1998). Human herpesvirus-6: neurologic implications of a newly-described viral pathogen [see comments]. *J Neurovirol* **4**, 474-85.

Knipe, D. M., Batterson, W., Nosal, C., Roizman, B. & Buchan, A. (1981). Molecular genetics of herpes simplex virus. VI. Characterization of a temperature-sensitive mutant defective in the expression of all early viral gene products. *J Virol* **38**, 539-47.

Knipe, D. M. & Smith, J. L. (1986). A mutant herpesvirus protein leads to a block in nuclear localization of other viral proteins. *Mol Cell Biol* **6**, 2371-81.

Koyama, A. H. & Uchida, T. (1987). The mode of entry of herpes simplex virus type 1 into Vero cells. *Microbiol Immunol* **31**, 123-30.

Krikorian, C. R. & Read, G. S. (1991). In Vitro mRNA degradation system to study the virion host shutoff function of herpes simplex virus. *J Virol* **65**, 112-122.

Kristie, T. M., LeBowitz, J. H. & Sharp, P. A. (1989). The octamer-binding proteins form multi-protein--DNA complexes with the HSV alpha TIF regulatory protein. *Embo J* **8**, 4229-38.

Kristie, T. M. & Roizman, B. (1986). DNA-binding site of major regulatory protein alpha 4 specifically associated with promoter-regulatory domains of alpha genes of herpes simplex virus type 1. *Proc Natl Acad Sci U S A* **83**, 4700-4.

Kristie, T. M. & Roizman, B. (1987). Host cell proteins bind to the cis-acting site required for virion- mediated induction of herpes simplex virus 1 alpha genes. *Proc Natl Acad Sci U S A* **84**, 71-5.

Kristie, T. M. & Roizman, B. (1988). Differentiation and DNA contact points of host proteins binding at the cis site for virion-mediated induction of alpha genes of herpes simplex virus 1. *J Virol* **62**, 1145-57.

Kristie, T. M. & Sharp, P. A. (1990). Interactions of the Oct-1 POU subdomains with specific DNA sequences and with the HSV alpha-trans-activator protein. *Genes Dev* **4**, 2383-96.

Krummenacher, C., Nicola, A. V., Whitbeck, J. C., Lou, H., Hou, W., Lambris, J. D., Geraghty, R. J., Spear, P. G., Cohen, G. H. & Eisenberg, R. J. (1998). Herpes simplex virus glycoprotein D can bind to poliovirus receptor- related protein 1 or herpesvirus entry mediator, two structurally unrelated mediators of virus entry. *J Virol* **72**, 7064-74.

Kuddus, R., Gu, B. & DeLuca, N. A. (1995). Relationship between TATA-binding protein and herpes simplex virus type 1 ICP4 DNA-binding sites in complex formation and repression of transcription. *J Virol* **69**, 5568-75.

Kwong, A. D. & Frenkel, N. (1987). Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs. *Proc Natl Acad Sci USA* **84**, 1926-1930.

Kwong, A. D., Kruper, J. A. & Frenkel, N. (1988). Herpes simplex virus virion host shutoff function. *J Virol* **62**, 912-21.

La Boissiere, S., Hughes, T. & O'Hare, P. (1999). HCF-dependent nuclear import of VP16. *Embo J* **18**, 480-9.

Lahat, E., Barr, J., Barkai, G., Paret, G., Brand, N. & Barzilai, A. (1999). Long term neurological outcome of herpes encephalitis. *Arch Dis Child* **80**, 69-71.

Lam, Q., Smibert, C. A., Koop, K. E., Lavery, C., Capone, J. P., Weinheimer, S. P. & Smiley, J. R. (1996). Herpes simplex virus VP16 rescues viral mRNA from destruction by the virion host shutoff function. *Embo J* **15**, 2575-81.

Lehtinen, M., Kulomaa, P., Kallioniemi, O. P., Paavonen, J. & Leinikki, P. (1989). Analysis of DNA synthesis in herpes simplex virus infected cells by dual parameter flow cytometry. *Arch Virol* **107**, 215-23.

Leib, D. A., Coen, D. M., Bogard, C. L., Hicks, K. A., Yager, D. R., Knipe, D. M., Tyler, K. L. & Schaffer, P. A. (1989). Immediate-early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. *J Virol* **63**, 759-68.

Leib, D. A., Harrison, T. E., Laslo, K. M., Machalek, M. A., Moorman, N. J. & Virgin, H. W. (1999). Interferons Regulate the Phenotype of Wild-type and Mutant Herpes Simplex Viruses In Vivo. *J Exp Med* **189**, 663-672.

Leopardi, R., Michael, N. & Roizman, B. (1995). Repression of the herpes simplex virus 1 alpha 4 gene by its gene product (ICP4) within the context of the viral genome

is conditioned by the distance and stereoaxial alignment of the ICP4 DNA binding site relative to the TATA box. *J Virol* **69**, 3042-8.

Leopardi, R., Ward, P. L., Ogle, W. O. & Roizman, B. (1997). Association of herpes simplex virus regulatory protein ICP22 with transcriptional complexes containing EAP, ICP4, RNA polymerase II, and viral DNA requires posttranslational modification by the U(L)13 proteinkinase. *J Virol* **71**, 1133-9.

Leslie, J., Rixon, F. J. & McLauchlan, J. (1996). Overexpression of the herpes simplex virus type 1 tegument protein VP22 increases its incorporation into virus particles. *Virology* **220**, 60-8.

Ligas, M. W. & Johnson, D. C. (1988). A herpes simplex virus mutant in which glycoprotein D sequences are replaced by beta-galactosidase sequences binds to but is unable to penetrate into cells. *J Virol* **62**, 1486-94.

Lin, Y. S., Ha, I., Maldonado, E., Reinberg, D. & Green, M. R. (1991). Binding of general transcription factor TFIIB to an acidic activating region. *Nature* **353**, 569-71.

Liu, Y., Gong, W., Huang, C. C., Herr, W. & Cheng, X. (1999). Crystal structure of the conserved core of the herpes simplex virus transcriptional regulatory protein VP16. *Genes Dev* **13**, 1692-1703.

Lodish, H. F. & Rose, J. K. (1977). Relative importance of 7-methylguanosine in ribosome binding and translation of vesicular stomatitis virus mRNA in wheat germ and reticulocyte cell-free systems. *J Biol Chem* **252**, 1181-8.

Long, M. C., Leong, V., Schaffer, P. A., Spencer, C. A. & Rice, S. A. (1999). ICP22 and the UL13 protein kinase are both required for herpes simplex virus-induced modification of the large subunit of RNA polymerase II. *J Virol* **73**, 5593-604.

Lukonis, C. J. & Weller, S. K. (1997). Formation of herpes simplex virus type 1 replication compartments by transfection: requirements and localization to nuclear domain 10. *J Virol* **71**, 2390-9.

Mackem, S. & Roizman, B. (1982a). Differentiation between alpha promoter and regulator regions of herpes simplex virus 1: the functional domains and sequence of a movable alpha regulator. *Proc Natl Acad Sci U S A* **79**, 4917-21.

Mackem, S. & Roizman, B. (1982b). Structural features of the herpes simplex virus alpha gene 4, 0, and 27 promoter-regulatory sequences which confer alpha regulation on chimeric thymidine kinase genes. *J Virol* **44**, 939-49.

Mackie, G. A. (1998). Ribonuclease E is a 5'-end-dependent endonuclease. *Nature* **395**, 720-3.

Martinez, R., Sarisky, R. T., Weber, P. C. & Weller, S. K. (1996). Herpes simplex virus type 1 alkaline nuclease is required for efficient processing of viral DNA replication intermediates. *J Virol* **70**, 2075-85.

Marton, R., Gotlieb-Stematsky, T., Klein, C., Lahat, E. & Arlazoroff, A. (1995). Mild form of acute herpes simplex encephalitis in childhood. *Brain Dev* **17**, 360-1.

Maul, G. G. (1998). Nuclear domain 10, the site of DNA virus transcription and replication. *Bioessays* **20**, 660-7.

Maul, G. G. & Everett, R. D. (1994). The nuclear location of PML, a cellular member of the C3HC4 zinc- binding domain protein family, is rearranged during herpes simplex virus infection by the C3HC4 viral protein ICP0. *J Gen Virol* **75**, 1223-33.

Maul, G. G., Guldner, H. H. & Spivack, J. G. (1993). Modification of discrete nuclear domains induced by herpes simplex virus type 1 immediate early gene 1 product (ICP0). *J Gen Virol* **74**, 2679-90.

Maul, G. G., Ishov, A. M. & Everett, R. D. (1996). Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. *Virology* **217**, 67-75.

Maul, G. G., Yu, E., Ishov, A. M. & Epstein, A. L. (1995). Nuclear domain 10 (ND10) associated proteins are also present in nuclear bodies and redistribute to hundreds of nuclear sites after stress. *J Cell Biochem* **59**, 498-513.

McCarthy, A. M., McMahan, L. & Schaffer, P. A. (1989). Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. *J Virol* **63**, 18-27.

McCullers, J. A., Lakeman, F. D. & Whitley, R. J. (1995). Human herpesvirus 6 is associated with focal encephalitis. *Clin Infect Dis* **21**, 571-6.

McGeoch, D. J., Dalrymple, M. A., Davison, A. J., Dolan, A., Frame, M. C., McNab, D., Perry, L. J., Scott, J. E. & Taylor, P. (1988). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J Gen Virol* **69**, 1531-74.

McGeoch, D. J., Dolan, A., Donald, S. & Rixon, F. J. (1985). Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. *J Mol Biol* **181**, 1-13.

McGregor, F., Phelan, A., Dunlop, J. & Clements, J. B. (1996). Regulation of herpes simplex virus poly (A) site usage and the action of immediate-early protein IE63 in the early-late switch. *J Virol* **70**, 1931-40.

McKnight, J. L., Kristie, T. M. & Roizman, B. (1987). Binding of the virion protein mediating alpha gene induction in herpes simplex virus 1-infected cells to its cis site requires cellular proteins. *Proc Natl Acad Sci U S A* **84**, 7061-5.

McLauchlan, J., Addison, C., Craigie, M. C. & Rixon, F. J. (1992). Noninfectious L-particles supply functions which can facilitate infection by HSV-1. *Virology* **190**, 682-8.

McLauchlan, J. & Rixon, F. J. (1992). Characterization of enveloped tegument structures (L-particles) produces by alphaherpesviruses: interrity of the tegument does not depend on the presence of capsid or envelope. *J Gen Virol* **73**, 269-276.

McNabb, D. S. & Courtney, R. J. (1992a). Analysis of the UL36 open reading frame encoding the large tegument protein (ICP1/2) of herpes simplex virus type 1. *J Virol* **66**, 7581-4.

McNabb, D. S. & Courtney, R. J. (1992b). Characterization of the large tegument protein (ICP1/2) of herpes simplex virus type 1. *Virology* **190**, 221-32.

Meredith, M., Orr, A., Elliott, M. & Everett, R. (1995). Separation of sequence requirements for HSV-1 Vmw110 multimerisation and interaction with a 135-kDa cellular protein. *Virology* **209**, 174-87.

Meredith, M., Orr, A. & Everett, R. (1994). Herpes simplex virus type 1 immediate-early protein Vmw110 binds strongly and specifically to a 135-kDa cellular protein. *Virology* **200**, 457-69.

Michael, N. & Roizman, B. (1989). Binding of the herpes simplex virus major regulatory protein to viral DNA. *Proc Natl Acad Sci U S A* **86**, 9808-12.

Michael, N., Spector, D., Mavromara-Nazos, P., Kristie, T. M. & Roizman, B. (1988). The DNA-binding properties of the major regulatory protein alpha 4 of herpes simplex viruses. *Science* **239**, 1531-4.

Mitchell, C., Blaho, J. A., McCormick, A. L. & Roizman, B. (1997). The nucleotidylylation of herpes simplex virus 1 regulatory protein alpha22 by human casein kinase II. *J Biol Chem* **272**, 25394-400.

Mitchell, C., Blaho, J. A. & Roizman, B. (1994). Casein kinase II specifically nucleotidylylates in vitro the amino acid sequence of the protein encoded by the alpha 22 gene of herpes simplex virus 1. *Proc Natl Acad Sci U S A* **91**, 11864-8.

Montgomery, R. I., Warner, M. S., Lum, B. J. & Spear, P. G. (1996). Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* **87**, 427-36.

Morgan, C., Rose, H. M. & Mednis, B. (1968). Electron microscopy of herpes simplex virus. I. Entry. *J Virol* **2**, 507-16.

Morrison, E. E., Stevenson, A. J., Wang, Y. F. & Meredith, D. M. (1998a). Differences in the intracellular localization and fate of herpes simplex virus tegument proteins early in the infection of Vero cells. *J Gen Virol* **79**, 2517-28.

Morrison, E. E., Wang, Y. F. & Meredith, D. M. (1998b). Phosphorylation of structural components promotes dissociation of the herpes simplex virus type 1 tegument. *J Virol* **72**, 7108-14.

Mullen, M. A., Gerstberger, S., Ciufo, D. M., Mosca, J. D. & Hayward, G. S. (1995). Evaluation of colocalization interactions between the IE110, IE175, and IE63 transactivator proteins of herpes simplex virus within subcellular punctate structures. *J Virol* **69**, 476-91.

Muller, M. T. (1987). Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site. *J Virol* **61**, 858-65.

Muller, S. & Dejean, A. (1999). Viral immediate-early proteins abrogate the modification by SUMO-1 of PML and Sp100 proteins, correlating with nuclear body disruption. *J Virol* **73**, 5137-43.

Muller, S., Matunis, M. J. & Dejean, A. (1998). Conjugation with the ubiquitin-related modifier SUMO-1 regulates the partitioning of PML within the nucleus. *Embo J* **17**, 61-70.

Murphy, F. A. (1996). Virus taxonomy. In *Fields Virology*, 3rd edn, pp. 15-58. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott-Raven Press.

Neipel, F., Albrecht, J. C. & Fleckenstein, B. (1998). Human herpesvirus 8--the first human Rhadinovirus. *J Natl Cancer Inst Monogr* **23**, 73-77.

Newcomb, W. W., Homa, F. L., Thomsen, D. R., Booy, F. P., Trus, B. L., Steven, A. C., Spencer, J. V. & Brown, J. C. (1996). Assembly of the herpes simplex virus capsid: characterization of intermediates observed during cell-free capsid formation. *J Mol Biol* **263**, 432-46.

Newcomb, W. W., Homa, F. L., Thomsen, D. R., Trus, B. L., Cheng, N., Steven, A., Booy, F. & Brown, J. C. (1999). Assembly of the herpes simplex virus procapsid from purified components and identification of small complexes containing the major capsid and scaffolding proteins. *J Virol* **73**, 4239-50.

Newcomb, W. W., Homa, F. L., Thomsen, D. R., Ye, Z. & Brown, J. C. (1994). Cell-free assembly of the herpes simplex virus capsid. *J Virol* **68**, 6059-63.

Newcomb, W. W., Trus, B. L., Booy, F. P., Steven, A. C., Wall, J. S. & Brown, J. C. (1993). Structure of the herpes simplex virus capsid. Molecular composition of the pentons and the triplexes. *J Mol Biol* **232**, 499-511.

Newton, A., Dendy, P., Smith, C. & Wildy, P. (1962). A pool size problem associated with the use of tritiated thymadine. *Nature* **194**, 886-.

Nishioka, Y. & Silverstein, S. (1977). Degradation of cellular mRNAs during infection by herpes simplex virus. *Proc Natl Acad Sci USA* **74**, 2370-2374.

Nishioka, Y. & Silverstein, S. (1978). Requirement of protein synthesis for the degradation of host mRNA in friend erythroleukemia cells infected with herpes simplex virus type 1. *J Virol* **27**, 619-627.

O'Hare, P. & Goding, C. R. (1988). Herpes simplex virus regulatory elements and the immunoglobulin octamer domain bind a common factor and are both targets for virion transactivation. *Cell* **52**, 435-45.

O'Hare, P., Goding, C. R. & Haigh, A. (1988). Direct combinatorial interaction between a herpes simplex virus regulatory protein and a cellular octamer-binding factor mediates specific induction of virus immediate-early gene expression. *Embo J* **7**, 4231-8.

O'Hare, P. & Hayward, G. S. (1985a). Evidence for a direct role for both the 175,000- and 110,000-molecular- weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. *J Virol* **53**, 751-60.

O'Hare, P. & Hayward, G. S. (1985b). Three trans-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. *J Virol* **56**, 723-33.

O'Hare, P. & Hayward, G. S. (1987). Comparison of upstream sequence requirements for positive and negative regulation of a herpes simplex virus immediate-early gene by three virus-encoded trans-acting factors. *J Virol* **61**, 190-9.

Oroskar, A. A. & Read, G. S. (1987). A mutant of herpes simplex virus type 1 exhibits increased stability of immediate-early (alpha) mRNAs. *J Virol* **61**, 604-606.

Oroskar, A. A. & Read, G. S. (1989). Control of mRNA stability by the virion host shutoff function of herpes simplex virus. *J Virol* **63**, 1897-1906.

Overton, H., McMillan, D., Hope, L. & Wong-Kai-In, P. (1994). Production of host shutoff-defective mutants of herpes simplex virus type 1 by inactivation of the UL13 gene. *Virology* **202**, 97-106.

Pak, A. S., Everly, D. N., Knight, K. & Read, G. S. (1995). The virion host shutoff protein of herpes simplex virus inhibits reporter gene expression in the absence of other viral gene products. *Virology* **211**, 491-506.

Panagiotidis, C. A., Lium, E. K. & Silverstein, S. J. (1997). Physical and functional interactions between herpes simplex virus immediate-early proteins ICP4 and ICP27. *J Virol* **71**, 1547-57.

Panagiotidis, C. A. & Silverstein, S. J. (1999). The host-cell architectural protein HMG I(Y) modulates binding of herpes simplex virus type 1 ICP4 to its cognate promoter. *Virology* **256**, 64-74.

Para, M. F., Baucke, R. B. & Spear, P. G. (1980). Immunoglobulin G(Fc)-binding receptors on virions of herpes simplex virus type 1 and transfer of these receptors to the cell surface by infection. *J Virol* **34**, 512-20.

Patel, A., Hanson, J., McLean, T. I., Olgiate, J., Hilton, M., Miller, W. E. & Bachenheimer, S. L. (1998). Herpes simplex type 1 induction of persistent NF-kappa B nuclear translocation increases the efficiency of virus replication. *Virology* **247**, 212-22.

Paterson, T. & Everett, R. D. (1988a). Mutational dissection of the HSV-1 immediate-early protein Vmw175 involved in transcriptional transactivation and repression. *Virology* **166**, 186-96.

Paterson, T. & Everett, R. D. (1988b). The regions of the herpes simplex virus type 1 immediate early protein Vmw175 required for site specific DNA binding closely correspond to those involved in transcriptional regulation. *Nucleic Acids Res* **16**, 11005-25.

Pellett, P. E. & Black, J. B. (1996). Human Herpesvirus 6. In *Fields Virology*, 3rd edn, pp. 2587-2608. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott-Raven.

Pellett, P. E., McKnight, J. L., Jenkins, F. J. & Roizman, B. (1985). Nucleotide sequence and predicted amino acid sequence of a protein encoded in a small herpes simplex virus DNA fragment capable of trans- inducing alpha genes. *Proc Natl Acad Sci U S A* **82**, 5870-4.

Peng, T., Ponce-de-Leon, M., Jiang, H., Dubin, G., Lubinski, J. M., Eisenberg, R. J. & Cohen, G. H. (1998). The gH-gL complex of herpes simplex virus (HSV) stimulates neutralizing antibody and protects mice against HSV type 1 challenge. *J Virol* **72**, 65-72.

Peter, J. & Ray, C. G. (1998). Infectious mononucleosis. *Pediatr Rev* **19**, 276-9.

Phelan, A. & Clements, J. B. (1997). Herpes simplex virus type 1 immediate early protein IE63 shuttles between nuclear compartments and the cytoplasm. *J Gen Virol* **78**, 3327-31.

Phelan, A., Dunlop, J. & Clements, J. B. (1996). Herpes simplex virus type 1 protein IE63 affects the nuclear export of virus intron-containing transcripts. *J Virol* **70**, 5255-65.

Phelan, A., Dunlop, J., Patel, A. H., Stow, N. D. & Clements, J. B. (1997). Nuclear sites of herpes simplex virus type 1 DNA replication and transcription colocalize at early times postinfection and are largely distinct from RNA processing factors. *J Virol* **71**, 1124-32.

Poffenberger, K. L., Raichlen, P. E. & Herman, R. C. (1993). In vitro characterization of a herpes simplex virus type 1 ICP22 deletion mutant. *Virus Genes* **7**, 171-86.

Post, L. E., Mackem, S. & Roizman, B. (1981). Regulation of alpha genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with alpha gene promoters. *Cell* **24**, 555-65.

Post, L. E. & Roizman, B. (1981). A generalized technique for deletion of specific genes in large genomes: alpha gene 22 of herpes simplex virus 1 is not essential for growth. *Cell* **25**, 227-32.

Powell, K. L. & Courtney, R. J. (1975). Polypeptide synthesized in herpes simplex virus type 2-infected HEp-2 cells. *Virology* **66**, 217-28.

Preston, C. M. (1979). Abnormal properties of an immediate early polypeptide in cells infected with the herpes simplex virus type 1 mutant tsK. *J Virol* **32**, 357-69.

Preston, C. M., Cordingley, M. G. & Stow, N. D. (1984). Analysis of DNA sequences which regulate the transcription of a herpes simplex virus immediate early gene. *J Virol* **50**, 708-16.

Preston, C. M. & Newton, A. A. (1976). The effects of herpes simplex virus type 1 on cellular DNA-dependent RNA polymerase activities. *J Gen Virol* **33**, 471-82.

Preston, V. G., Darling, A. J. & McDougall, I. M. (1988). The herpes simplex virus type 1 temperature-sensitive mutant ts1222 has a single base pair deletion in the small subunit of ribonucleotide reductase. *Virology* **167**, 458-67.

Preston, V. G., Rixon, F. J., McDougall, I. M., McGregor, M. & al Kobaisi, M. F. (1992). Processing of the herpes simplex virus assembly protein ICP35 near its carboxy terminal end requires the product of the whole of the UL26 reading frame. *Virology* **186**, 87-98.

Prod'hon, C., Machuca, I., Berthomme, H., Epstein, A. & Jacquemont, B. (1996). Characterization of regulatory functions of the HSV-1 immediate-early protein ICP22. *Virology* **226**, 393-402.

Purves, F. C., Ogle, W. O. & Roizman, B. (1993). Processing of the herpes simplex virus regulatory protein alpha 22 mediated by the UL13 protein kinase determines the accumulation of a subset of alpha and gamma mRNAs and proteins in infected cells. *Proc Natl Acad Sci U S A* **90**, 6701-5.

Purves, F. C. & Roizman, B. (1992). The UL13 gene of herpes simplex virus 1 encodes the functions for posttranslational processing associated with phosphorylation of the regulatory protein alpha 22. *Proc Natl Acad Sci U S A* **89**, 7310-4.

Quinlan, M. P. & Knipe, D. M. (1985). Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. *Mol Cell Biol* **5**, 957-63.

Read, G. S. & Frenkel, N. (1983). Herpes simplex virus mutants defective in the virion-associated shutoff of host polypeptide synthesis and exhibiting abnormal synthesis of α (immediate early) viral polypeptides. *J Virol* **46**, 498-512.

Read, G. S., Karr, B. M. & Knight, K. (1993). Isolation of a herpes simplex virus type 1 mutant with a deletion in the virion host shutoff gene and identification of multiple forms of the vhs (UL41) polypeptide. *J Virol* **67**, 7149-60.

Rice, S. A., Long, M. C., Lam, V., Schaffer, P. A. & Spencer, C. A. (1995). Herpes simplex virus immediate-early protein ICP22 is required for viral modification of host RNA polymerase II and establishment of the normal viral transcription program. *J Virol* **69**, 5550-9.

Rice, S. A., Long, M. C., Lam, V. & Spencer, C. A. (1994). RNA polymerase II is aberrantly phosphorylated and localized to viral replication compartments following herpes simplex virus infection. *J Virol* **68**, 988-1001.

Rice, S. A., Su, L. S. & Knipe, D. M. (1989). Herpes simplex virus alpha protein ICP27 possesses separable positive and negative regulatory activities. *J Virol* **63**, 3399-407.

Richter-Cook, N. J., Dever, T. E., Hensold, J. O. & Merrick, W. C. (1998). Purification and characterization of a new eukaryotic protein translation factor. Eukaryotic initiation factor 4H. *J Biol Chem* **273**, 7579-87.

Rickinson, A. B. & Kieff, E. (1996). Epstein-Barr virus. In *Fields Virology*, 3rd edn. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott-Raven.

Rixon, F. J., Addison, C. & McLauchlan, J. (1992). Assembly of enveloped tegument structures (L particles) can occur independently of virion maturation in herpes simplex virus type 1- infected cells. *J Gen Virol* **73**, 277-84.

Rixon, F. J. & McNab, D. (1999). Packaging-competent capsids of a herpes simplex virus temperature- sensitive mutant have properties similar to those of in vitro-assembled procapsids. *J Virol* **73**, 5714-21.

Roberts, M. S., Boundy, A., O'Hare, P., Pizzorno, M. C., Ciufo, D. M. & Hayward, G. S. (1988). Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (alpha 4) promoter and a specific binding site for the IE175 (ICP4) protein. *J Virol* **62**, 4307-20.

Rogers, G. W., Jr., Richter, N. J. & Merrick, W. C. (1999). Biochemical and kinetic characterization of the RNA helicase activity of eukaryotic initiation factor 4A. *J Biol Chem* **274**, 12236-44.

Roizman, B. (1996). Herpesviridae. In *Fields Virology*, 3rd edn, pp. 2221-2230. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott-Raven Press.

Roizman, B., Borman, G. S. & Rousta, M. K. (1965). Macromolecular synthesis in cells infected with herpes simplex virus. *Nature* **206**, 1374-5.

Roizman, B. & Roane, P. (1964). The multiplication of HSV. II. The relation between protein synthesis and the duplication of viral DNA in infected HEp-2 cells. *Virology* **22**, 262.

Roizman, B. & Sears, A. E. (1996). Herpes simplex viruses and their replication. In *Fields Virology*, 3rd edn, pp. 2231-2295. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott-Raven Press.

Roop, C., Hutchinson, L. & Johnson, D. C. (1993). A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H. *J Virol* **67**, 2285-97.

Rosenthal, K. S., Killius, J., Hodnichak, C. M., Venetta, T. M., Gyurgyik, L. & Janiga, K. (1989). Mild acidic pH inhibition of the major pathway of herpes simplex virus entry into HEp-2 cells. *J Gen Virol* **70**, 857-67.

Sacks, W. R., Greene, C. C., Aschman, D. P. & Schaffer, P. A. (1985). Herpes simplex virus type 1 ICP27 is an essential regulatory protein. *J Virol* **55**, 796-805.

Sacks, W. R. & Schaffer, P. A. (1987). Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. *J Virol* **61**, 829-39.

Sadowski, I., Ma, J., Triezenberg, S. & Ptashne, M. (1988). GAL4-VP16 is an unusually potent transcriptional activator. *Nature* **335**, 563-4.

Samaniego, L. A., Neiderhiser, L. & DeLuca, N. A. (1998). Persistence and expression of the herpes simplex virus genome in the absence of immediate-early proteins. *J Virol* **72**, 3307-20.

Samaniego, L. A., Webb, A. L. & DeLuca, N. A. (1995). Functional interactions between herpes simplex virus immediate-early proteins during infection: gene expression as a consequence of ICP27 and different domains of ICP4. *J Virol* **69**, 5705-15.

Sambrook, J., Fritsch, E. & Maniatis, T. (1989). Molecular Cloning, a laboratory manual, 2nd edn. Edited by Nolan. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Sandri-Goldin, R. M. (1994). Properties of an HSV-1 regulatory protein that appears to impair host cell splicing. *Infect Agents Dis* **3**, 59-67.

Sandri-Goldin, R. M. (1998a). ICP27 mediates HSV RNA export by shuttling through a leucine-rich nuclear export signal and binding viral intronless RNAs through an RGG motif. *Genes Dev* **12**, 868-79.

Sandri-Goldin, R. M. (1998b). Interactions between a herpes simplex virus regulatory protein and cellular mRNA processing pathways. *Methods* **16**, 95-104.

Sandri-Goldin, R. M. & Mendoza, G. E. (1992). A herpesvirus regulatory protein appears to act post-transcriptionally by affecting mRNA processing. *Genes Dev* **6**, 848-63.

Sarrias, M. R., Whitbeck, J. C., Rooney, I., Spruce, L., Kay, B. K., Montgomery, R. I., Spear, P. G., Ware, C. F., Eisenberg, R. J., Cohen, G. H. & Lambris, J. D. (1999). Inhibition of herpes simplex virus gD and lymphotoxin-alpha binding to HveA by peptide antagonists [In Process Citation]. *J Virol* **73**, 5681-7.

Schek, N. & Bachenheimer, S. L. (1985). Degradation of cellular mRNAs induced by a virion-associated factor during herpes simplex virus infection of Vero cells. *J Virol* **55**, 601-10.

Schrag, J. D., Prasad, B. V., Rixon, F. J. & Chiu, W. (1989). Three-dimensional structure of the HSV1 nucleocapsid. *Cell* **56**, 651-60.

Schwartz, J. & Roizman, B. (1969). Concerning the egress of herpes simplex virus from infected cells: electron and light microscope observations. *Virology* **38**, 42-9.

Sears, A. E., Halliburton, I. W., Meignier, B., Silver, S. & Roizman, B. (1985). Herpes simplex virus 1 mutant deleted in the alpha 22 gene: growth and gene expression

in permissive and restrictive cells and establishment of latency in mice. *J Virol* **55**, 338-46.

Sekulovich, R. E., Leary, K. & Sandri-Goldin, R. M. (1988). The herpes simplex virus type 1 alpha protein ICP27 can act as a trans- repressor or a trans-activator in combination with ICP4 and ICP0. *J Virol* **62**, 4510-22.

Severini, A., Morgan, A. R., Tovell, D. R. & Tyrrell, D. L. (1994). Study of the structure of replicative intermediates of HSV-1 DNA by pulsed-field gel electrophoresis. *Virology* **200**, 428-35.

Severini, A., Scraba, D. G. & Tyrrell, D. L. (1996). Branched structures in the intracellular DNA of herpes simplex virus type 1. *J Virol* **70**, 3169-75.

Shao, L., Rapp, L. M. & Weller, S. K. (1993). Herpes simplex virus 1 alkaline nuclease is required for efficient egress of capsids from the nucleus. *Virology* **196**, 146-62.

Sheldrick, P., Laithier, M., Lando, D. & Ryhiner, M. L. (1973). Infectious DNA from herpes simplex virus: infectivity of double- stranded and single-stranded molecules. *Proc Natl Acad Sci U S A* **70**, 3621-5.

Shepard, A. A. & DeLuca, N. A. (1989). Intragenic complementation among partial peptides of herpes simplex virus regulatory protein ICP4. *J Virol* **63**, 1203-11.

Shepard, A. A. & DeLuca, N. A. (1991). Activities of heterodimers composed of DNA-binding- and transactivation- deficient subunits of the herpes simplex virus regulatory protein ICP4. *J Virol* **65**, 299-307.

Shieh, M. T., WuDunn, D., Montgomery, R. I., Esko, J. D. & Spear, P. G. (1992). Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *J Cell Biol* **116**, 1273-81.

Shlomai, J., Friedmann, A. & Becker, Y. (1976). Replication intermediates of herpes simplex virus DNA. *Virology* **69**, 647-59.

Slobedman, B., Zhang, X. & Simmons, A. (1999). Herpes simplex virus genome isomerization: origins of adjacent long segments in concatemeric viral DNA. *J Virol* **73**, 810-3.

Smibert, C. A., Johnson, D. C. & Smiley, J. R. (1992). Identification and characterization of the virion-induced host shutoff product of herpes simplex virus gene UL41. *J Gen Virol* **73**, 467-70.

Smibert, C. A., Popova, B., Xiao, P., Capone, J. P. & Smiley, J. R. (1994). Herpes simplex virus VP16 forms a complex with the virion host shutoff protein vhs. *J Virol* **68**, 2339-46.

Smibert, C. A. & Smiley, J. R. (1990). Differential regulation of endogenous and transduced beta-globin genes during infection of erythroid cells with a herpes simplex virus type 1 recombinant. *J Virol* **64**, 3882-94.

Smiley, J., Panning, B. & Smibert, C. (1991). Regulation of cellular genes by HSV products. In *Herpesvirus transcription and its regulation*, 1st edn, pp. 151-180. Edited by E. Wagner. Boca Raton: CRC Press, Inc.

Smiley, J. R. & Duncan, J. (1992). The herpes simplex virus type 1 immediate-early polypeptide ICP4 is required for expression of globin genes located in the viral genome. *Virology* **190**, 538-41.

Smiley, J. R. & Duncan, J. (1997). Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 produces a phenotype similar to that of the in1814 linker insertion mutation. *J Virol* **71**, 6191-3.

Smiley, J. R., Duncan, J. & Howes, M. (1990). Sequence requirements for DNA rearrangements induced by the terminal repeat of herpes simplex virus type 1 KOS DNA. *J Virol* **64**, 5036-50.

Smiley, J. R., Fong, B. S. & Leung, W. C. (1981). Construction of a double-jointed herpes simplex viral DNA molecule: inverted repeats are required for segment inversion, and direct repeats promote deletions. *Virology* **113**, 345-62.

Smiley, J. R., Johnson, D. C., Pizer, L. I. & Everett, R. D. (1992). The ICP4 binding sites in the herpes simplex virus type 1 glycoprotein D (gD) promoter are not essential for efficient gD transcription during virus infection. *J Virol* **66**, 623-31.

Smiley, J. R., Smibert, C. & Everett, R. D. (1987). Expression of a cellular gene cloned in herpes simplex virus: rabbit beta-globin is regulated as an early viral gene in infected fibroblasts. *J Virol* **61**, 2368-77.

Smith, C. A., Bates, P., Rivera-Gonzalez, R., Gu, B. & DeLuca, N. A. (1993). ICP4, the major transcriptional regulatory protein of herpes simplex virus type 1, forms a tripartite complex with TATA-binding protein and TFIIB. *J Virol* **67**, 4676-87.

Smith, I. L., Hardwicke, M. A. & Sandri-Goldin, R. M. (1992). Evidence that the herpes simplex virus immediate early protein ICP27 acts post-transcriptionally during infection to regulate gene expression. *Virology* **186**, 74-86.

Sodeik, B., Ebersold, M. W. & Helenius, A. (1997). Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J Cell Biol* **136**, 1007-21.

Soliman, T. M., Sandri-Goldin, R. M. & Silverstein, S. J. (1997). Shuttling of the herpes simplex virus type 1 regulatory protein ICP27 between the nucleus and cytoplasm mediates the expression of late proteins. *J Virol* **71**, 9188-97.

Sorenson, C. M., Hart, P. A. & Ross, J. (1991). Analysis of herpes simplex virus-induced mRNA destabilizing activity using an in vitro mRNA decay system. *Nucleic Acids Res* **19**, 4459-65.

Spear, P. G. (1993a). Entry of alphaherpesvirus into cells. *semin virol* **4**, 167-180.

Spear, P. G. (1993b). Membrane fusion induced by herpes simplex virus. In *Viral Fusion Mechanisms*, pp. 201-232. Edited by J. Bentz. Boca Raton, Fla.: CRC Press.

Spear, P. G. & Roizman, B. (1972). Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. *J Virol* **9**, 143-59.

Spector, D., Purves, F. & Roizman, B. (1990). Mutational analysis of the promoter region of the alpha 27 gene of herpes simplex virus 1 within the context of the viral genome. *Proc Natl Acad Sci U S A* **87**, 5268-72.

Spector, D., Purves, F. & Roizman, B. (1991). Role of alpha-transinducing factor (VP16) in the induction of alpha genes within the context of viral genomes. *J Virol* **65**, 3504-13.

Spencer, C. A., Dahmus, M. E. & Rice, S. A. (1997). Repression of host RNA polymerase II transcription by herpes simplex virus type 1. *J Virol* **71**, 2031-40.

Stackpole, C. W. (1969). Herpes-type virus of the frog renal adenocarcinoma. I. Virus development in tumor transplants maintained at low temperature. *J Virol* **4**, 75-93.

Stannard, L. M., Fuller, A. O. & Spear, P. G. (1987). Herpes simplex virus glycoproteins associated with different morphological entities projecting from the virion envelope. *J Gen Virol* **68**, 715-25.

Stern, S., Tanaka, M. & Herr, W. (1989). The Oct-1 homoeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16. *Nature* **341**, 624-30.

Sternsdorf, T., Jensen, K. & Will, H. (1997). Evidence for covalent modification of the nuclear dot-associated proteins PML and Sp100 by PIC1/SUMO-1. *J Cell Biol* **139**, 1621-34.

Stevens, A. (1998). Endonucleolytic cleavage of RNA at 5' endogenous stem structures by human flap endonuclease 1. *Biochem Biophys Res Com* **251**, 501-508.

Stow, N. D. (1982). Localization of an origin of DNA replication within the TRS/IRS repeated region of the herpes simplex virus type 1 genome. *Embo J* **1**, 863-7.

Stow, N. D. (1992). Herpes simplex virus type 1 origin-dependent DNA replication in insect cells using recombinant baculoviruses. *J Gen Virol* **73**, 313-21.

Stow, N. D., McMonagle, E. C. & Davison, A. J. (1983). Fragments from both termini of the herpes simplex virus type 1 genome contain signals required for the encapsidation of viral DNA. *Nucleic Acids Res* **11**, 8205-20.

Stow, N. D. & Stow, E. C. (1986). Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. *J Gen Virol* **67**, 2571-85.

Strelow, L. I. & Leib, D. A. (1995). Role of the virion host shutoff (vhs) of herpes simplex virus type 1 in latency and pathogenesis. *J Virol* **69**, 6779-86.

Stringer, K. F., Ingles, C. J. & Greenblatt, J. (1990). Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. *Nature* **345**, 783-6.

Strom, T. & Frenkel, N. (1987). Effect of herpes simplex virus on mRNA stability. *J Virol* **61**, 2198-2207.

Su, L. & Knipe, D. M. (1989). Herpes simplex virus alpha protein ICP27 can inhibit or augment viral gene transactivation. *Virology* **170**, 496-504.

Subramanian, G., McClain, D. S., Perez, A. & Fuller, A. O. (1994). Swine testis cells contain functional heparan sulfate but are defective in entry of herpes simplex virus. *J Virol* **68**, 5667-76.

Sullivan-Bolyai, J. Z., Fife, K. H., Jacobs, R. F., Miller, Z. & Corey, L. (1983). Disseminated neonatal herpes simplex virus type 1 from a maternal breast lesion. *Pediatrics* **71**, 455-457.

Svennerholm, B., Jeansson, S., Vahlne, A. & Lycke, E. (1991). Involvement of glycoprotein C (gC) in adsorption of herpes simplex virus type 1 (HSV-1) to the cell. *Arch Virol* **120**, 273-9.

Svitkin, Y. V., Ovchinnikov, L. P., Dreyfuss, G. & Sonenberg, N. (1996). General RNA binding proteins render translation cap dependent. *EMBO J* **15**, 7174-7155.

Svitkin, Y. V., Pause, A. & Sonenberg, N. (1994). La autoantigen alleviates translational repression by the 5' leader sequence of the human immunodeficiency virus type 1 mRNA. *J Virol* **68**, 7001-7.

Sydiskis, R. J. & Roizman, B. (1966). Polysomes and protein synthesis in cells infected with a DNA virus. *Science* **153**, 76-78.

Sydiskis, R. J. & Roizman, B. (1967). The disaggregation of host polyribosomes in productive and abortive infection with herpes simplex virus. *Virology* **32**, 678-686.

Sydiskis, R. J. & Roizman, B. (1968). The sedimentation profiles of cytoplasmic polyribosomes in mammalian cells productively and abortively infected with herpes simplex virus. *Virology* **34**, 562-565.

Szilagyi, J. F. & Cunningham, C. (1991). Identification and characterization of a novel non-infectious herpes simplex virus-related particle. *J Gen Virol* **72**, 661-8.

Tal-Singer, R., Peng, C., Ponce De Leon, M., Abrams, W. R., Banfield, B. W., Tufaro, F., Cohen, G. H. & Eisenberg, R. J. (1995). Interaction of herpes simplex virus glycoprotein gC with mammalian cell surface molecules. *J Virol* **69**, 4471-83.

Tal-Singer, R., Pichyangkura, R., Chung, E., Lasner, T. M., Randazzo, B. P., Trojanowski, J. Q., Fraser, N. W. & Triezenberg, S. J. (1999). The transcriptional activation domain of VP16 is required for efficient infection and establishment of latency by HSV-1 in the murine peripheral and central nervous systems. *Virology* **259**, 20-33.

Taus, N. S., Salmon, B. & Baines, J. D. (1998). The herpes simplex virus 1 UL 17 gene is required for localization of capsids and major and minor capsid proteins to intranuclear sites where viral DNA is cleaved and packaged. *Virology* **252**, 115-25.

Thomsen, D. R., Newcomb, W. W., Brown, J. C. & Homa, F. L. (1995). Assembly of the herpes simplex virus capsid: requirement for the carboxyl-terminal twenty-five amino acids of the proteins encoded by the UL26 and UL26.5 genes. *J Virol* **69**, 3690-703.

Tomazin, R., Hill, A. B., Jugovic, P., York, I., van Endert, P., Ploegh, H. L., Andrews, D. W. & Johnson, D. C. (1996). Stable binding of the herpes simplex virus ICP47 protein to the peptide binding site of TAP. *Embo J* **15**, 3256-66.

Tomazin, R., van Schoot, N. E., Goldsmith, K., Jugovic, P., Sempe, P., Fruh, K. & Johnson, D. C. (1998). Herpes simplex virus type 2 ICP47 inhibits human TAP but not mouse TAP. *J Virol* **72**, 2560-3.

Torre, D., Speranza, F., Martegani, R., Ferrante, P., Omodeo-Zorini, E., Mancuso, R. & Fiori, G. P. (1998). Meningoencephalitis caused by human herpesvirus-6 in an immunocompetent adult patient: case report and review of the literature. *Infection* **26**, 402-4.

Torrisi, M. R., Di Lazzaro, C., Pavan, A., Pereira, L. & Campadelli-Fiume, G. (1992). Herpes simplex virus envelopment and maturation studied by fracture label. *J Virol* **66**, 554-61.

Triezenberg, S. J., Kingsbury, R. C. & McKnight, S. L. (1988a). Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. *Genes Dev* **2**, 718-29.

Triezenberg, S. J., LaMarco, K. L. & McKnight, S. L. (1988b). Evidence of DNA: protein interactions that mediate HSV-1 immediate early gene activation by VP16. *Genes Dev* **2**, 730-42.

Trus, B. L., Booy, F. P., Newcomb, W. W., Brown, J. C., Homa, F. L., Thomsen, D. R. & Steven, A. C. (1996). The herpes simplex virus procapsid: structure, conformational changes upon maturation, and roles of the triplex proteins VP19c and VP23 in assembly. *J Mol Biol* **263**, 447-62.

Trus, B. L., Homa, F. L., Booy, F. P., Newcomb, W. W., Thomsen, D. R., Cheng, N., Brown, J. C. & Steven, A. C. (1995). Herpes simplex virus capsids assembled in insect cells infected with recombinant baculoviruses: structural authenticity and localization of VP26. *J Virol* **69**, 7362-6.

Uprichard, S. L. & Knipe, D. M. (1997). Assembly of herpes simplex virus replication proteins at two distinct intranuclear sites. *Virology* **229**, 113-25.

van Genderen, I. L., Brandimarti, R., Torrisi, M. R., Campadelli, G. & van Meer, G. (1994). The phospholipid composition of extracellular herpes simplex virions differs from that of host cell nuclei. *Virology* **200**, 831-6.

Varmuza, S. L. & Smiley, J. R. (1985). Signals for site-specific cleavage of HSV DNA: maturation involves two separate cleavage events at sites distal to the recognition sequences. *Cell* **41**, 793-802.

Ward, P. L., Ogle, W. O. & Roizman, B. (1996). Assemblons: nuclear structures defined by aggregation of immature capsids and some tegument proteins of herpes simplex virus 1. *J Virol* **70**, 4623-31.

Warner, M. S., Geraghty, R. J., Martinez, W. M., Montgomery, R. I., Whitbeck, J. C., Xu, R., Eisenberg, R. J., Cohen, G. H. & Spear, P. G. (1998). A cell surface protein with herpesvirus entry activity (HveB) confers susceptibility to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2, and pseudorabies virus. *Virology* **246**, 179-89.

Watson, R. J. & Clements, J. B. (1978). Characterization of transcription-deficient temperature-sensitive mutants of herpes simplex virus type 1. *Virology* **91**, 364-79.

Watson, R. J. & Clements, J. B. (1980). A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis. *Nature* **285**, 329-30.

Weinheimer, S. P., Boyd, B. A., Durham, S. K., Resnick, J. L. & O'Boyle, D. R. d. (1992). Deletion of the VP16 open reading frame of herpes simplex virus type 1. *J Virol* **66**, 258-69.

Weller, S. K., Seghatoleslami, M. R., Shao, L., Rowse, D. & Carmichael, E. P. (1990). The herpes simplex virus type 1 alkaline nuclease is not essential for viral DNA synthesis: isolation and characterization of a lacZ insertion mutant. *J Gen Virol* **71**, 2941-52.

Weller, S. K., Spadaro, A., Schaffer, J. E., Murray, A. W., Maxam, A. M. & Schaffer, P. A. (1985). Cloning, sequencing, and functional analysis of oriL, a herpes simplex virus type 1 origin of DNA synthesis. *Mol Cell Biol* **5**, 930-42.

Whitbeck, J. C., Peng, C., Lou, H., Xu, R., Willis, S. H., Ponce de Leon, M., Peng, T., Nicola, A. V., Montgomery, R. I., Warner, M. S., Soulika, A. M., Spruce, L. A., Moore, W. T., Lambris, J. D., Spear, P. G., Cohen, G. H. & Eisenberg, R. J. (1997). Glycoprotein D of herpes simplex virus (HSV) binds directly to HVEM, a member of the tumor necrosis factor receptor superfamily and a mediator of HSV entry. *J Virol* **71**, 6083-93.

Whitley, R. (1996). Herpes simplex viruses. In *Fields Virology*, 3rd edn, pp. 2297-2342. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott-Raven.

Whitley, R. J. (1983). Interim summary of mortality in herpes simplex encephalitis and neonatal herpes simplex virus infections: vidarabine versus acyclovir. *J Antimicrob Chemother* **12 Suppl B**, 105-12.

Whitley, R. J. (1988). Herpes simplex virus infections of the central nervous system. A review. *Am J Med* **85**, 61-7.

Whitley, R. J. (1993). Neonatal herpes simplex virus infections. *J Med Virol* **Suppl**, 13-21.

Whitley, R. J. & Kimberlin, D. W. (1999). Viral Encephalitis. *Pediatr Rev* **20**, 192-198.

Williams, R. K. & Straus, S. E. (1997). Specificity and affinity of binding of herpes simplex virus type 2 glycoprotein B to glycosaminoglycans. *J Virol* **71**, 1375-80.

Willis, S. H., Rux, A. H., Peng, C., Whitbeck, J. C., Nicola, A. V., Lou, H., Hou, W., Salvador, L., Eisenberg, R. J. & Cohen, G. H. (1998). Examination of the kinetics of herpes simplex virus glycoprotein D binding to the herpesvirus entry mediator, using surface plasmon resonance. *J Virol* **72**, 5937-47.

Wilson, A. C., Freiman, R. N., Goto, H., Nishimoto, T. & Herr, W. (1997). VP16 targets an amino-terminal domain of HCF involved in cell cycle progression. *Mol Cell Biol* **17**, 6139-46.

Wittels, M. & Spear, P. G. (1991). Penetration of cells by herpes simplex virus does not require a low pH- dependent endocytic pathway. *Virus Res* **18**, 271-90.

Wolontis, S. & Jeansson, S. (1977). Correlation of herpes simplex virus types 1 and 2 with clinical features of infection. *J Infect Dis* **135**, 28-33.

WuDunn, D. & Spear, P. G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J Virol* **63**, 52-8.

Xiao, H., Pearson, A., Coulombe, B., Truant, R., Zhang, S., Regier, J. L., Triezenberg, S. J., Reinberg, D., Flores, O., Ingles, C. J. & et al. (1994). Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53. *Mol Cell Biol* **14**, 7013-24.

Xiao, P. & Capone, J. P. (1990). A cellular factor binds to the herpes simplex virus type 1 transactivator Vmw65 and is required for Vmw65-dependent protein-DNA complex assembly with Oct-1. *Mol Cell Biol* **10**, 4974-7.

Xiao, W., Pizer, L. I. & Wilcox, K. W. (1997). Identification of a promoter-specific transactivation domain in the herpes simplex virus regulatory protein ICP4. *J Virol* **71**, 1757-65.

Yao, F. & Schaffer, P. A. (1994). Physical interaction between the herpes simplex virus type 1 immediate- early regulatory proteins ICP0 and ICP4. *J Virol* **68**, 8158-68.

Yao, Q. Y., Croom-Carter, D. S., Tierney, R. J., Habeshaw, G., Wilde, J. T., Hill, F. G., Conlon, C. & Rickinson, A. B. (1998). Epidemiology of infection with Epstein-Barr virus types 1 and 2: lessons from the study of a T-cell-immunocompromised hemophilic cohort. *J Virol* **72**, 4352-63.

York, I. A., Roop, C., Andrews, D. W., Riddell, S. R., Graham, F. L. & Johnson, D. C. (1994). A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. *Cell* **77**, 525-35.

Young, J. C., Ursini, J., Legate, K. R., Miller, J. D., Walter, P. & Andrews, D. W. (1995). An amino terminal domain containing hydrophobic and hydrophilic sequences binds the signal recognition particle receptor alpha subunit to the bata subunit on the endoplasmic reticulum membrane. *J Biol Chem* **270**, 15650-15657.

Zelus, B. D., Stewart, R. S. & Ross, J. (1996). The virion host shutoff protein of herpes simplex virus type1: messenger ribonucleolytic activity in vitro. *J Virol* **70**, 2411-2419.

Zhang, X., Efstathiou, S. & Simmons, A. (1994). Identification of novel herpes simplex virus replicative intermediates by field inversion gel electrophoresis: implications for viral DNA amplification strategies. *Virology* **202**, 530-9.

Zhou, Z. H., Chen, D. H., Jakana, J., Rixon, F. J. & Chiu, W. (1999). Visualization of tegument-capsid interactions and DNA in intact herpes simplex virus type 1 virions. *J Virol* **73**, 3210-8.

Zhou, Z. H., He, J., Jakana, J., Tatman, J. D., Rixon, F. J. & Chiu, W. (1995). Assembly of VP26 in herpes simplex virus-1 inferred from structures of wild-type and recombinant capsids. *Nat Struct Biol* **2**, 1026-30.

Zhou, Z. H., Macnab, S. J., Jakana, J., Scott, L. R., Chiu, W. & Rixon, F. J. (1998). Identification of the sites of interaction between the scaffold and outer shell in herpes simplex virus-1 capsids by difference electron imaging. *Proc Natl Acad Sci U S A* **95**, 2778-83.

Zhou, Z. H., Prasad, B. V., Jakana, J., Rixon, F. J. & Chiu, W. (1994). Protein subunit structures in the herpes simplex virus A-capsid determined from 400 kV spot-scan electron cryomicroscopy. *J Mol Biol* **242**, 456-69.

Zhu, Z., Cai, W. & Schaffer, P. A. (1994). Cooperativity among herpes simplex virus type 1 immediate-early regulatory proteins: ICP4 and ICP27 affect the intracellular localization of ICP0. *J Virol* **68**, 3027-40.

Zhu, Z., DeLuca, N. A. & Schaffer, P. A. (1996). Overexpression of the herpes simplex virus type 1 immediate-early regulatory protein, ICP27, is responsible for the aberrant localization of ICP0 and mutant forms of ICP4 in ICP4 mutant virus-infected cells. *J Virol* **70**, 5346-56.

Zhu, Z. & Schaffer, P. A. (1995). Intracellular localization of the herpes simplex virus type 1 major transcriptional regulatory protein, ICP4, is affected by ICP27. *J Virol* **69**, 49-59.