mRNA DECAPITATION INDUCED BY VHS

mRNA DECAPITATION INDUCED BY THE HERPES SIMPLEX VIRUS VIRION HOST SHUTOFF PROTEIN

By

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ABSTRACT

Cells infected with herpes simplex virus show a rapid cessation of protein synthesis and a dramatic decline in the levels of mRNA; a process known as host shutoff. This effect is attributed to a viral tegument protein called the virion host shutoff protein, or vhs. The mechanism by which vhs induces mRNA degradation is not yet understood. It is not known whether vhs possesses RNase activities or if it acts in combination with other cellular factors. To gain a better understanding of the function of vhs, I examined RNA degradation in detail by analyzing the RNA decay products generated in the presence of vhs. *In vivo* experiments, performed by infecting murine erythroid leukemia cells with HSV, revealed that beta-globin mRNA is rapidly degraded, in the presence of vhs, without being converted to detectable decay intermediates. The half-life of this mRNA was 15 and 60 minutes for HSV-2 and HSV-1, respectively. Using vhs translated in a rabbit reticulocyte lysate system, I found that vhs induced rapid decay at the 5' end of a capped RNA molecule. The decay event was endonucleolytic and occurred at preferred sites downstream of the cap, generating capped oligonucleotides. Unlike influenza RNA polymerase, the cleavage event did not occur at a fixed distance from the cap since capped oligos of differing size were generated from different RNA substrates. My data indicate that vhs induced cleavage exhibits a strong, but not absolute preference for RNAs possessing an m^7G cap, which may account for vhs' specificity for mRNAs *in vivo*.

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(d)ATP or A	(deoxy)adenosine triphosphate
µCi	microCurie
µL	microlitre
µM	micromolar
cDNA	complementary deoxyribonucleic acid
cm2	square centimetres
CTP or C	cytosine triphosphate
E	early
g	gravitional force
GTP or G	guanosine triphosphate
hrs	hours
HSV-1	herpes simplex type 1
HSV-2	herpes simplex type 2
IE	immediate early
kb	kilobases
kDa	kilodaltons
L	late
L	litre
M	molar
MEL	murine erythroid leukemia

min	minutes
mL	millilitre
mm	millimetre
mM	millimolar
mRNA	messenger ribonucleic acid
ng	nanograms
ORF	open reading frame
PFU	plaque forming units
RNA	ribonucleic acid
rpm	revolutions per minute
TTP or T	thymidine triphosphate
U	units
UTP or U	uridine triphosphate
UTR	untranslated region
UV	ultraviolet
V	volts

I. INTRODUCTION

1.1 Eukaryotic mRNA turnover

It is becoming increasingly clear that an important aspect in the regulation of gene expression is the post-transcriptional control of mRNA stability. The decay rates of eukaryotic mRNAs vary greatly. For example, the half-life of beta globin mRNA in terminally differentiated murine erythroid leukemia cells is approximately 17 hours (Lowenhaupt and Lingrel, 1978) compared to less than 30 minutes for the unstable c-fos oncogene mRNA (Shaw and Kamen, 1986). Although transcription rates largely account for mRNA steady-state levels, the importance of mRNA turnover in eukaryotic regulation has resulted in much research in this area.

1.1.1 Specific Sequence Determinants Affecting mRNA Stability.

What determines whether an mRNA is stable or unstable? Recent advances in mRNA stability have revealed that many eukaryotic mRNAs possess specific *cis* sequences that influence the rate at which they decay (reviewed in, Ross, 1988; Ross, 1995 and Caponigro and Parker, 1996). Sequences have been identified in all regions of mRNA molecules that target them for rapid degradation or increase their stability through interaction with *trans*-acting elements (discussed below).

Many unstable mRNAs have been studied and a majority of them have been shown to possess decay signals within the 3' untranslated region (UTR), indicating the importance of this region to the half-life of an mRNA. Studies on the yeast MFA2 mRNA have revealed that a signal motif within the 3' UTR promotes rapid degradation of the mRNA (Muhlrad and Parker, 1992). The exact sequence of the decay signal(s) is not known however, one possibility is $Y_{(6-8)}$CAU (where Y= C or U), as there are six copies of this sequence within the 3' UTR. Single mutations and small deletions in this region have little effect on stabilizing the MFA2 mRNA but double mutations and larger deletions of the above sequence result in an increased half-life (Muhlrad and Parker, 1992).

Another sequence motif within the 3' UTR of MFA2 that may induce rapid turnover has been identified in several unstable mammalian mRNAs (Muhlrad and Parker, 1992). This sequence is the so-called AU-rich element (AURE); a stretch of AUUUA repeats. Analysis of this sequence has demonstrated that it induces degradation of *c-fos* (Wilson and Treisman, 1988), *c-myc* (Brewer and Ross, 1988) and granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA (Aharon and Schneider, 1993), in a process that involves rapid loss of the poly(A) tail and decay of the mRNA body (see below). Furthermore, insertion of the AURE of *c-fos* mRNA into the stable beta globin transcript causes the chimeric mRNA to decay at a much faster rate, indicating that the AURE sequences are important in triggering degradation (Shyu et al., 1991). Several studies have identified cytosolic proteins that are capable of binding the AUUUA motifs of unstable mRNAs, suggesting that these binding factors are involved in

the regulation of mRNA half-life (Gillis and Malter, 1991); (Vakalopoulou et al., 1991).

During iron deprivation in mammalian cells, human transferrin receptor mRNA (hTR) increases in abundance, though the transcription rate remains unaltered . The levels of this mRNA have shown to be regulated through a specific sequence in the 3' UTR (Mullner and Kuhn, 1988). This sequence, termed the iron-responsive element (IRE), forms a stem-loop structure that is capable of binding an iron-regulatory protein (IRP) which acts to stabilize the mRNA at times of low intracellular iron concentration (Koeller et al., 1991).

Sequences influencing stability have also been identified in other regions of mRNA molecules. In addition to the AUREs, c-fos mRNA possesses an instability determinant within the protein coding region. Using chimeric mRNAs containing the 5' and 3' UTRs of beta globin and the coding region of c-fos, it was established that a 0.32 Kb region of the coding region contains sequences that induce rapid deadenylation of the c-fos mRNA (Shyu et al., 1991). Finally, the 5' UTR has been shown to influence mRNA half-lives (reviewed in Caponigro and Parker, 1996) Though it is unknown how these sequences promote mRNA decay it is believed that they influence the translation initiation machinery which may induce removal of the cap structure.

1.1.2 Pathways of mRNA Decay

The majority of research on mRNA stability has been performed in yeast, and from those studies several pathways of mRNA degradation have been proposed (reviewed in Beelman and Parker, 1995). Despite differences in half-lives many mRNAs undergo decay via the same process. The most common and best understood of these pathways is deadenylation-dependent decay. As the name suggests, an important aspect of this turnover pathway is deadenylation, that is, exonucleolytic decay of the poly(A) tail in a 3' to 5' direction. For many mRNAs, deadenylation represents the first detectable decay event (Brewer and Ross, 1988; Shyu et al., 1991; Decker and Parker, 1993). By inserting strong RNA secondary structures into the 3' UTR in an attempt to slow down exonucleolytic decay and trap intermediates, it has been shown that loss of the poly(A) tail must occur before the body of the mRNA is degraded (Decker and Parker, 1993). However, deadenylation does not result in complete degradation of the poly(A) tail, but rather, the tails are reduced to an oligo(A) form. In yeast, the poly(A) tail of some mRNAs is shortened to a length of 10 to 12 adenosine residues before decay of the transcript body occurs (Decker and Parker, 1993). Degradation of oligoadenylated mRNA transcripts in mammals occurs once the poly(A) tail is reduced to approximately 25 to 60 adenosine residues (Shyu et al., 1991). As stated earlier, the rate at which deadenylation occurs varies from mRNA to mRNA and seems to be controlled by specific sequences and trans-acting factors. However, the deadenylation rate is not entirely responsible for the difference in half-lives. The oligoadenylated form of the stable PGK1 mRNA decays at a much slower rate than oligoadenylated

MFA2, implying that other factors or events contribute to the stability of an mRNA (Decker and Parker, 1993).

Once shortening of the poly(A) tail has taken place, the next process in the deadenylation-dependent decay pathway involves removal of the cap structure and subsequent 5' to 3' exonucleolytic decay. Recall that the important functions of the m^{7}G cap of eukaryotic mRNAs are to protect the transcript from cellular nucleases and to provide a target site for translation initiation (reviewed in Banerjee, 1980). Evidence supporting removal of the cap following deadenylation stems from experiments using XRN1p deficient yeast strains (Hsu and Stevens, 1993). The XRN1 gene encodes a major 5' to 3' exonuclease (Larimer et al., 1992). In this study Hsu et al. analyzed the decay intermediates of several yeast mRNAs and found that the most abundant species were transcripts that were essentially full-length yet were poly(A) minus and lacked a 5' cap structure. Neither the transcription nor deadenylation rates were abnormal in the *xrn1* cells (Hsu and Stevens, 1993). Furthermore, mRNAs possessing strong secondary structures (through insertion of a poly(G) site) that inhibit exonucleases give rise to decay intermediates that have been deadenylated to an oligo(A) state and are degraded at the 5' end up to the site of secondary structure (Decker and Parker, 1993; Muhlrad et al., 1994). Finally, yeast strains that lack an enzyme responsible for decapping, Dcp1p, have accumulated levels of full-length, capped mRNAs that were deadenylated. Taken in combination with the above studies showing that deadenylation occurs prior to decay of the mRNA body, these findings demonstrate that decapping follows the

deadenylation of an mRNA transcript resulting in a susceptible 5' end that is rapidly degraded at least in part by the yeast exonuclease, XRN1p. A similar pathway may also be present in mammalian cells, since c-myc and c-fos mRNAs are deadenylated before decay of the transcript body occurs (Brewer and Ross, 1988; Shyu et al., 1991). Furthermore, a decapping enzyme (Kumagai et al., 1992) and a 5' exoribonuclease (Coutts and Brawerman, 1993) have been identified in mammalian cells. Although it is unknown whether these proteins play a role in mRNA turnover, the existence of similar activities between yeast and higher eukaryotes suggests the possibility of common mRNA decay pathways.

There is evidence that following deadenylation, some mRNAs undergo 3' to 5' exonucleolytic decay. Studies using the stable PGK1 mRNA identified low levels of decay intermediates that possessed truncated 3' ends, which became more abundant in cells deficient in 5' to 3' decay (Muhlrad et al., 1995). This suggests that although 5' to 3' degradation is a more rapid pathway for PGK1 turnover, there is a system that degrades mRNAs in a 3' to 5' direction following deadenylation.

Eukaryotic cells are capable of inducing rapid decay of mRNAs that contain premature translation initiation codons, by a pathway that does not require prior deadenylation (reviewed in Beelman and Parker, 1995). This pathway has been termed deadenylation-independent decapping, as the first detectable decay event on the mRNA is the cleavage of the cap structure. Using

the stable yeast PGK1 mRNA, Muhlrad and Parker (Muhlrad and Parker, 1994) demonstrated that the insertion of an nonsense codon into the coding region resulted in immediate degradation of the transcript following transcription. Degradation intermediates of this pathway have long poly(A) tails yet are lacking sequences from the 5' end and in *xrn1* strains, full length, capless transcripts are stabilized (Muhlrad and Parker, 1994). The rapid decay associated with deadenylation-independent decapping raises the hypothesis that this pathway serves as an mRNA surveillance system that removes nonsense transcripts that would result in truncated proteins. Support for this theory stems from studies using mutant C. elegans stains that are unable to degrade nonsense mRNAs (Pulak and Anderson, 1993). In these strains some mutations that are recessive in wild-type become dominant-negative, due to the presence of aberrant proteins.

1.1.3 The Poly(A)-Binding Protein

The poly(A)-binding protein (PABP) plays an important role in the control of both deadenylation and decapping (reviewed in Ross, 1988; Caponigro and Parker, 1996). As far as deadenylation is concerned, there is some controversy as to the function of the PABP. In mammalian in vitro extracts, there is evidence that the PABP acts to protect poly(A) tails from degradation since depletion of PABP from polyribosomes results in the rapid deadenylation of beta-globin mRNA (Bernstein and Ross, 1989) A model proposed from this study suggests that binding of PABP to the poly(A) tail prevents degradation by cellular nucleases. For stable mRNAs the PABP-poly(A) complex remains intact while

for unstable transcripts the PABP either disassociates from the tail or it migrates to other sites within the 3' UTR thus exposing the poly(A) tail (Bernstein and Ross, 1989).

A different role for the PABP has been identified from studies with yeast. Using strains that contain a mutation in the *pab1* gene (encodes yeast PABP), it was determined that the PABP was not required for deadenylation however, in its absence the rates of poly(A) decay were significantly reduced, suggesting that the yeast PABP plays a role in controlling deadenylation (Caponigro and Parker, 1995). Furthermore, in *pab1* conditional mutant yeast, mRNAs are rapidly decapped without first being deadenylated, implying that the PABP inhibits decapping (Caponigro and Parker, 1995). The fact that the absence of the PABP results in decapping suggests that an interaction may exist between the 5' and 3' ends of an mRNA. The presence of a poly(A) tail and PABP has been shown to enhance translation initiation in vitro (Grossi de Sa et al., 1988), providing evidence for such an interaction.

The increase in the rate of deadenylation caused by the PABP has been linked to a poly(A)-binding protein dependent poly(A) nuclease or PAN. This enzyme forms a complex with the PABP and catalyzes the exonucleolytic decay of the adenosine tail (Lowell et al., 1992). PAN is influenced by sequences within the 3' UTR that switch the enzyme from a distributive to a more processive form, which may account for the difference in decay rates between yeast mRNAs (Lowell et al., 1992).

1.2 Inhibition of Macromolecular Synthesis by Virus Infection.

Many viruses are capable of altering or interfering with the regulation of gene expression. Such viruses have unique systems that enable them to selectively inhibit the production of proteins from cellular mRNAs in an attempt to switch the cell's machinery into one devoted to the synthesis of new virus. This process is known as host shutoff. The site of interference of gene expression varies for different virus (reviewed in Kozak, 1986) but the end result is a decrease in cellular macromolecular synthesis as viral production takes over.

Several viruses induce shutoff by interfering with cellular transcription of mRNA. One example is vesicular stomatitis virus (VSV). VSV is an RNA virus that, upon cell entry, begins to transcribe five capped and polyadenylated mRNAs through the action of the virion associated transcriptase (reviewed in Kaariainen and Ranki, 1984). Therefore, the virus does not require the host's transcription machinery for synthesis of viral mRNAs. In fact, VSV inhibits the production of RNA polymerase II transcripts. This is achieved by a major virion structural protein called the viral matrix (M) protein (Black and Lyles, 1992). Cotransfection of an M protein expression vector and a chloramphenicol acetyltransferase (CAT) target gene vector resulted in decrease in CAT activity in a M protein dose-dependent manner. Nuclear run-off assays demonstrated that the reduction in activity was due to a decrease in transcription (Black and Lyles, 1992). Another element of VSV that has been implicated in host transcription inhibition is a small viral encoded RNA called the positive-strand leader (Knipe,

1996). This RNA is capable of inhibiting transcription in vitro possibly by binding to a cellular factor that is required for transcription.

Adenoviruses (Ad) induce host shutoff at a different site than VSV. In Ad infected cells, cellular mRNA transcripts are synthesized and processed normally however, they are prevented from accumulating in the cytoplasm. Two virally encoded proteins have been linked to this phenomenon. Both the E1B-55K and the E4-34K proteins are required for decreased cellular mRNA transport, yet promote the accumulation of viral transcripts in the cytoplasm (Pilder et al., 1986). These proteins have been shown to localize at the sight where viral transcription is believed to take place (Ornelles and Shenk, 1991) and although the exact mechanism of transport inhibition is not known, perhaps these proteins cause a redistribution of a cellular factor required for transport of cellular mRNAs.

One of the most interesting mechanisms of viral induced shutoff occurs in cells infected with poliovirus (reviewed in Sonenberg, 1987). Poliovirus, a member of the picornaviridiae family, induces a rapid cessation of cellular protein synthesis upon infection. This effect is limited to cellular mRNAs as viral transcripts are actively translated however, cellular mRNAs still remain intact in the cytoplasm. The selective nature of this inhibition is a result of the alteration of a cellular factor required for translation initiation. The factor in question is a component of the cap-binding protein (CBP) complex or eIF4F (reviewed in Thach, 1992). Proteins that form a part of this complex include: 1) CBP (eIF4E), responsible for binding to the m^7G cap structure of mRNAs; 2) eIF4A and eIF4B,

which possess RNA helicase activities that reduce secondary structure in the 5' UTR; and 3) p220 whose function is unknown. p220 is however, essential for translation initiation by the CBP complex and is the target for shutoff induced by poliovirus. CBP complexes purified from polio-infected cells reveal that the virus causes proteolytic cleavage of the p220 factor (Buckley and Ehrenfield, 1987). Polyacrylamide gel electrophoresis of the purified complex demonstrates that a band migrating at 220kDa in uninfected cells is replaced with bands between 100 and 130 kDa (Buckley and Ehrenfield, 1987). The cleavage of p220 is mediated by the poliovirus 2A protease, which may not catalyze the reaction but requires the presence of other cellular factors, including eIF3 (Wyckoff et al., 1990).

Proteolysis of p220 results in a non-functional CBP complex and thus translation initiation involving m^7G-capped mRNAs is prevented. This would also result in inhibition of poliovirus protein synthesis if the virus required cap-dependent translation. However, poliovirus has developed a unique system that allows for the internal binding of ribosomes to polio RNA rather than the conventional scanning model. The internal binding is mediated by a specific sequence within the 5' UTR of poliovirus mRNAs that allows for translation initiation and elongation, independent of cap recognition (Pelletier and Sonenberg, 1988) and thus, accounts for the selective synthesis of viral proteins.

A similar mechanism also accounts for the inhibition of cellular protein synthesis during late phases of adenovirus infection. In addition to the ability to prevent cellular mRNA transport, adenovirus, like poliovirus, causes inactivation

of the CBP complex. This is achieved by viral-mediated dephosphorylation of the CBP or eIF4E, which must be phosphorylated to be in its active state (Huang and Schneider, 1991). Adenovirus late mRNAs possess a common 200 nucleotide tripartite leader that enables the mRNA to be translated in the absence of the CBP complex (Dolph et al., 1991).

More relevant to this introduction are the viruses that inhibit protein synthesis by altering or degrading pre-existing cellular mRNAs. One such example is the L-A virus of *Saccharomyces cerevisiae.* This double-stranded RNA virus produces viral transcripts that are both uncapped and unadenylated (Thiele et al., 1984), which under normal circumstances would be degraded by the yeast 5' exonuclease, XRN1p (see above). However, the L-A virus possesses a mechanism for decoying the yeast degradation system that partially alleviates the attack on the viral mRNA allowing synthesis of protein (Masison et al., 1995). This is achieved by the major viral coat protein, Gag, which covalently binds to the m^7GMP cap in the presence of magnesium. Following binding Gag mediates cleavage of the m^7G cap, thus releasing the uncapped yeast mRNA (Masison et al., 1995). These capless mRNAs then undergo degradation by XRN1p (Larimer et al., 1992) providing some time for viral transcripts to be translated.

During influenza virus infection, the induction of host shutoff is required for the transcription of viral mRNAs (reviewed in Katze and Krug, 1990). Influenza is a negative-strand RNA virus that encodes its own polymerase necessary for the

synthesis of viral transcripts in the nucleus of infected cells. Initiation of influenza transcription involves a novel mechanism that results in degradation of host pre-mRNAs in the nucleus. For viral mRNA synthesis to occur, influenza requires RNA primers for transcription elongation from the viral template. The source of the RNA primers is the 5' end of RNA polymerase II capped and methylated host transcripts (Plotch et al., 1981). A virion-encoded endonuclease (PB2), that forms part of the virus polymerase complex, cleaves m^7G-capped mRNAs nine to fifteen nucleotides downstream of the cap structure, thus generating a capped primer with a 3' hydroxyl end (Plotch et al., 1981). The polymerase complex then uses the oligoribonucleotide as a primer, whereby the nucleotide complementary to the penultimate 3' base of the template is added to the 3' end of the primer and elongation of the viral mRNA proceeds (Plotch et al., 1981). As a result, the cleaved cellular mRNAs are now exposed to cellular nucleases that degrade the host transcripts and therefore, diminish their levels as viral mRNAs accumulate, enter the cytoplasm and are translated efficiently as capped mRNAs.

The last virus that I will describe in this introduction is the herpes simplex virus (HSV). Much like L-A and influenza viruses, HSV induces inhibition of protein synthesis by affecting the integrity of cellular mRNAs. Infection with HSV results in a rapid decline in the levels of host mRNAs. This effect has been accredited to a herpes virion protein, called the virion host shutoff protein or vhs (discussed in more detail below), and the mechanism by which mRNA degradation is induced will be the prime focus of this thesis.

1.3 HSV- A General Description

HSV-1 and HSV-2 are two of the eight human herpes viruses in a group that includes Epstein-Barr virus (EBV), human cytomegalovirus (HCMV) and varicella-zoster virus (VZV) (reviewed in Roizman, 1990). *Herpes,* which means "to creep or crawl" describes the spreading nature of the lesions caused by lytic infection with this virus. Primary sites of infection are usually mucosal epithelial cells (especially the mouth, lips and labia), where the virus replicates and then travels to local ganglia via sensory neurons. HSV can then become latent in infected neurons, where it can remain as a circular DNA molecule for the lifetime of the individual. Recurring infections occur through reactivation of the virus as it tracks back along the sensory neurons to the epithelium. Although quite rare, disseminated HSV infections can cause encephalitis.

HSV is a member of the alphaherpesvirinae sub-family (reviewed in Roizman, 1990). General structural features of the virus include: an electron opaque core; an icosadeltahedral capsid, surrounded by an "amorphous" tegument structure; and an outer envelope possessing glycoprotein spikes. The HSV core contains a linear double-stranded DNA molecule that is approximately 150 Kbp in length . The genome is composed of two linked segments, L (long) and S (short), each containing a unique region (U_L, U_S) flanked at either end by a stretch of inverted repeats, designated as *b* and *c* respectively. The *a* sequence located at the ends of the genome, and between the L and S regions, accounts for the terminal redundancy. The *a* sequence contains the packaging signals for DNA during viral replication. HSV has two serotypes, namely type 1

and type 2, which possess relatively similar sequences, but differ in restriction endonuclease sites and sizes of viral proteins.

The DNA core is surrounded by the capsid, which is a proteinaceous structure made of pentameric capsomeres. There are several forms of capsids that differ in their protein content and whether they contain DNA (Gibson and Roizman, 1972). Surrounding the capsid is the tegument. The tegument has been described as an amorphous region that possesses no structure however, recent studies have shown that the tegument retains its structural integrity in the absence of the capsid or envelope (McLauchlan and Rixon, 1992), suggesting that its formation does not depend on the other virion structures. Included in this region is the immediate early gene transactivator VP16 (α-TIF, Vmw65, ICP25), which is believed to form complexes with the structural protein VP22 (Elliott et al., 1995) and vhs (Smibert et al., 1994) within the tegument and glycoprotein B (gB) on the inside of the envelope (Zhu and Courtney, 1994).

The envelope of HSV is comprised of patches of altered cellular membrane derived from the host cell, with characteristic "spikes" protruding (Morgan et al., 1959). The spikes represent the HSV glycoproteins, of which there are eleven. These proteins play key roles in infection of cells by HSV. Glycoproteins mediate attachment of the virion to the cell membrane through a process that is not well understood. Next, the envelope fuses with and penetrates the cell membrane by a process that involves glycoproteins gB, gD and gH. This results in the release of the tegument and capsid into the

cytoplasm. The capsid is transported to the nuclear pores where docking and subsequent injection of the viral genome into the nucleus occurs. The fate of the tegument is not understood however, it is believed that the vhs-VP16 complex dissociates allowing each protein to perform its function (discussed below). After viral DNA replication takes place (by rolling circle synthesis), packaging of the DNA into empty capsids occurs which requires the *a* sequences. Two models exist describing the envelopment of capsids and virion egress of herpesviruses. One model proposed by Johnson and Spear (1983), suggests that encapsidated DNA obtains its envelope and immature glycoproteins by budding into the perinuclear space. Glycosylation and maturation of the glycoproteins then take place as the enveloped virions travel through the endoplasmic reticulum (ER) and Golgi apparatus. If Golgi-derived vesicles are prevented from transporting to the cell membrane, large vacuoles accumulate in the cytoplasm that contain enveloped capsids (Johnson and Spear, 1982). On the contrary, Jones and Grose (1988) proposed that VZV capsids do not acquire an envelope from the nuclear membrane. Instead they pass through the nuclear membrane into the cytoplasm as naked capsids and bud into transport vesicles that contain mature glycoproteins, thus obtaining their envelope. Analysis of the phospholipid composition of extracellular virions, demonstrated that the viral envelope resembled more that of Golgi rather than nuclear membranes(van Genderen et al., 1994), providing support for the latter theory. The mechanism and time of tegument formation is unknown.

1.4 HSV Gene Regulation

HSV gene expression occurs in a temporally regulated cascade. Viral genes are expressed in three tiers that are designated immediate early (IE) or α, early (β) and late (γ) (rewieved by Roizman and Sears, 1990). The IE genes were first characterized as HSV genes that could be transcribed soon after infection, in the absence of de novo protein synthesis . There are five IE genes that give rise to infected cell proteins (ICP) 0, 4, 22, 27 and 47. All but ICP47 have been shown to possess regulatory functions (see below). Following the production of IE proteins, is the expression of the early (E, β). E gene expression requires prior IE protein synthesis, demonstrating IE regulatory properties. The majority of E gene products are involved in viral DNA synthesis, such as ribonucleotide reductase (ICP6), thymidine kinase (TK) and HSV DNA polymerase. The final stage in the cascade involves the production of the late (L, γ) genes, whose expression is dependent on IE proteins and somewhat on DNA synthesis. Late proteins are, for the most part, involved in virion production and include the major capsid protein, glycoproteins, VP16 and vhs. The tight regulation of HSV genes and the viral trans-activating factors that are involved has made herpes a good model of eukaryotic gene expression.

As in most eukaryotes, the regulation of viral genes is greatly influenced by the presence of cis-acting sequences that are upstream of the coding region. In addition to the basal TATA box promoter, many genes possess specific sites that cause enhanced transcription. All IE gene promoters contain one or more copies of the sequence TAATGARAT (where R is a purine) (Mackem and Roizman, 1982). Presence of this sequence allows binding of the virion protein

VP16 (Batterson and Roizman, 1983), which acts, in combination with cellular proteins (Kristie and Roizman, 1986b; McKnight et al., 1987; Xiao and Capone, 1990), to activate the specific transcription of the IE genes. Transfer of the IE upstream region to that of the TK gene results in TK being regulated as an IE gene (Post et al., 1981), further demonstrating that specific sequence elements are responsible for IE expression.

VP16 is a 65kDa phosphoprotein that forms part of the incoming HSV tegument (Heine et al., 1974), and thus is active in the absence of protein synthesis. The transactivation properties of VP16 were first noted as a structural component of the virus capable of inducing the expression of chimeric genes with IE gene promoters, in the presence of cycloheximide (Post et al., 1981). It was identified from an HSV DNA fragment that contained an open reading frame that was capable of transactivating cotransfected IE promoters (Campbell et al., 1984). Sequence analysis of the protein revealed that VP16 possesses an extremely acidic carboxy terminus (Pellett et al., 1985) that was required for transactivation (Triezenberg et al., 1988). Key evidence indicating the transactivating capability of this region came from studies using a chimeric protein consisting of the GAL4 DNA binding domain and the VP16 C terminal domain (Sadowski et al., 1988). This chimera was capable of inducing high level expression of a reporter gene. However, VP16 is unable to directly bind to DNA possessing the TAATGARAT sequence (McKnight et al., 1987; Triezenberg et al., 1988). Instead it interacts indirectly through a ubiquitous cellular protein known as Oct-1 that has a strong affinity for an ATGCTAAT (octamer binding

site) sequence that overlaps the TAATGARAT (O'Hare et al., 1988). This interaction occurs through the POU domain of Oct-1 and amino acids 378 to 389 of VP16 (Stern and Herr, 1991). Alteration of the octamer binding site without disrupting the TAATGARAT sequence results in loss of Oct-1 and VP16 binding, further demonstrating that VP16 does not bind directly (Spector et al., 1990). Another protein, called HCF or host cell factor, is able to bind VP16 in the absence of DNA (Kristie and Sharp, 1990; Xiao and Capone, 1990). HCF is required for VP16/ Oct-1 mediated binding to the TAATGARAT sequence (Kristie and Sharp, 1990; Kristie and Sharp, 1993) and together these proteins function as a promoter recognition complex.

In addition to its role as a transactivator, VP16 is important in virion assembly. Approximately 500 to 1000 copies of VP16 are present in HSV virions (Heine et al., 1974). Deletion of the VP16 open reading frame results in non-viable mutants, which can be rescued by propagation on a cell line that expresses wild-type VP16 (Weinheimer et al., 1992). The viral progeny, which contain cell-derived VP16, are capable of infecting cells lacking VP16, and can induce IE gene expression and promote normal levels of capsid production (Weinheimer et al., 1992). However, at late times when VP16 expression usually occurs, viral protein synthesis ceases, preventing the formation of infectious virions (Lam et al., 1996). VP16 mutants that lack the transactivation domain, generate lower levels of IE mRNAs but are viable, suggesting that VP16 is an essential component for virion assembly (Lam et al., 1996).

The gene products of most of the IE genes possess trans-regulatory functions. The most well studied and understood of the IE genes is ICP4 (Roizman, 1990). ICP4 is approximately 175 kDa in size (Honess and Roizman, 1974) and has been designated as the major transactivator of HSV genes. It is required for activation and transcription of E and L genes and the downregulation of IE genes (Ackermann et al., 1984; DeLuca et al., 1985; Pereira et al., 1977; Watson and Clements, 1980). ICP4's activating ability was first shown using temperature sensitive (ts) mutants that overproduced IE genes yet failed to express most E and L genes at the non-permissive temperature (Courtney et al., 1976; Preston, 1979; Watson and Clements, 1978). ICP4 is a DNA binding protein that seems to act in the form of a homodimer, which recognizes a ATCGTCNNNN(T/C)cg-(A/G)C consensus sequence (Faber and Wilcox, 1986; Kristie and Roizman, 1986; Muller, 1987). Subsequent studies have revealed that ICP4 is capable of binding to numerous other sites that differ from the consensus (Kristie and Roizman, 1986; Kristie and Roizman, 1986; Michael et al., 1988).

As stated above, ICP4 acts to trans-repress the expression of IE genes. Repression is mediated by the presence of the ICP4 consensus sequence. This sequence is present, primarily, in the 5' upstream regions of both ICP4 and ICP0 genes. The ICP4 noncoding domain contains three ICP4 binding sites (Kristie and Roizman, 1986; Michael et al., 1988; Muller, 1987). Binding of ICP4 to the IE genes causes downregulation of their expression (Kristie and Roizman, 1986; Mackem and Roizman, 1982). Autoregulation of ICP4 is mediated by an ICP4

binding site that spans across the transcription initiation site of its own gene, thus preventing expression when bound (Muller, 1987). ICP4 binding sites that are inserted either upstream or downstream of TATA boxes, result in decreased gene expression from those promoters; an effect that is alleviated following viral DNA synthesis (Koop et al., 1993). This may be due to an alteration in the phosphorylation state of ICP4. Papavassiliou et al. (1991) demonstrated that only phosphorylated forms of ICP4 bind to E and L gene promoters and induce expression. Perhaps unphosphorylated ICP4 binds only IE gene promoters and after DNA synthesis ICP4 is modified to a transactivating state.

A few studies have provided some evidence as to the mechanism of ICP4-induced transactivation. Smith et al. (1993) demonstrated that ICP4 can interact with two transcription factors, namely the TATA binding protein (TBP) and TFIIB. These factors form a part of the basal transcription machinery that initiates at TATA box promoters. Binding of ICP4 to the factors produced a tripartite protein complex that showed increased affinity for their respective DNA binding sites (Smith et al., 1993). This suggests that ICP4 may increase transcription initiation, thus affecting expression. ICP4 has been shown to possess DNA bending ability when bound to its recognition site (Everett et al., 1992); a feature associated with other transcription factors. Further studies of ICP4 may elucidate more information about its regulatory mechanism and provide a good model of eukaryotic transcription regulation.

ICP0 is another IE protein that possesses regulatory activities (reviewed in Everett et al., 1991). It is a 110 kDa nuclear phosphoprotein (Honess and Roizman, 1974) that is capable of enhancing the expression of all three classes of viral genes (Cai and Schaffer, 1992; Everett, 1986; Mavromara-Nazos et al., 1986; O'Hare and Hayward, 1985). ICP0 is also capable of transactivating the expression of a number of heterologous promoters, such as SV40 early promoter and the HIV LTR (Everett, 1988; Mosca et al., 1987) and thus, ICP0 has been termed a promiscuous transactivator. The method of transactivation is unknown however, its activity increases in the presence of ICP4 (Everett, 1984; O'Hare and Hayward, 1985), with which it has been shown to interact (Yao and Schaffer, 1994). Both ICP0 and ICP4 have been identified in the HSV tegument (Yao and Courtney, 1989; Yao and Courtney, 1992) and may initiate trans-activation upon infection. ICP0 is not essential for viral replication but is required for full viral gene expression during low multiplicity infections (Sacks and Schaffer, 1987). It is also required for efficient expression of ICP4 in the absence of VP16 (Cai and Schaffer, 1992), a property which is over-shadowed during normal infection.

The remaining two IE gene products that have regulatory functions are ICP22 and ICP27. ICP22 is not essential for viral production in Vero cells however, in certain cell lines ICP22 deletion mutants show low levels of late gene expression and reduced viral growth (Sears et al., 1985). Thus, it has been proposed that a cellular factor may be able to complement the activity of ICP22

(Sears et al., 1985). More recently ICP22 has been linked to the modification of host RNA polymerase II (Rice et al., 1995).

Deletion mutants of ICP27 show increased levels of IE proteins and a dramatic reduction in levels of viral DNA and L gene products (McCarthy et al., 1989) and thus is essential for viral growth. ICP27 appears to regulate the transition between E and L gene synthesis which seems to be modulated by an acidic region in the amino-terminal region of the protein (Rice et al., 1993). ICP27 also acts post-transcriptionally by affecting the processing of mRNA (Sandri-Goldin and Mendoza, 1992). This ability of ICP27 partially accounts for the late phase of host-shutoff. ICP27 preferentially selects for non-intronic mRNAs that possess certain poly- adenylation sequences, in a promoter-independent manner (Sandri-Goldin and Mendoza, 1992). This seems to be modulated by ICP27s ability to bind to the 3' ends of mRNAs (Brown et al., 1995). Since most mammalian genes contain introns and E and L HSV genes do not, ICP27 has a negative regulatory effect on host mRNA processing.

To date, no regulatory function has been accredited to ICP47. Instead, this protein has been shown to play a key role in HSV evasion of the host immune system during infection (York et al., 1994). This is achieved through blocking the presentation of viral peptides by MHC class I molecules. The gene product of ICP47 has been shown to bind to a transporter protein (TAP) that shuttles peptide fragments into the endoplasmic reticulum (ER) (Hill et al., 1995). Prevention of transport of peptide fragments results in the retention of MHC

class I molecules in the ER, and thus inhibition of antigen presentation to cytotoxic T cells (Hill et al., 1995).

1.5 Inhibition of Host Protein Synthesis by HSV

As described above, many viral infections result in a decrease in the metabolism of cellular macromolecules. In each case, the mechanism of host shutoff provides an advantage for a more productive infection, at the expense of the cell. Cells infected with HSV exhibit a profound inhibition of host macromolecular synthesis that initiates at the onset of infection (reviewed in Fenwick, 1984). Early studies demonstrated that the amount of tritiated uridine incorporated into host RNA, declined rapidly following infection with HSV (Roizman et al., 1965). Reduction of RNA synthesis was accompanied by an immediate decrease in the production of protein. The shutoff effect seemed to occur in two distinct cycles; the first between zero and three hours and the second from six hours postinfection. It was then postulated that the initial phase was a result of a viral factor present early in infection, whereas the later phase followed viral DNA synthesis (Roizman et al., 1965).

It was later observed that infection with HSV resulted in a rapid decrease in the stability of mRNAs associated with a disaggregation of cellular poly-ribosomes (Fenwick and Walker, 1978; Nishioka and Silverstein, 1977; Nishioka and Silverstein, 1978; Schek and Bachenheimer, 1985). Nishioka and Silverstein (1977) demonstrated that at four hours after infection of murine erythroid leukemia (MEL) cells with HSV, only 15% of globin mRNA sequences

remained. The ratio of globin mRNA to the total amount of polyA+ RNA remained constant, indicating that all cellular mRNAs were subject to degradation by HSV. Polyribosomes were reduced to monosomes by two hours post-infection however, their dissociation was not the cause of mRNA degradation, since mRNAs remained stable in uninfected cells treated with NaF (which dissociates polyribosomes) (Nishioka and Silverstein, 1978). From these initial experiments, it was suggested that mRNA degradation required expression of the HSV genome, for the following reasons: UV irradiated virus was incapable of host shutoff and cells infected in the presence of cycloheximide showed an increase in the globin mRNA levels up to six hours post-infection. Furthermore, infection of cycloheximide treated cells showed that polyribosomes dissociated while mRNA remained intact (Nishioka and Silverstein, 1978). It was concluded, therefore, that a virion component was responsible for polyribosome disaggregation but degradation of globin mRNA in MEL cells required expression of HSV genes. In contrast to these findings, Smibert et al. (Smibert and Smiley, 1990) demonstrated that loss of globin mRNA in MEL cells occurred in the presence of actinomycin D. Since no viral transcription can take place under this condition, the shutoff inducing factor must be present prior to viral gene expression.

There is much evidence suggesting that the initial phase of host shutoff is caused by a component of the infecting virion. Degradation of mRNAs, polyribosome dissociation and inhibition of translation have been shown to occur in the absence of *de novo* transcription and protein synthesis in infected Vero

cells (Fenwick and McMenamin, 1984; Fenwick and Walker, 1978; Schek and Bachenheimer, 1985). Furthermore, Vero cells that have been enucleated or infected with UV irradiated HSV-2 show shutoff comparable to that of wild type infection (Fenwick and Walker, 1978). Thus, no viral expression is required indicating the involvement of a virion factor. Infection of cells with purified L particles (contain no DNA or capsid) demonstrated that host shutoff activity was equal to that of purified virions containing DNA (McLauchlan et al., 1992) providing further evidence that a component of the virion (tegument or envelope) is responsible for degradation of mRNAs and inhibition of protein synthesis.

The isolation of mutant HSV strains that are incapable of host shutoff has proven to be useful in identifying and characterizing the virion factor that is responsible. Read and Frenkel (1983) isolated six mutants that were defective in inhibiting protein synthesis in the presence of actinomycin D; these mutants were designated as *vhs*. These vhs mutants underwent proper viral entry and all of them were viable (Read and Frenkel, 1983) indicating that vhs induced shutoff was not essential for growth of the virus. Viral yields however, were reduced two- to threefold compared to wild type HSV infection. Cells infected with the vhs mutants without addition of actinomycin D showed a general shutoff later during infection that required the synthesis of E or L proteins, which is in agreement with earlier findings that a second phase of shutoff occurs (Nishioka and Silverstein, 1978; Roizman et al., 1965). Of the mutants vhs1 has been most characterized. Using marker rescue, vhs1 was mapped to a region of the HSV genome that contains the UL41 ORF that encodes a protein with a

predicted molecular weight of 55 kDa (Kwong et al., 1988). Direct inactivation of the UL41 ORF by insertion of a beta-galactosidase expression cassette also results in viruses that are deficient in the ability to degrade cellular mRNAs and inhibit protein synthesis (Fenwick and Everett, 1990; Smibert and Smiley, 1990) providing clear evidence that UL41 is involved in shutoff. The protein encoded by UL41 was identified by Smibert et al. (Smibert et al., 1992). Using antiserum raised against a synthetic peptide derived from UL41 sequences, they identified a 58 kDa phosphoprotein that associates with HSV virions.

In addition to destabilizing host mRNAs, vhs also induces the degradation of HSV viral mRNAs (Kwong and Frenkel, 1987; Oroskar and Read, 1989; Read and Frenkel, 1983; Strom and Frenkel, 1987). Cells infected with vhs1 overproduced IE proteins and there was an increase in the functional stability of IE mRNAs . Superinfection of these cells with wild-type HSV restored host shutoff and caused a pronounced decrease in the level of IE polypeptides (Read and Frenkel, 1983). The half-life of E and some L mRNAs was also prolonged during infection with vhs1 (Kwong and Frenkel, 1987), suggesting that a functional UL41 ORF results in destabilization of viral mRNAs. Comparison of the kinetics of mRNA degradation revealed that vhs has little, if any, selectivity of target mRNAs (Oroskar and Read, 1989).

Why would vhs act to decrease the level of HSV mRNAs and proteins during infection? The answer to this question lies in the role vhs plays in viral regulation. As stated earlier, HSV genes are expressed in a temporally

regulated cascade. Optimal gene expression requires two factors: transcriptional activation and repression by proteins such as VP16, ICP4 and ICP27; and a rapid transition between gene subsets. It is believed that vhs is responsible for such a transition (Kwong and Frenkel, 1987). Functional vhs induces rapid degradation of viral mRNAs in an effort to rid the translation system of IE transcripts (for example) following their transcriptional repression, allowing efficient synthesis of E proteins. Although vhs is not essential for viral growth, in its absence HSV DNA replication is delayed and viral yields are lower (Fenwick and Everett, 1990) illustrating the importance of rapid transition.

Some L proteins involved in virion structure continue to accumulate at late times during infection. In fact, gC mRNA levels in vhs1 infected cells, lagged behind those in wild-type infected cells (Oroskar and Read, 1987). It has been proposed that the activity of vhs is downregulated at late times during infection through the action of another HSV protein (Fenwick and Everett, 1990; Fenwick and Everett, 1990; Fenwick and Owen, 1988; Smibert et al., 1994). Cells infected with HSV-1 strain F that were superinfected at three hours post infection with HSV-2 strain G, were resistant to further shutoff (Fenwick and Owen, 1988). Fenwick and co-workers suggested that an E or L protein conferred such resistance. More recent evidence comes from studies using a VP16 null mutant (8MA) constructed by Weinheimer et al. (1992). Cells infected with 8MA virus (first grown on complementing cell line) suffer a severe decline in protein synthesis by eleven hours (Lam et al., 1996), which resembles host shutoff. The discovery that vhs and VP16 form a complex in infected cells (Smibert et al.,

1994) raised the possibility that VP16 somehow modulates vhs activity. This theory is supported by the fact that the 8MA phenotype can be rescued by an 8MA recombinant possessing a truncated form of VP16 that is transcriptionally inactive yet retains the domain required for binding to vhs (Lam et al., 1996). Furthermore, deleting a region of the UL41 ORF from 8MA thus generating a VP16/ vhs double mutant, allows viral protein synthesis to occur at a similar rate to wild type (Lam et al., 1996). Finally, cells that constitutively express VP16 are resistant to virion-induced shutoff, which can only be overcome at high MOIs (Lam et al., 1996). It is not known how VP16 dampens vhs activity however, it may be possible that binding of VP16 to vhs masks a functional domain required for shutoff or VP16 causes relocalization of vhs to the nucleus, for assembly into virions.

As of yet, there is little information about the mechanism by which vhs induces degradation of mRNAs and inhibition of protein synthesis. Several possibilities exist (Strom and Frenkel, 1987): 1) vhs could encode an RNase that enters the cell as part of the virion; 2) vhs could activate a pre-existing cellular RNase or degradation pathway; or 3) vhs could act to disable a cellular factor that is responsible for protecting mRNAs from degradation. Two pieces of evidence suggest the first possibility to be unlikely. First, disaggregation of polyribosomes caused by in vitro treatment with RNase results in monosomes that are more stable than those isolated from infected cells (Fenwick and Walker, 1978). Second, using infected cell lysates as an in vitro assay, mRNA degradation was not inhibited by addition of placental RNase inhibitor (Krikorian

and Read, 1991). However, several studies have shown that vhs is the only viral protein required for degradation of mRNA. Jones et al. (1995) used cotransfection assays in which wild-type and mutant vhs constructs were assayed for their ability to inhibit protein synthesis from a reporter gene. In this case, whenever an active form of vhs was present, expression of a *lacZ* reporter gene was suppressed. Furthermore studies using in vitro translated vhs in rabbit reticulocyte lysate assays demonstrate that the presence of functional vhs causes destruction of reporter mRNAs added and thus inhibition of their translation (Elgadi et al., in preparation; Zelus et al., 1996). Thus the shutoff ability seen with HSV is mediated, at least in part, by the vhs protein which is capable of degrading mRNAs and inhibiting protein synthesis in the absence of other viral proteins.

1.6 Experimental Rationale

At the onset of this project there was very little known about the mechanism of degradation of mRNAs induced by vhs. Although exhaustive data was available describing the decrease in mRNA stability during HSV infection, the pathway of mRNA destruction was unexplained at this time. To investigate such a pathway, I initiated my project by tracking the destruction of beta globin mRNA during infection of MEL cells with HSV1 and HSV2. The mRNA was analyzed, at various time points during infection, by Northern hybridization with a radio-labeled DNA probe complementary to beta globin mRNA. I demonstrated that, as expected, infection with HSV resulted in a decrease in the level of globin mRNA which was more pronounced during infection with HSV-2 333.

Degradation intermediates arising from globin mRNA were never detected suggesting that degradation occurs very rapidly with the rate limiting step being the targeting of the mRNA for destruction.

The development of an in vitro assay for vhs activity by Mabrouk Elgadi allowed for continued investigation of the vhs induced mRNA degradation pathway. Using this assay I generated numerous radiolabeled mRNAs that served as substrates for in vitro translated vhs. The fate of these substrates was tracked and analyzed in various ways. Results from these experiments indicate that vhs initiated decay through endonucleolytic cleavage of the reporter mRNAs within a region close to the 5' end of the mRNA. The proximity of cleavage to the 5' end raised the possibility that the cap structure of mRNAs may serve as a target for vhs induced cleavage. I, therefore, tested whether the presence of a cap influenced the rate of degradation of the reporter RNAs. The presence of endonucleolytic cleavage products detected early from capped mRNAs were profoundly delayed when uncapped RNAs were used as substrates. Thus vhs induces an endonucleolytic pathway that preferentially causes the destruction of capped mRNAs.

II. MATERIALS AND METHODS

2.1 Mammalian Tissue Culture

Murine erythroleukemia cells (MEL, line 745aJ56, provided by A Bernstein, Mount Sinai Research Institute, Toronto, ON Canada) were used throughout the *in vivo* section of this study. Cells were grown in suspension culture in 1L Corning roller bottles in a 37°C, in a humidified incubator containing 5% CO_2. Cells were grown in complete alpha minimal essential medium (α-MEM) supplemented with 10% fetal calf serum (FCS, Gibco), 2mM L-glutamine, 100U/mL penicillin G (Gibco) and 100U/mL streptomycin sulfate (Gibco). This is now designated complete 10% α-MEM (percent indicates amount of FCS).

For infection with HSV, MEL cells were diluted to a concentration of $2x10^4$ cells/mL in 10% α-MEM containing 5mM hexamethylene bisacetamide (HMBA) and allowed to grow to a density of $2x10^5$ cells/mL. The addition of HMBA induced terminal erythroid differentiation. Three and one half days post induction, MEL cells were pelleted in an IEC tabletop centrifuge at 2500rpm at 4°C for 10 min. Cells were resuspended in complete 0% α-MEM and recentrifuged. The pelleted cells were then resuspended to final concentration of $2x10^7$ cells/mL in complete 0% α-MEM containing 10 PFU/cell of the appropriate virus.

African Green Monkey Kidney (Vero) cells were used for the propagation of viral stocks. Vero cells were grown in 150cm^2 Corning tissue culture flasks as monolayers in complete 10% α-MEM.

2.2 Growth and Titration of Viral Stocks

Viruses used in the *in vivo* section of this study were HSV2 333 (wild type strain, type 2), HSV2 vhsB333 (mutated form of vhs containing lacZ cassette), HSV1 PAAr5 (Hall et al., 1984) and HSV1 vhsA (containing lacZ cassette). All viruses were propagated on confluent monolayers of Vero cells grown in 150cm^2 Corning culture dishes. Vero cells were washed with phosphate buffered saline (PBS) and infected with 10^6 PFU/ flask in 5mL of complete 0% α-MEM. Two hours post-infection the cells were overlaid with 15 mL of complete α-MEM supplemented with 2% FCS. The infection was allowed to progress in a humidified incubator containing 5% CO_2 until approximately 3/4 of the cells had exhibited cytopathic effect (CPE). At this point, cells were scraped from the plates, transferred to a 15mL Corning centrifuge tube and frozen in liquid N_2. The cells were then thawed in a 37°C water bath and refrozen a total of 5 times, in order to lyse the cells. Cell debris was pelleted in an IEC tabletop centrifuge at 3000 rpm for 10 min at 4°C. The supernatant containing virus was then aliquoted into Nunc cryovials and stored at -70°C.

Viral stocks were titrated on Vero monolayers grown on Corning 35mm 6-well pates. Serial dilutions of the viral stocks, from 10^{-2} to 10^{-7}, were made in complete 0% α-MEM and 200μL was used to infect the Vero monolayers. Two

hours post-infection the cells were overlaid with 2mL of complete α-MEM supplemented with 2% FCS and 0,05% human immune serum (Connaught Laboratories). After visible plaques had appeared, the monolayer was stained with 1% crystal violet in 61% ethanol, 8.7% formalin and 4.3% acetic acid. Plaques were then counted using a light microscope and the titre was calculated from the appropriate dilution.

2.3 RNA Extraction from MEL cells

In experiments involving infection of MEL cells, total RNA was extracted from cells using guanidium isothiocyanate and phenol (Trizol, Gibco). In this procedure, cells were pelleted in 1.5 mL microcentrifuge tubes (Diamed), resuspended in complete 0% α-MEM and repelleted. The cells were then resuspended in 1mL of Trizol and lysed by repeat pipetting. 200µL of chloroform was then added, the lysate was vortexed, placed on ice for 5 min and centrifuged in a microcentrifuge (Eppendorf) for 15 min. at 15000g. The aqueous phase was extracted with 600µL of choroform, transferred to a fresh tube at which point 10µg of tRNA was added and the RNA was precipitated with isopropanol, centrifuged and dried. The RNA preparationwas then made 0.3M with Na-Acetate and the RNA reprecipitated in ethanol, centrifuged and dried.

2.4 Formaldehyde-Agarose gel electrophoresis

Dried RNA samples were dissolved in MOPS buffer (pH 7, 20mM 3-n-morpholinopropanesulfonic acid, 5mM Na-Acetate, 0.5mM EDTA), 50% deionized formamide and 6% formaldehyde. The samples were then heated to

70°C for 10 minutes at which point RNA loading dye (50% glycerol, 10mg/mL xylene cyanol, 15mg/mL bromophenyl blue, 5mM EDTA (pH 7.5)) was added just prior to loading on a formaldehyde agarose gel. A formaldehyde-agarose gel was then prepared by dissolving Seakem agarose (S&S) to 1% in MOPS buffer and 6% formaldehyde. The heated RNA samples were loaded onto the gel which was subjected to electrophoresis at 110V for 2-3 hrs, in 6% formaldehyde, MOPS running buffer.

2.5 Northern blotting and hybridization

RNA samples were separated by electrophoresis on a formaldehyde-agarose gel were treated in the following manner. The gel was soaked in 500mL of water then 500mL of 50mM NaOH, 10mM NaCl and finally in 500mL of 100mM Tris-HCl (pH7.5), each for 10 minutes. The RNA was then transferred with 20X SSC (3M NaCl, 0.3M Na-Citrate, pH 6.35) to Nytran membrane (S&S) using a PosiBlot pressure blotter (Stratagene) for 3-4 hrs. The RNA was fixed to the membrane with a UV crosslinker (Statagene). The resulting membrane was then prehybridized for 30 minutes, in Church buffer (250mM Na-Phosphate (pH7.2), 7% SDS, 1% BSA, 1mM EDTA)(Church and Gilbert, 1984) . At this point the denatured DNA random labeled probe was then added and hybridization occurs in a 65°C oven (Techne) overnight. The membrane was then washed with 500mL of 0.3M NaCl, 30mM Na-Citrate and 0.1% SDS at 60°C for 10 minutes and exposed to X-ray film (Dupont-NEN).

2.6 Random primer labeling of plasmid DNA

Plasmid DNA was labeled using random hexamer primers (Pharmacia) in the following reaction. Approximately 200ng of plasmid DNA was combined with 5mM random DNA primers in a 38µL volume and boiled for five minutes. The reaction was then immediately placed on ice for five minutes. The reaction was then made 50mM dATP, 50mM dGTP, 50mM dTTP, 800µCi/mL a ^{32}P-dCTP, and 0.3U/µL Klenow polymerase (NEB) in a final volume of 60µL and incubated at room temperature from one to 24 hrs. 100µL of water was then added and the unincorporated nucleotides are removed over a 0.8mL column bed of a G-50 Sephadex (Pharmacia) spin column. The labeled plasmid DNA was then denatured at 95°C prior to use as a probe.

2.7 *In vitro* transcription of RNA

RNAs to be used in the *in vitro* degradation assay were generated from various plasmid constructs (see section 2.12) obtained from Dr. David Andrews. The reporter RNAs were produced from run-off transcripts after the plasmids were linearized with various restriction endonucleases, as described in the Promega protocol manual. Approximately 5µg of linearized plasmid DNA was incubated in a 50µL reaction containing 1X transcription optimized buffer (Promega, 40 mM Tris-HCl pH 7.5, 6mM $MgCl_2$, 2mM spermidine, 10mM NaCl), 10mM DTT, 1U/µL RNasin (Promega), 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.5 mM GTP and 40U SP6 RNA polymerase (Promega). The reaction was placed at 30 °C for 60 -120 minutes. In order to remove the template DNA, 5U of RQ1 DNase (Promega) was added and incubated at 37 °C for 15 minutes.

Following phenol: chloroform: isoamyl alcohol and chloroform extraction, the mRNA was made 2.5 mM ammonium acetate and precipitated with 95% ethanol. After centifugation, the RNA pellet was washed with 70% ethanol, dried and resuspended in 20 µL of RNase free water.

The above reaction generates RNA transcripts bearing a triphosphate GTP end. To generate *in vitro* capped transcripts the reaction was modified by adding 0.5 mM of the cap primer, m7GpppG and reducing the GTP to 50 µM.

2.7a Internal labeling of RNA

RNA was internally labeled by including 1µCi of α^{32}P-GTP in the above transcription reaction.

2.7b Cap labeling of RNA

RNAs used for cap labeling were *in vitro* transcribed as in section 2.7, however, the cap primer m7GpppG was omitted and replaced with 500µM GTP, thus producing uncapped transcripts. Cap labeling was achieved in a 30µL reaction containing 1µg uncapped RNA, 50mM Tris-HCl (pH7.9), 1.25 mM $MgCl_2$, 6mM KCl, 2.5mM DTT, 0.1mg/mL RNase-free acetylated BSA, 1U/ul RNasin, 0.1mM S-adenosyl-L-methionine, 30µCi α^{32}P-GTP and 1-3 units of vaccinia virus guanylyltransferase (Gibco-BRL). The reaction was incubated at 37°C for 45 minutes and cap labeled RNA was recovered by phenol: chloroform: isoamyl alcohol extraction followed by chloroform:isoamyl alcohol extraction. The RNA was made 2.5M ammonium acetate and precipitated with ethanol.

2.7c 5' end labeling of RNA

This was achieved by essentially the same reaction as in section 2.7. Linearized plasmid was incubated as above except the 500μM GTP was replaced with 50μCi of γ^{32}P-GTP and incubated at 30°C for 10 minutes. At this point the reaction was made 500μM with GTP and allowed to continue for 30 minutes at 30°C.

2.7d 3' end labeling of RNA

The three prime end labeling of 19X capped RNA was performed as follows. Approximately 15pmol of capped 19X RNA was combined with 10pmol of oligo AB5688 (CCCCCTGAACATTCTGGAAATCAAA) in an 18μL transcription reaction (same as in 2.7 however, replacing the 10 mM GTP with 50μCi α^{32}P-GTP). This oligo annealed to the penultimate 16 bases of 19X RNA (389-404) leaving a 5' DNA overhang which was then used as a template for continued transcription of 19X RNA using SP6 phage RNA polymerase. The oligo and RNA were annealed at 37°C for 30 minutes at which point 40U (2μL) of SP6 RNA polymerase were added and transcription was allowed to continue for 45 minutes. 20μL of sequencing loading dye was added (see section 2.11) and the labeled 19X RNA (now 413 bases) was run on an 8% sequencing gel and eluted from a gel slice as in section 2.14. The eluted RNA was combined with 4μL of 2% linear polyacrylamide and precipitated twice with ethanol. The purified RNA was then used as a substrate for the *in vitro* degradation assay (section 2.8)

2.8 *In vitro* degradation assay in rabbit reticulocyte lysate (RRL)

Approximately 200ng of *in vitro* transcribed capped vhs or vhs1 RNA was translated in a 50µL reaction containing 25µL reticulocyte lysate (Promega), 20nM amino acid mix, and 0.4U/µL RNasin (Promega). The reaction was incubated at 30°C for 20 minutes. At this point various RNA substrates were added and aliquots were taken at varied times. The samples were extracted with 200µL Trizol: 40µL chloroform followed by 130µL chloroform. The aqueous phase was then supplemented with 10µg carrier tRNA and precipitated in isopropanol . The dried RNA was than analyzed by various forms of gel electrophoresis. RNA samples destined to be used in primer extension reactions were reprecipitated in 0.3M Na-Acetate and ethanol.

2.9 RNase H cleavage of RNA

RNA samples (following Trizol extraction, see above) of 10µg, to be used in the oligo-directed RNaseH cleavage experiments, were dissolved in 10µL of 10mM Tris-HCl (pH7.9), 1mM EDTA, 250mM KCl containing 0.5µg DNA oligo (various oligos, complimentary to RNA). The reaction was placed at 60°C for 30 minutes to allow annealing of the oligo to the RNA. The reaction was then made 28mM $MgCl_2$ and 1 U of RNaseH (Promega) was added and incubated at 37°C for 30 minutes. Water was added to a volume of 200µL, and the RNA was extracted with phenol:chloroform:isoamyl alcohol and then precipitated with 0.3M Na-Acetate and ethanol. The resulting products were then analyzed by formaldehyde-agarose gel electrophoresis.

2.10 Thin layer chromatography using polyethylene imine cellulose

Rabbit reticulocyte samples aliquoted in section 2.8 were spotted directly onto polyethylene imine cellulose plates, along with nucleotide and cap analog standards, and allowed to dry. The chromatogram was then placed in a chamber containing 1M LiCl allowing migration of the solvent front. The solvent front was marked and the location of the standards was determined by fluorescence using a hand-held UV illuminator. The chromatogram was exposed to x-ray film and developed. The chromatogram was then aligned with the marked standards.

2.11 Polyacrylamide gel electrophoresis

Both 8% and 20% sequencing gels were used throughout this study and were prepared as follows. 42g of urea was dissolved in 1X TBE (89mM Tris, 89mM boric acid and 2.5mM EDTA) containing either 8% or 20% acrylamide (diluted from a 40% stock solution of 38:2 acrylamide: N,N'-methylenebisacrylamide). The gel solution was then made 0.04% APS (ammonium persulphate) and polymerization was initiated by the addition of 100µL of N,N,N',N -tetramethylethylenediamide (TEMED). Both 8% and 20% gels were run at constant power (100W) with voltages of ca. 2000V and 3000V respectively. Samples to be analyzed through the above gels were resuspended in sequencing loading dye (80% deionized formamide, 0.1% bromophenol blue and 0.1% xylene cyanol).

2.12 Plasmids used for *in vitro* transcription

The plasmic constructs used in the *in vitro* section of this study were obtained from David Andrews. All plasmids contained the consensus sequence of the SP6 phage RNA polymerase promoter. The backbone of the plasmids was derived from the pGEM cloning vector (Promega). In all cases (except #1, pSPSR10), the cloned cDNAs contained an artificial 5' leader sequence that was linked to the appropriate open reading frame at the AUG start codon through an Nco1site. Coding regions that did not already begin with an Nco1 site were modified to do so, making all the translational initiation sites identical. pSPSR10 was not modified and possessed the natural leader sequence of the canine signal recognition particle α (SRPα). The sequences of each of the seven different leader sequences used is shown in Table 3.2 and 3.3.

The plasmids used to transcribe vhs and vhs1 RNAs were pSPvhs and pSPvhs1, respectively. These plasmids were constructed by Mabrouk Elgadi by ligating a 1.8 kb fragment bearing the vhs (vhs1) ORF from pCMVvhs (pCMVvhs1) (Jones et al., 1995), between the Nco1 and EcoR1 sites of pSPUTK (Falcone and Andrews, 1991). UTK refers to a modified Xenopus beta globin 5' untranslated region which possesses an engineered consensus Kozak initiation signal (Falcone and Andrews, 1991). The plasmid used to generate 19 and 19X RNAs was pSPSR19N. It contains the complete ORF of the SRPα which was inserted into the Nco1 site of pSPUTK.

2.13 Large scale plasmid preparation using Qiagen

All plasmids received from David Andrews were transformed into E. coli DH5α and grown for large scale plasmid preparations. The bacteria were made competent for transformation using the following method. 100 mL of TB (1.2% bactotryptone, 2.4% yeast extract, 0.4% glycerol, and 88.7 mM potassium phosphate (pH7.4)) was inoculated with a 1 mL of an overnight culture of DH5α and incubated for 2.5 hours at 37C in a shaker. The bacteria were centrifuged at 1300g for 10 minutes at 4°C and the pellet was resuspended in 20mL of MOPS I buffer (50mM 3-N-morpholinopropanesulfonic acid; pH7.0, 10mM RbCl). The bacteria were again centrifuged and resuspended (on ice) in MOPS II buffer (100mM MOPS; pH 6.5, 70mM $CaCl_2$, 10mM RbCl). A final centrifugation of the bacteria was done and the pellet was resuspended in 2mL of MOPS II buffer. The cells are now "competent". 1µg of plasmid DNA was added to 100ul of competent cells, and placed on ice for 30 minutes. The cells were then heat shocked at 37°C for 45 seconds and placed on ice for two minutes. 1mL of LB (Luria-Bernoulli broth; 1% bactotryptone, 0.5% yeast extract, 0.5% NaCl, 10mM Tris-HCl (pH7.6), 0.4% glucose) was then added and the cells were agitated in a shaker at 37°C for 30 minutes. 5µL of the transformation mix was then spread onto LB agar-Amp plates (LB supplemented with 1.5% bactoagar and 50µg/mL ampicillin) and incubated overnight at 37°C.

Colonies arising from the transformation of plasmid DNA were grown in 5mL overnight cultures of TB from which 1mL was used to inoculate 250mL of TB. The large cultures were grown overnight and centrifuged the following day

and plasmid DNA was purified from bacteria using Qiagen columns according to the manufacturer's instructions. Purified plasmid DNA was then quantified using fluorometry.

2.14 5' end labeling of DNA oligos

Oligos used for primer extension studies were 5' labeled in a 50µL reaction containing 1ul of 1OD/mL oligo, kinase buffer (supplied by enzyme manufacturer), 50µCi γ^{32}P-dATP and 20U of polynucleotide kinase (NEB). The reaction was incubated at 37°C for one hour and then stopped using 200µL of 2.5M ammonium acetate. 20µg of carrier tRNA was then added followed by precipitation in 1mL of ethanol. The dried oligo was resuspended in 20µL sequencing dye (see section 2.11) and run on an 8% sequencing gel at 100W for one hour. The gel was then exposed to film and the bands corresponding to the labeled full length oligo (25 bases) was cut out of the gel and placed in 300µL of Elution buffer (500mM ammonium acetate, 10mM Mg-Acetate, 1mM EDTA and 0.1% SDS) overnight at 37°C. The gel slice was then washed with 200µL of Elution buffer and 10µg of carrier tRNA was added to the combined eluate and precipitated twice in ethanol and dried.

2.15 Primer extension

Dried RNA samples were resuspended in 10µL of 10mM Tris-HCl (pH 7.9), 1mM EDTA, 250 mM KCl, and 50,000 Cerenkov cpm of 5' end labeled (see section 2.14) primer (5' GGTGAAGAAGTCGACCATGGTAGAT 3'), complementary to residues 60-84 of the SRPα transcript generated by *in vitro*

transcription of pSP^{2}SR19N. The reaction was incubated at 62°C for one hour to allow for annealing. After cooling to room temperature, 25µL of 10mM $MgCl_2$, 20 mM Tris-HCl (pH8.7), 5mM DTT, 330µM of each dNTP, 10 µg/mL actinomycin D and 10U of SuperScript (Gibco-BRL) reverse transcriptase was added to the RNA:oligo reaction. Extension took place at 42°C for one hour at which point 300 µL of ethanol was added and the samples were precipitated. The resulting primer extension products were analyzed by electrophoresis through an 8% polyacrylamide sequencing gel run at 100W (~1800V) for one to two hours. The gel was then exposed to X-ray film for varied times.

2.16 Oligo blocking

The experiments which describe the use of DNA oligos to block vhs cleavage were performed as follows. Cap labelled RNA (see 2.7b) was incubated with 0.1 µg of various DNA oligos (see Table 3.4) in a 5µL volume of reticulocyte buffer (2mM EGTA, 0.25mM ATP, 0.1mM DTT, 2mM Mg acetate) and allowed to anneal at 60°C for 45 minutes. The annealed complex was then used in the *in vitro* degradation assay (sect 2.8). Reticulocyte buffer is equivalent to the salt composition of rabbit reticulocyte lysate.

III. RESULTS

As stated previously, the mechanism by which the virion host-shutoff (vhs) protein induces mRNA decay, is not known. The primary goal of this project was to determine the decay process of mRNAs, targetted by the vhs system. Two approaches were used in addressing this goal. The first section of this report describes *in vivo* experiments in which beta globin mRNA levels were followed in HSV-1 and HSV-2 infected tissue culture cells. The second approach involved the use of an *in vitro* assay for vhs activity developed by Mabrouk Elgadi. In both cases it was our hope that analyzing mRNA decay products, generated in the presence of vhs, would help elucidate the mechanism of action of vhs and perhaps provide information about some of the factors that control cellular mRNA half-lives and surveillance.

3.1 vhs-induced degradation of mRNA in tissue culture cells.

I initially set out to learn more about vhs induced mRNA degradation by attempting to detect decay intermediates of cellular mRNAs during infection with HSV. If intermediates are generated, then they may be analyzed to determine if the decay process has any apparent polarity, that is, is either end of the mRNA molecules selectively targeted for degradation before the other. I chose to track the degradation of a stable and abundant cellular mRNA as this would allow for easier detection. Using this rationale, I analyzed the decay of beta globin mRNA

in terminally differentiated murine erythroid leukemia cells (MELs, see Materials and Methods). MEL cells were infected at an MOI of 10 PFU/ cell with HSV2 strain 333 and the vhs null mutant strain vhsB333 or 10 PFU/ cell of HSV1 strain PAAr5 and the vhs mutant vhsA. After three and a half days in conditions inducing differentiation, the cells were infected and at various time points, aliquots were removed and cells were lysed in Trizol (see Materials and Methods). 10 ug of purified total RNA was loaded on a 1% agarose formaldehyde gel and subjected to electrophoresis. RNA was then transferred and fixed to Nytran membrane (S&S). In order to visualize the mRNA, a randomly labeled DNA plasmid (pM 2.6) containing a genomic copy of the mouse beta major globin gene, was used to probe for beta globin mRNA present on the membrane.

Cells infected with either wild type strain showed a marked decrease in the levels of beta globin mRNA (Fig. 3.1A) with the majority of the signal lost by 90 minutes for 333 and 180 minutes for PAAr5. Degradation intermediates were not seen. The loss of signal was not seen with cells infected with either of the vhs null mutants and their RNA levels were similar to that of mock infected cells. The Northern blot was exposed to a phosphoimager screen in order to quantitate the loss of signal and it was determined that the half-life of globin mRNA from 333 infected cells was 30 min. and 60 min. for cells infected with PAAr5 (Fig 3.1B).

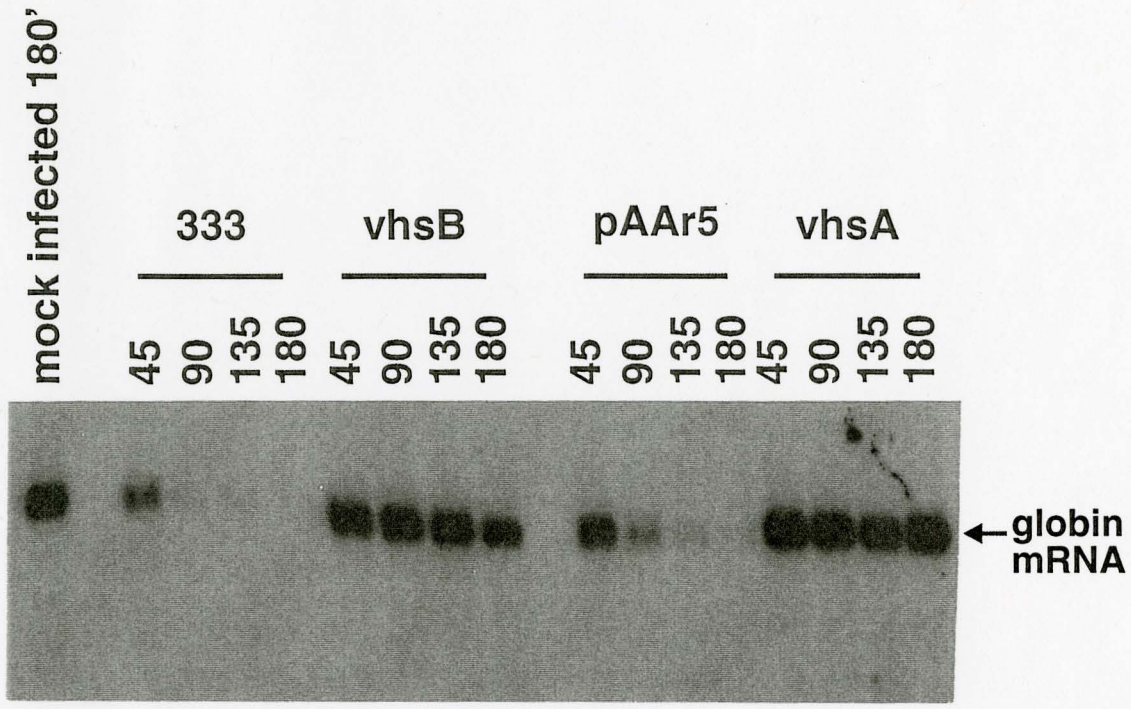
Since no decay intermediates were observed using the above time scale, I decided to examine the loss of the beta globin mRNA more closely by sampling at shorter time intervals. Again, mRNA levels in cells infected with wild type 333 declined over time (Fig 3.2A) with a noticeable decrease apparent at eight minutes post-infection. Quantification revealed that the half-life of globin mRNA, in this case was 15 min (Fig. 3.2B). Examining mRNA at earlier time points, however, did not lead to the detection of decay intermediates, and thus degradation polarity could not be addressed. In all cases, the beta globin mRNA was either in its intact form or not present. This suggests that the total digestion of beta globin mRNA is a very rapid process.

Tracking the global decay of beta globin mRNA (as above) may not allow for detection of small changes occurring at the ends of the transcript. For instance, a loss of a few RNA sequences at the 5' end would yield a product which would not resolve from the intact signal and thus go undetected. I, therefore, decided to focus on smaller regions of globin mRNA in an attempt to see such small alterations. To do this, I used a technique that involves cleaving globin mRNA, purified from HSV infected cells, into fragments of different size (adapted from Brewer and Ross, 1988). Each fragment can then be analyzed for small changes. This is achieved by asymmetrically annealing a DNA oligonucleotide to a specific site of the mRNA molecule. Incubating the DNA/ mRNA hybrid with purified RNase H results in cleavage of the mRNA into two fragments that are electrophoretically distinct. The oligos used, the sites they anneal to and the fragments they generate are summarized in Table 3.1. The

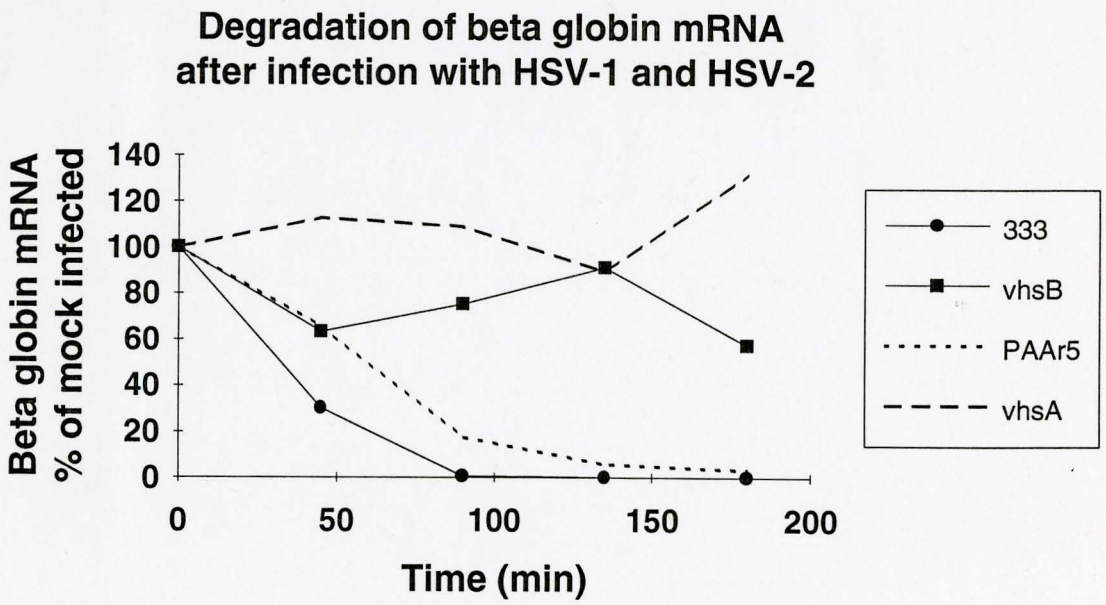
Figure 3.1 Effect of HSV-1 and HSV-2 infection of beta globin mRNA levels in MEL cells.

Terminally differentiated MEL cells were infected with 10 PFU per cell of each virus. Total RNA samples were extracted by Trizol at the times indicated (in minutes) and 10µg of each sample were analyzed by formaldehyde-agarose gel electrophoresis. The RNA was transferred to Nytran membrane and probed with randomly labeled (α^{32}P-dCTP) pUMβ2.6 (genomic copy of beta globin)(A). The signal represents cellular beta globin mRNA. MI refers to cells that were mock infected. The Northern blot was then quantified using the Phosphoimager (B) and beta globin RNA levels were reported as a percentage of mock infected (MI, lane 1).

A


mock infected 180'
333
vhsB
pAAr5
vhsA
45
90
135
180
globin mRNA

B


Degradation of beta globin mRNA after infection with HSV-1 and HSV-2
Beta globin mRNA % of mock infected
140
120
100
80
60
40
20
0
0
50
100
150
200
Time (min)
333
vhsB
PAAr5
vhsA

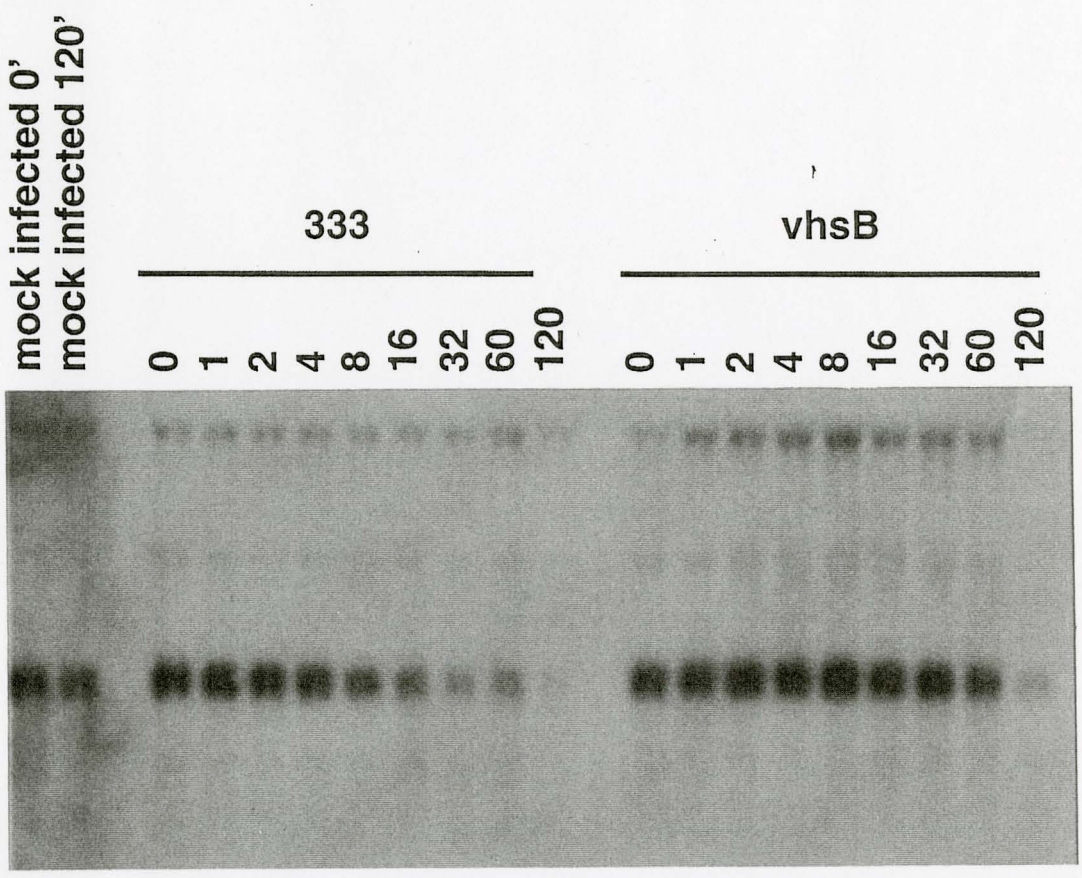
products of the RNAse H reaction were analyzed by Northern hybridization. However, contamination with phenol made loading equal amounts of RNA difficult and although many attempts were made, none of the globin mRNA fragments were converted to smaller molecular weight products that would indicate small changes (Fig. 3.3).

To ask whether vhs induced degradation *in vivo* resulted in loss of sequences at the 5' end of globin mRNA, I analyzed RNA samples using primer extension. This technique uses the enzyme reverse transcriptase and an oligo primer to produce a DNA copy of the RNA extending from the site of the annealed oligo to the 5' end of the RNA. Although activity at the 3' end would go undetected, primer extension would allow us to investigate the decay process of the 5' end of the RNA. I tested to see if vhs triggers degradation at or near the 5' end by infecting MEL cells with 333 and vhsB333 and extracting total RNA at various times. The purified RNA was incubated with a 5' end labeled oligo complementary to residues 65 through 89 of globin mRNA, at 62 °C for one hour. Reverse transcriptase was then added and extension proceeded for one hour at 42 °C. The products were then analyzed on a 7M urea- 8% polyacrylamide sequencing gel. The results show that in the presence of wild type vhs, the full length primer extension product decreases in abundance over time (Fig. 3.4). This corresponds to a decrease in the level of beta globin mRNA. Cells infected with vhsB333 showed comparable levels of RNA to mock infected cells. However, there were no smaller primer extension products detected, at any time points, that would indicate a degradation of mRNA at the 5' end. There

Figure 3.2. Effect of HSV-2 infection of MEL cells at smaller time intervals.

Terminally differentiated MEL cells were infected with 10 PFU per cell of each virus. Total RNA samples were extracted by Trizol at the times indicated (in minutes) and 10μg of each sample were analyzed by formaldehyde-agarose gel electrophoresis. The RNA was transferred to Nytran membrane and probed with randomly labeled (α^{32}P-dCTP) pUMβ2.6 (genomic copy of beta globin)(A). The signal represents cellular beta globin mRNA. MI refers to cells that were mock infected. 0 minutes indicates globin mRNA levels at the time of infection. The Northern blot was then quantified using the Phosphoimager (B) and beta globin RNA levels were reported as a percentage of mock infected (MI, lane 1 and 2).

A


mock infected 0'
mock infected 120'
333
vhsB
0
1
2
4
8
16
32
60
120
0
1
2
4
8
16
32
60
120

B

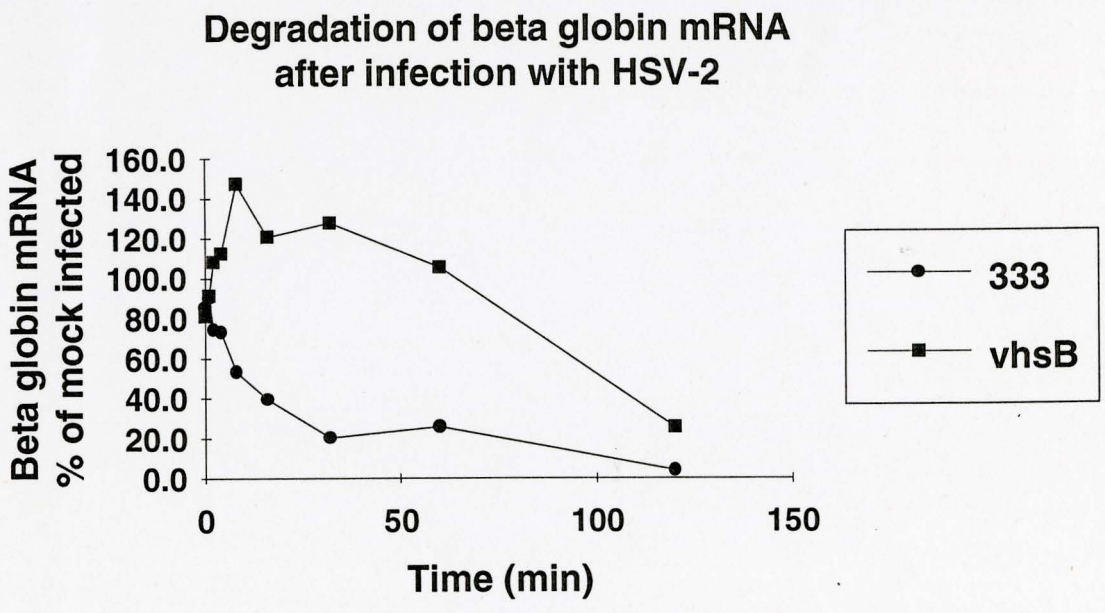

Degradation of beta globin mRNA
after infection with HSV-2
Beta globin mRNA
% of mock infected
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
0
50
100
150
Time (min)
333
vhsB

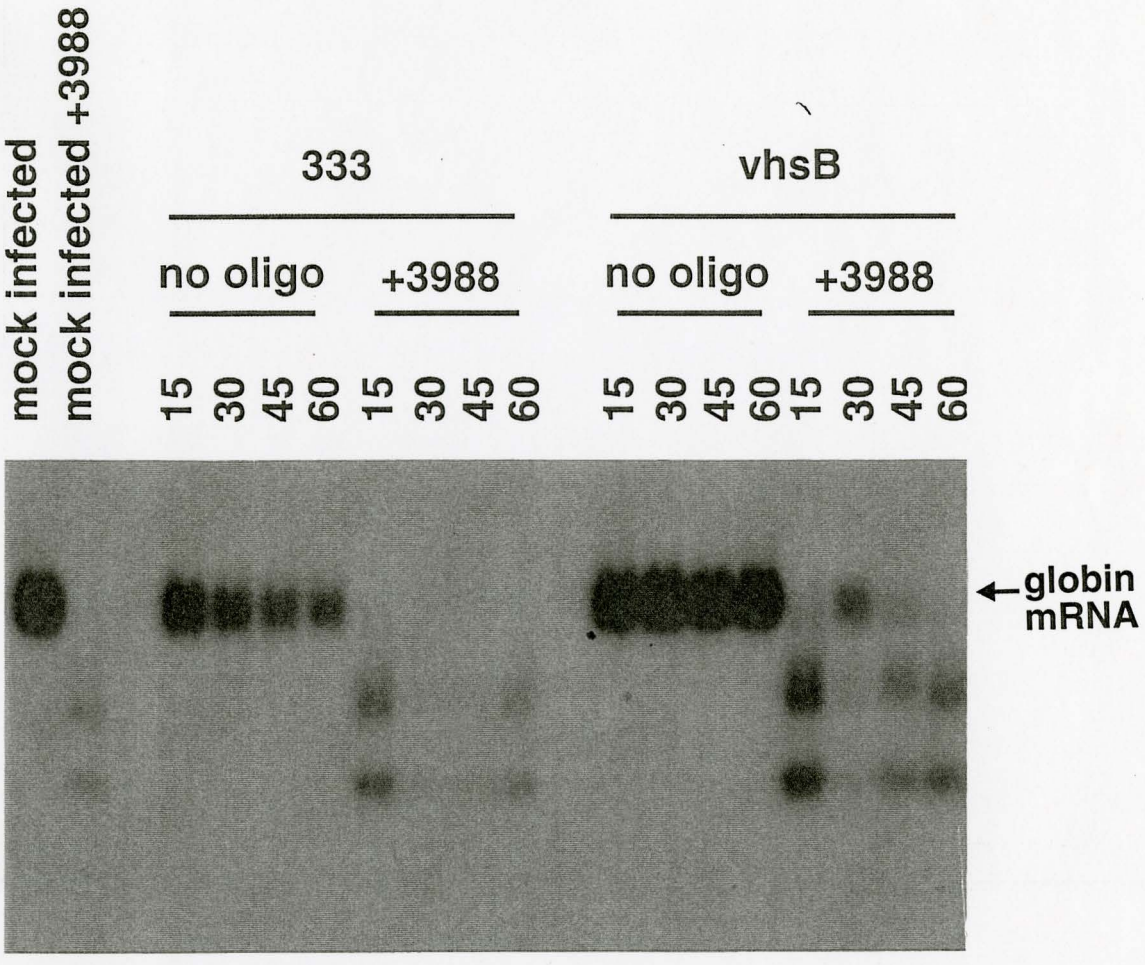
Table 3.1 Oligos used for RNaseH cleavage of MEL cells.

Purified RNA samples that were destined for RNase H cleavage were annealed to the oligos listed. The sequence of the oligos are shown with dNTPs represented by G, C, A, T. The annealing site refers to the site downstream of the 5' end of beta globin mRNA. The fragments generated are listed in nt. with the 5' and 3' referring to the separate ends of the molecule. Oligos were synthesized by MOBIX, McMaster University, Hamilton, ON.

OLIGO	SEQUENCE	ANNEALING SITE	FRAGMENTS GENERATED
AB3988	GCCTTCACTTTGGCATTACC	221 - 240	5' 220 nt 3' ca. 500 nt
AB3989	CAATCACGATCATATTGCCC	311 - 330	5' 310 nt 3' ca. 400 nt

Figure 3.3 Oligo-directed RNaseH cleavage of globin mRNA from MEL cells infected with HSV-1 and HSV-2.

MEL cells were infected, as usual with 10 PFU / cell of each virus and total RNA was extracted at the indicated times. 10µg of the RNA samples were annealed with oligo AB3988 (GCCTTCACTTTGGCATTACC) and then treated with RNase H (lanes indicated with +3988). The reactions were pelleted and dried and subjected to formaldehyde-agarose gel electrophoresis and Northern hybridization. MI: mock infected.


mock infected
mock infected +3988
333
vhsB
no oligo
+3988
no oligo
+3988
15 30 45 60 15 30 45 60 15 30 45 60 15 30 45 60
globin mRNA

are two possibilities for this result. First, degradation could occur 3' to 5', resulting in decay through the oligo priming site, which would lead to detection of only intact mRNA. Second, 5' end degradation could occur so rapidly that primer extension products smaller than the intact 89 bases could not be detected.

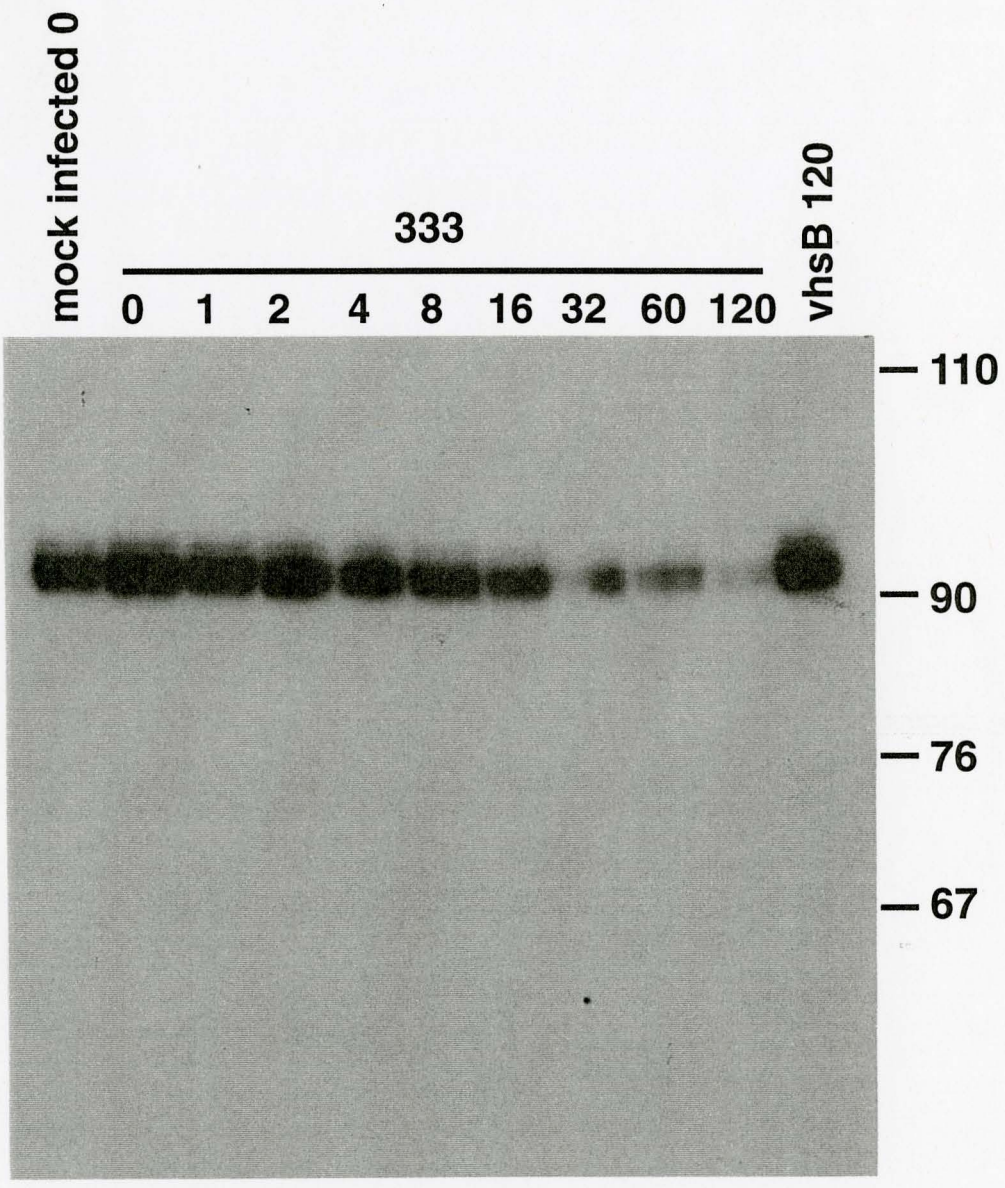
The results obtained from the above tissue culture experiments suggest that degradation of beta globin mRNA, induced by vhs, is extremely rapid. The fact that decay intermediates could not be detected implies that once an mRNA is targeted for degradation its sequences are very short lived. The difference in activity of HSV-1 and HSV-2 would appear to be in the targeting stage, since intermediates were absent in both cases.

3.2 vhs-induced degradation in a rabbit reticulocyte lysate system

Realizing that the investigations performed using tissue culture cells and whole virus, were not yielding conclusive information regarding the decay process induced by vhs, I decided to focus our efforts on an *in vitro* assay developed by Mabrouk Elgadi in our lab. Mabrouk was attempting to purify biologically active vhs made in a rabbit reticulocyte lysate *in vitro* translation system. This involved programming reticulocyte lysates with *in vitro* transcribed vhs RNA made from a high expression SP6 promoter driven plasmid, called pSPvhs (see Materials and Methods). Mabrouk discovered that *in vitro* translated vhs was capable of inhibiting the synthesis of protein from other RNAs added to the reticulocyte lysate (data not shown). This was performed by

Figure 3.4 Analysis of beta globin mRNA degradation by primer extension.

Total RNA samples were obtained from cells infected with HSV-2 333, 333vhsB or from mock infected cells. 10µg of total RNA from each sample were annealed with saturating amounts of 5' end labeled AB180 (AAGAGACAGCA-GCCTTCTCAGCATA). AB180 anneals to beta globin mRNA from nucleotides 65 to 89. Primer extension was carried out using reverse transcriptase at 42°C for one hour. The products of the reaction were then analyzed on an 8% urea-polyacrylamide gel. Molecular weight markers (M) were 5' end-labeled Hpa II fragments of pBR322, shown in nucleotides. MI: mock infected.


mock infected 0
333
0 1 2 4 8 16 32 60 120
vhsB 120
110
90
76
67

allowing vhs RNA to be translated, in the presence of 35S-methionine, for twenty minutes at which point a 2.4 kb reporter RNA termed 19 , generated from the plasmid pSPSR19N (see Materials and Methods), was added. Translation was allowed to continue for 60 minutes and the products were analyzed by SDS-polyacrylamide gel electrophoresis. Mabrouk's results demonstrated that active vhs protein prevented the production of protein from the reporter RNA. This was only seen when an active form of vhs was pretranslated as neither bovine preprolactin nor the vhs point mutant, vhs1 were capable of translation inhibition. As the protein shut-off effect mimicked the effect of vhs *in vivo*, Mabrouk tested whether *in vitro* translated vhs was capable of inducing degradation of mRNA. For this experiment, he added reporter α^{32}P-GTP internally labeled 19 RNA to pretranslated vhs and analyzed the RNA products on an 1% agarose-formaldehyde gel (results not shown). The results revealed that the reporter RNA was markedly degraded in the presence of active vhs. The intact signal was rapidly converted to a heterogeneous set of high molecular weight products which slowly reduced in size as time progressed. These results demonstrated that *in vitro* translated vhs possessed shut-off activity very similar to the *in vivo* phenotype, and that vhs seemed to be the only viral protein required to see this effect.

This discovery prompted me to re-examine the process by which vhs induces degradation of mRNA, using the above *in vitro* assay. This assay gave rise to degradation intermediates which could not be detected by Northern blot

analysis during the *in vivo* experiments, and therefore, may prove useful in testing the polarity of vhs induced degradation.

3.3 vhs induces rapid loss of the 5' end of an mRNA

The generation of an *in vitro* assay for vhs action now gave us the opportunity to manipulate the reaction conditions and present the system with RNA substrates that I constructed. In an attempt to determine if vhs induced decay had any apparent polarity, I decided to introduce an mRNA that was selectively radiolabeled at one of the RNA ends, to pretranslated vhs in rabbit reticulocyte lysate. For this experiment, I chose to add mRNA that had been labeled within the 5' m^7G cap structure. This was performed by incubating *in vitro* transcribed 19 RNA, possessing a tri-phosphate 5' end, with α^{32}P-GTP and S-adenosyl methionine in the presence of the vaccinia virus enzyme guanylyltransferase. This enzyme catalyses the formation of a bond between the GTP and the 5' end of the RNA thus generating an RNA possessing an m^7GpppX cap structure, with the boldface p representing the labeled phosphate. The resulting "cap-labeled" 19 RNA was used as a substrate for *in vitro* translated vhs.

If the mRNA decay, induced by vhs, occurred by a 3' to 5' exonuclease, then the intact 2.4 Kb cap labeled signal would be lost as progressively smaller degradation intermediates accumulated over time. A similar result would be obtained if degradation occurred via a random endonuclease. However, if degradation occurs at the 5' end of the mRNA first, then the intact 2.4Kb signal

would be reduced to low molecular weight products that did not arise from larger intermediates. To test this, approximately 200ng of vhs RNA was added to the reticulocyte lysate and translation proceeded for 20 minutes. Cap labeled 19 RNA was then added and samples were taken at various time points. The RNA was purified and the samples were analyzed by agarose-formaldehyde gel electrophoresis and transferred onto Nytran membrane. In the presence of an active form of *in vitro* translated vhs, the 2.4 Kb intact signal, corresponding to 19 RNA, was rapidly lost (Fig. 3.5A) with the majority of the signal gone four minutes after the addition of cap labeled 19 RNA. The intact RNA did not seem to degrade through high molecular weight intermediates and may have been rapidly converted to small products not apparent on this membrane. This rapid decay was not seen when the 19 RNA was incubated with either rabbit reticulocyte lysate alone or lysate containing pretranslated vhs1. Some loss of the intact signal was seen with vhs1 samples compared with lysate alone, suggesting that this point mutated form of vhs may not be completely inactive in our *in vitro* system.

To determine if the rapid loss of RNA was due to degradation at the 5' end I wished to examine the condition of the body of the RNA, to see in what form it existed. To accomplish this, the above membrane was kept aside for a month, to allow the ^{32}P signal to decay. The membrane was then probed with randomly labeled pSPSR19N plasmid (contains the entire cDNA corresponding to 19 RNA) which is complementary to full length 19 RNA. The condition of the mRNA body, as seen in figure 3.5B, differs greatly from the results obtained from the cap

labeled experiment. In this case, an appreciable fraction of the intact signal corresponding to 19 RNA remained apparently intact at 4 minutes. The intact signal was converted into a series of intermediates ranging in size, thus producing a smear. The intermediates are gradually reduced to small molecular weight products at a much slower rate than seen with cap labeled RNA. Again samples taken from
pretranslated vhs1 and reticulocyte lysate alone showed little degradation of the intact 19 RNA, demonstrating that the decay is due to vhs. This result, taken in combination with those from the cap labeled experiment, suggests that degradation induced by vhs, commences at the 5' end while the body of the mRNA decays via a slow but gradual process. However, upon learning that the cap labeling reaction (see Materials and Methods) was only about 8 to 12 percent efficient (i.e. only 8 to 12 percent of the RNAs were capped)(Gehrke, 1986), the results from the Northern hybridization of 19 RNA (Fig 3.5B) may not represent the fate of the cap labeled RNAs and in fact represent all RNAs (capped or uncapped). The interpretation that the 5' end is degraded before the RNA body thus depends on whether a cap affects the decay process (addressed in sect. 3.7). If the presence of a cap alters the reaction then the Northern results would illustrate the decay process of the predominant uncapped species and can therefore not be compared with the cap labeled results.

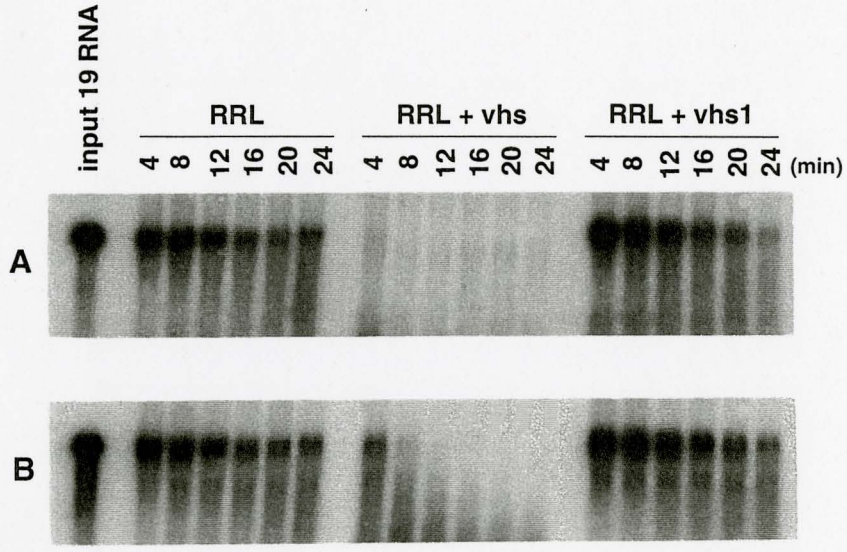
In order to circumvent the above problem, the cap labeled experiment was repeated, however, this time the fate of the cap labeled 19 RNA was compared to the fate of internally labeled capped 19 RNA. Internally labeled RNA was

Figure 3.5 Comparison of the degradation products of cap labeled RNA to Northern hybridization of the same RNA.

19 RNA was cap labeled using vaccinia guanylyltransferase and α^{32}P-GTP and added to reticulocyte lysate alone or containing pretranslated vhs and vhs1 RNA. RNA samples were taken at the indicated times (in minutes), purified by Trizol method, analyzed on a 1% formaldehyde-agarose gel and transferred to nylon membrane. The resulting autoradiogram is depicted in panel A. The membrane was then stored for 40 days and then hybridized to ^{32}P-internally labeled pSPSR19N fragment (containing 19 DNA) which probed for the RNA body (B). RRL: naive rabbit reticulocyte lysate; RRL+vhs: RRL containing pretranslated vhs; RRL+vhs1: RRL with the pretranslated vhs1 mutant.

A. Cap labeled signal after 14 hour exposure.

B. Hybridization signal after 7.5 minutes (40 days later).


input 19 RNA
RRL
RRL + vhs
RRL + vhs1
4 8 12 16 20 24
4 8 12 16 20 24
4 8 12 16 20 24
(min)
A
B

produced by transcribing 19 RNA in the presence of α ^{32}P-GTP and the cap primer m^{7}GpppG. This results in approximately 99 percent of the 19 RNAs being capped (data not shown) and labeled within the body of the RNA. To eliminate any differences between separate vhs reticulocyte lysate reactions, one batch of pretranslated vhs was prepared and equal amounts were added to cap labeled and internally labeled RNA. Samples were taken at various time points from both reactions and analyzed by agarose-formaldehyde gel electrophoresis. In the presence of pretranslated vhs the cap labeled 19 RNA was rapidly degraded and (in this case) converted to low molecular weight products (Fig 3.6A). Analogous to the Northern hybridization results, internally labeled 19 RNA was gradually degraded from large to small molecular weight intermediates (Fig 3.6B). In either reaction, the controls were not subject to rapid degradation. By direct comparison of capped 19 RNAs (internally or cap labeled), these results demonstrate that vhs induces rapid degradation at, or near, the 5' end of an mRNA.

3.4 Decay at the 5' end, induced by vhs, is due to endonucleolytic cleavage

Thus far, the data presented illustrates that the polarity of vhs induced degradation involves rapid decay at the 5' end of an mRNA. I next wanted to determine through what process was the 5' end of 19 RNA being degraded. The approach I took to answer this question was to attempt to track the loss of the cap labeled signal to determine in what form it existed. Three obvious possibilities existed. Perhaps vhs induces cleavage of one of the pyrophosphate bonds linking the cap to the RNA (decapping). The released cap moiety

(m^7Gp?) could then exist in either of two forms: as a free structure or associated with protein. The third possibility is that vhs induces cleavage shortly downstream of the cap structure thus generating a cap labeled RNA oligomer. At first, I attempted to test the first possibility by using thin layer chromatography to detect a free cap labeled structure. Polyethylene imine (PEI) cellulose was employed for this experiment. This form of chromatography allows mono- and di-nucleotides to migrate with the solvent, lithium chloride, while protein and larger nucleic acids are retained at the origin. If vhs does induce pyrophosphate bond cleavage within the cap, then with the cap being radioactively labeled it may migrate up the chromatogram.

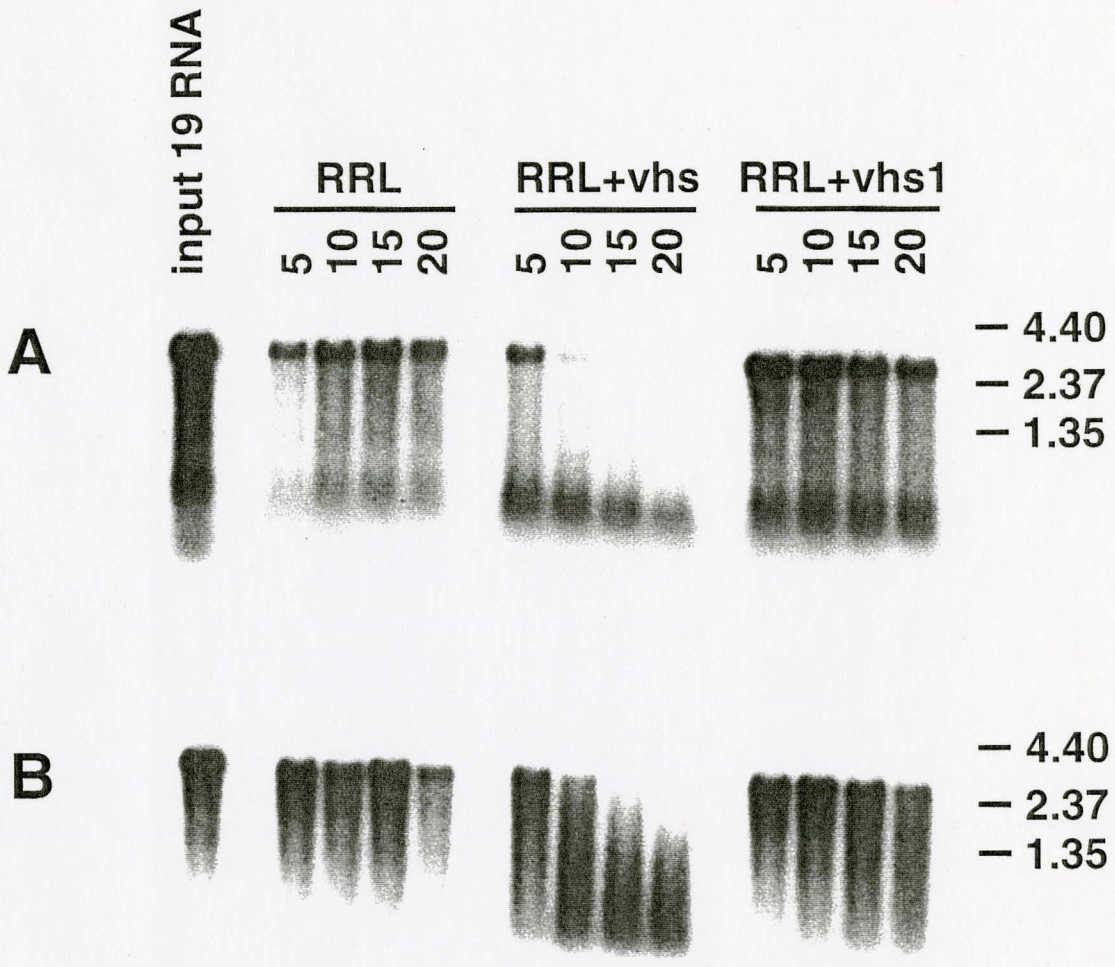
Cap labeled 19 RNA was added to naïve reticulocyte lysate or lysate containing pretranslated vhs or vhs1. Samples, taken at various times, were spotted directly onto the PEI cellulose along side various nucleotide and cap analog standards and the chromatogram was placed into a solvent chamber containing 1M lithium chloride. The chromatogram was then illuminated with UV light to locate the standards and exposed to X-ray film. As seen in figure 3.7, samples from reticulocyte lysate containing vhs did not give rise to any apparent labeled cap analog that differed from those generated from the controls. In all lanes, the majority of the radiolabel remained at the origin. The one product which was present in all samples did not increase in abundance over time and was not considered to be a product of degradation (possibly unincorporated α ^{32}P-GTP from the cap labeling reaction). This result left two possibilities: either

Figure 3.6 Comparison of cap labeled decay products and internally labeled intermediates of 19 RNA subjected to pretranslated vhs.

19 RNA was cap labeled and internally labeled in separate reactions and added to RRL, RRL +vhs and RRL+vhs1 (see Fig 3.5 legend). RNA samples were withdrawn at the times indicated (in minutes), subjected to formaldehyde-agarose gel electrophoresis and transferred to a nylon membrane. RNA marker sizes are indicated (kb) and were obtained by staining the membrane with methylene blue dye.

A. Cap labeled 19 RNA

B. Internally labeled capped 19 RNA

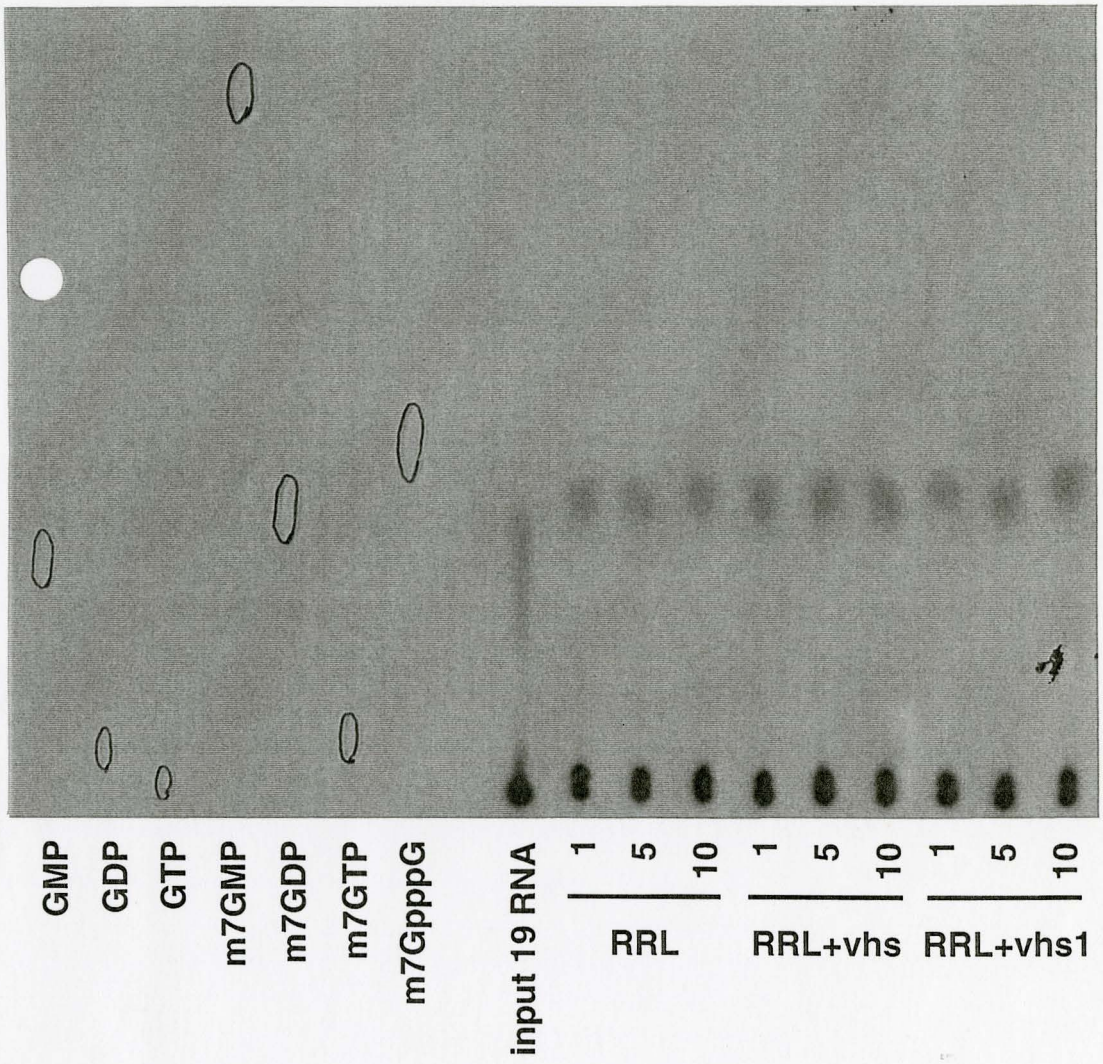

input 19 RNA
RRL
RRL+vhs
RRL+vhs1
5
10
15
20
5
10
15
20
5
10
15
20
A
B
4.40
2.37
1.35
4.40
2.37
1.35

the label becomes associated with protein or it exists as a larger nucleic acid, not capable of migrating on PEI cellulose.

I decided to test if the label exists as a larger nucleic acid by repeating the experiment, however, this time analyzing the purified RNA (see Materials and Methods) on a 7M urea-20% polyacrylamide sequencing gel. This high percentage gel allows for good separation of low molecular weight nucleic acids. Figure 3.8A reveals that pretranslated vhs induced the formation of two major labeled products that migrate around 30 and 40 nt. and several minor products. These products
increase in abundance over time and seem to remain intact rather than themselves being degraded, implying that they are end products of a reaction induced by vhs. The full length 19 RNA is shown to decline over time but does not seem to be converted into any other high molecular weight products that differ from the controls. This is consistent with the agarose gel analysis of cap labeled RNA (Fig 3.5, 3.6). However, with 19 RNA being 2.4 Kb, degradation products larger than 600 nt. would not migrate into the 20% sequencing gel and thus, would go undetected. To test if other degradation intermediates are produced I generated a truncated RNA from pSPSR19N that possessed the first 404 nt., which was termed 19X RNA. This was achieved by linearizing the plasmid with Xmn1. Any large products generated from this RNA substrate would be able to migrate into the gel. 19X RNA was then used as a substrate for pretranslated vhs. As seen earlier, degradation due to vhs resulted in the production of two major cap labeled oligos (Fig 3.8B) ca. 30 and 40 nt. long,

Figure 3.7 Analysis of degradation products obtained from cap labeled RNA, by thin layer chromatography.

Cap labeled RNA was added to RRL, RRL +vhs and RRL + vhs1. RNA samples were taken from the lysate at 1, 5 and 10 minutes and spotted directly onto polyethylene imine cellulose. Nucleotide and cap standards were also spotted. The samples were fractionated in 1M lithium chloride. Standards were visualized using a hand held UV light and their positions were scored. Lysate samples were visualized by autoradiography.


GMP
GDP
GTP
m7GMP
m7GDP
m7GTP
m7GpppG
input 19 RNA
1
5
10
RRL
1
5
10
RRL+vhs
1
5
10
RRL+vhs1

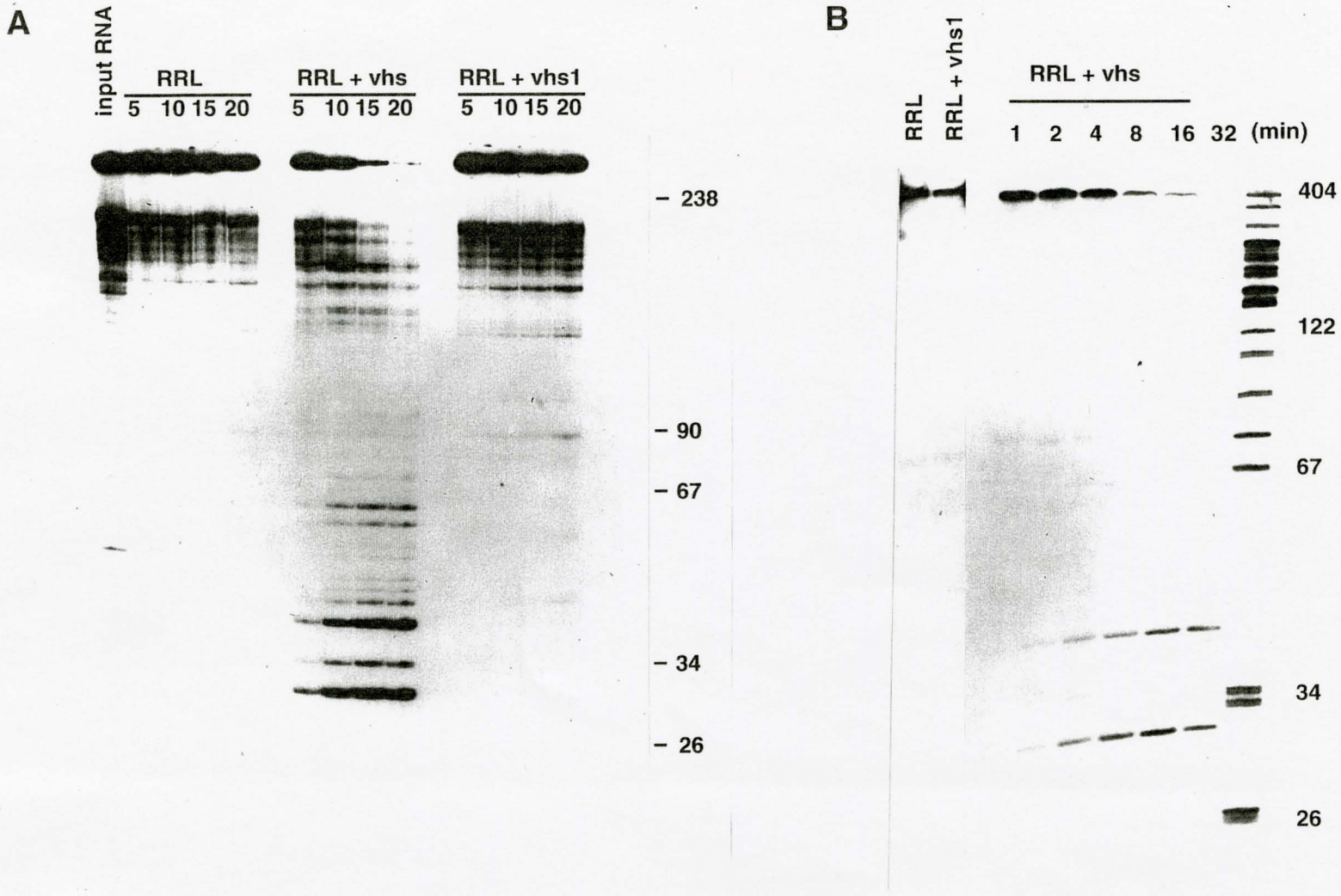
which were detectable as early as one minute post addition of 19X RNA. These products became more prevalent over time as the intact signal 19X signal decreased. No large products, specific to the vhs lanes, were evident on this gel suggesting that the cap labeled oligos were the result of an early, if not the first, degradation event.

Two alternative mechanisms could account for the production of the cap labeled oligos. The easiest interpretation is that the oligos are a result of an endonucleolytic cleavage at either 30 or 40 nucleotides downstream of the cap. However, the oligos could be generated by action of a rapid 3' to 5' exonuclease that tends to stop at these sites. The experiments tracking the decay of internally
labeled 19 RNA (Fig. 3.6B) provide preliminary evidence that the latter mechanism is unlikely, since the RNA body was present in high molecular weight form at times when the intact cap labeled signal was completely consumed. On the contrary, if vhs induces endonucleolytic cleavage producing the cap labeled oligos, then this would result in two new 5' ends being generated corresponding to the body of the RNA, 3' to the cleavage site. I decided to test this possibility using primer extension to detect if new 5' ends are evident. I had an oligo constructed (see Materials and Methods) that was complementary to residues 60 through 84 of 19 RNA thus yielding a primer extension product of 85 nt. This oligo was 5' end labeled and annealed at 62°C to RNA purified from a reticulocyte lysate reaction containing vhs. Reverse transcriptase was then added to the RNA:oligo complex and incubated at 40 °C for one hour. The

Figure 3.8 Analysis of cap labeled degradation products using high percentage polyacrylamide gel electrophoresis.

A. Cap labeled 19 RNA (2.4 kb) was added to lysate containing vhs (and controls) and RNA samples were taken at the times indicated (in minutes). The RNA was purified and analyzed on a 20% polyacrylamide gel.

B. Same as above using a 404 nt derivative of 19 RNA (termed 19X). This substrate was obtained from runoff transcripts of pSPSR19N linearized with Xmn1. End labeled pBR322 HpaII marker sizes are indicated in nt.


A
input RNA
RRL
5 10 15 20
RRL + vhs
5 10 15 20
RRL + vhs1
5 10 15 20
238
90
67
34
26
B
RRL
RRL + vhs1
RRL + vhs
1 2 4 8 16 32 (min)
404
122
67
34
26

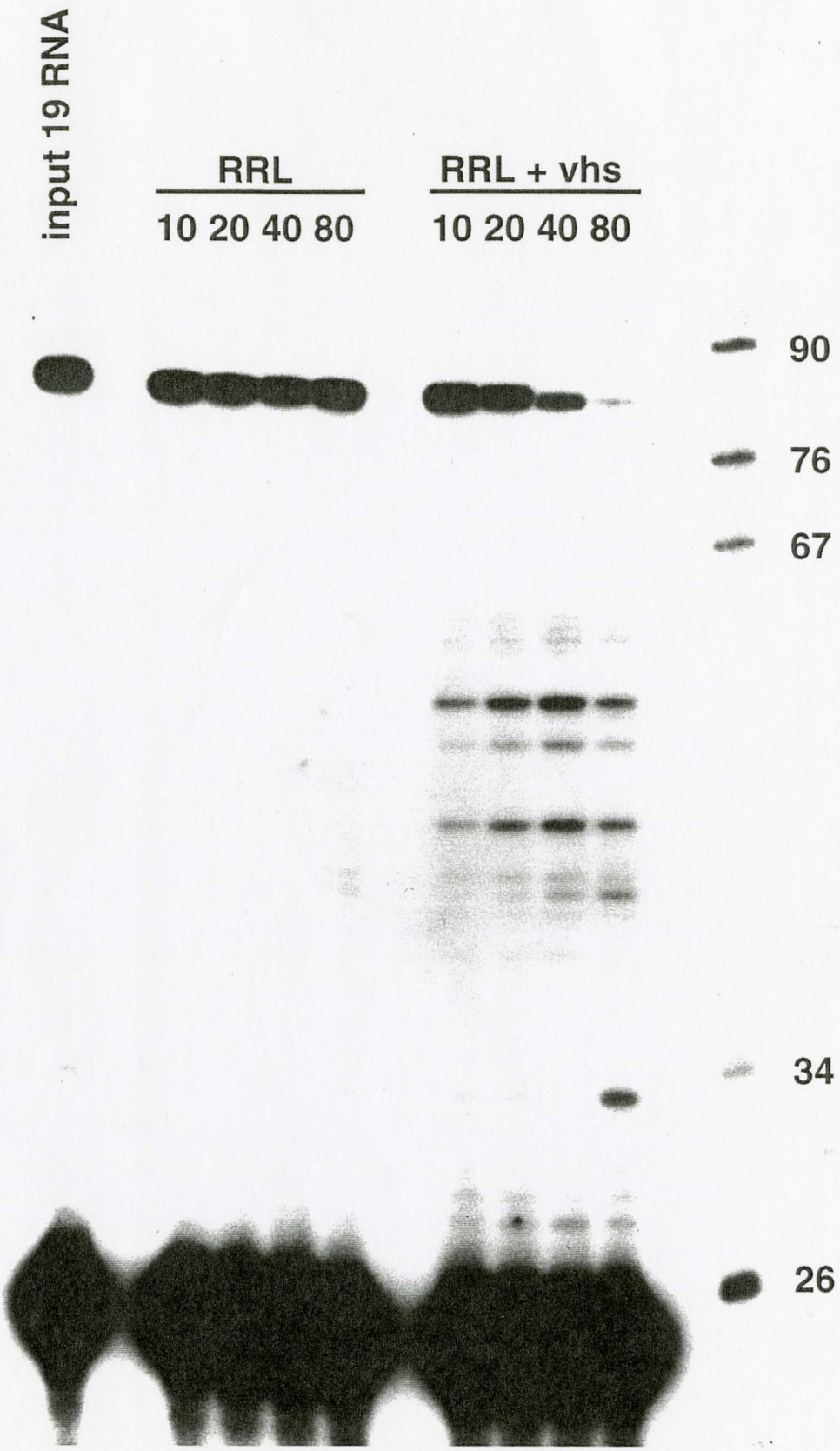
resulting primer extension products were analyzed on an 8% sequencing gel. Figure 3.9 illustrates the production of the predicted new 5' ends of 44 and 55 nt. These ends correspond to a loss of 30 and 40 nt. from the intact product of 85 nt., demonstrating that vhs induces loss of the 5' end of 19 mRNA through endonucleolytic cleavage 30 and 40 nt. downstream of the cap. I use the term *decapitation* to describe the cleavage events. Unlike the cap labeled oligos, these primer extension products are unstable. They were never detected in abundance relative to the input RNA, and they declined in concentration at later points during the reaction (see 80 minutes). In fact during the later stages new products appeared of ca. 40 and 34 nt. which may indicate secondary decay events following the decapitation. Whether vhs is invlolved in these susequent events is yet to be determined.

3.5 vhs induced cleavage is not a function of distance from the cap

Since all experiments, thus far, used 19 or 19X as a substrate for *in vitro* translated vhs, I wished to test if vhs induced the same cleavage events using a variety of other substrates. Perhaps vhs cleaves mRNAs at a fixed distance from the 5' cap or at a specific sequence or secondary structure. Using RNAs generated from plasmids that contain different 5' leader sequences or different coding regions I hoped to determine any site specificity. All plasmids used in these experiments were graciously donated by Dr. David Andrews. At first I obtained plasmids that possessed the same SRPα coding region but had different 5' untranslated regions (UTR). The UTRs differed through insertions and deletions made within the UTK leader sequence described in Materials and

Figure 3.9 Primer extension analysis of 19 RNA degradation products.

30 ng of unlabeled, capped 19 RNA (using cap primers) was added to lysate alone and containing pretranslated vhs. RNA samples were taken at the indicate times (in minutes) and were annealed with a 5' end labeled oligo complementary to residues 60 to 84 (GGTGAAGAAGTCGACCATGGTAGAT). Following the primer extension reaction, the products were analyzed on an 8% polyacrylamide gel. Marker sizes are indicated in nt.


input 19 RNA
RRL
10 20 40 80
RRL + vhs
10 20 40 80
90
76
67
34
26

Methods. One plasmid, denoted #1, possessed a 40nt UTR from the native SRP gene, that is completely different in sequence to the UTK leader of 19 RNA. The various structures of the plasmids are briefly summarized in Table 3.2. As done previously, RNAs were transcribed *in vitro*, cap labeled and used as substrates for reticulocyte lysate translated vhs. The cap labeled products generated from the various substrates are evident in figure 3.10. Cap labeled 19 RNA gave rise to the expected oligos of 30 and 40 nt. Virtually identical cleavage products were generated from cap labeled #2 and #13 RNA. However, when the proposed cleavage sites were lined up with the known sequences of these RNAs it was discovered that 19, #2 and #13 all possessed identical sequences surrounding the sites. Different cleavage patterns were generated from #1 and #47 RNAs. #1 RNA, produced a slightly modified pattern, although it possessed a completely different UTR sequence to 19 RNA. This tends to imply that distance is a factor in the site of cleavage since the sequence was
different yet the cap labeled oligos produced were roughly similar. The pattern generated from #47 RNA indicated preferential cleavage sites around 65 and 75 nt. downstream of the cap. As summarized in Table 3.2, #47 is identical in sequence to #13 RNA except for a 20 nt. insertion between the SP6 promoter and the UTK leader. This insertion results in a shift of cleavage sites approximately 35 nt. Since the cleavage products did not increase in size by 20 nt., sequence specificity seems unlikely. Distance from the cap also seems to be ruled out since the cleavage sites did not remain at 30 and 40 nt.

Table 3.2 First set of substrates used for site specificity experiments.

The following table lists the RNA substrates that were cap labelled and used to determine vhs site specificity. All plasmids were obtained from D. Andrews, McMaster University, Hamilton, Ontario. Leader designation refers to the type of untranslated region. Native refers to the natural UTR of SRPa. In this case, all the ORFs were canine signal recognition particle alpha (SRPa). The sequence of the UTK leader is as follows:

GAATACAAGCTTGCTTGTTCTTTTTGCAGAAGCTCAGAATAAACGCTCACTTTGGCAGATCT

UTK-40nt 3' -- UTK sequence with last 40 nt replaced with vector seq.

UTK-20nt 3, -- UTK sequence with last 20 nt replaced with vector seq.

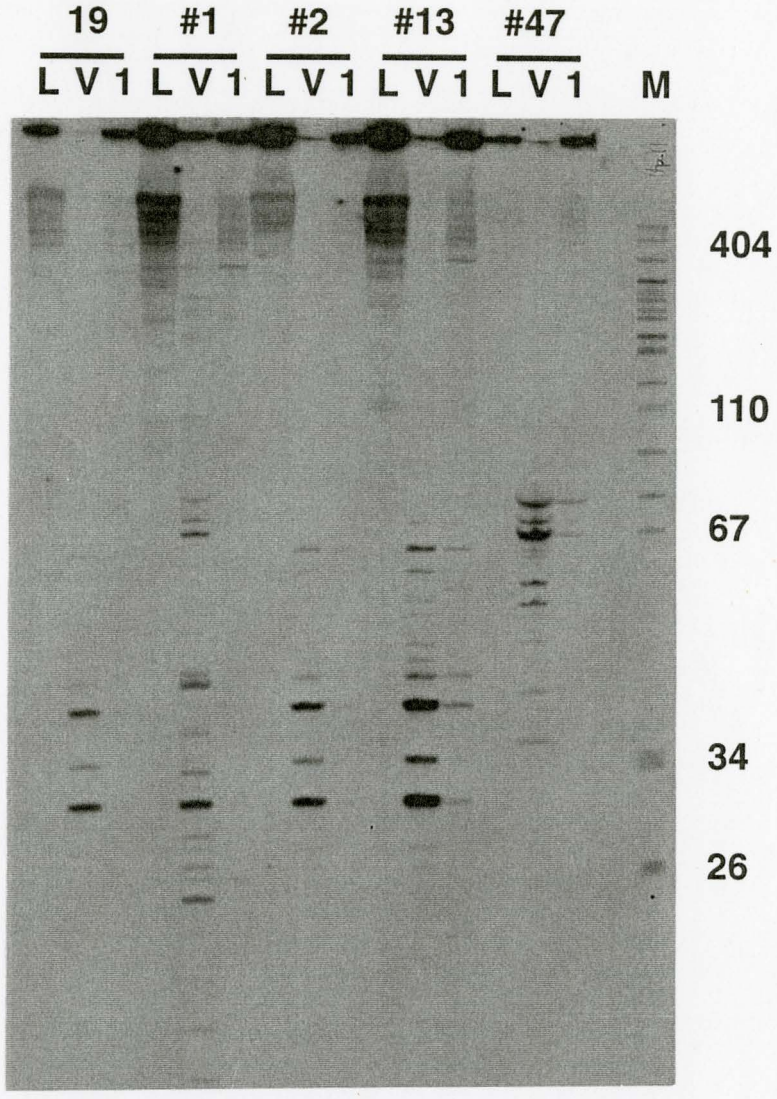
UTK-20nt 3' +20nt 5' -- same as above with 20 nt of polylinker inserted between the SP6 promoter and UTK.

RNA	PLASMID	5' LEADER DESIGNATION	ORF
19/ 19X	pSPSR19N	UTK	SRPa
#1	pSPSR10	NATIVE	SRPa
#2	pSPSR19	UTK - 40nt 3'	SRPa
#13	pSPSR19P	UTK - 20nt 3'	SRPa
#47	pSPSR19P5	UTK - 20nt 3' + 20nt 5'	SRPa

Figure 3.10 Cap labeled cleavage products generated from different RNA substrates.

The substrates described in Table 3.2 were *in vitro* transcribed and cap labeled in the same manner as 19 RNA. The cap labeled substrates were added to reticulocyte lysate containing pretranslated vhs, vhs1 and lysate alone. An attempt was made to add equal amount of ^{32}P signal. (A) The products of the reaction were analyzed (following RNA extraction) on a 20% sequencing gel. (B) The proposed cleavage sites (indicated by arrows) for each substrate were then mapped to the known sequence of the RNAs. The shaded boxes represent the 5' leader regions. Different sequences are represented by different colours. L: RNA samples from lysate alone; V: from pretranslated vhs; 1: from pretranslated vhs1; SRa: signal recognition particle alpha ORF. Marker sizes indicated in nt.

A


19
#1
#2
#13
#47
L V 1 L V 1 L V 1 L V 1 L V 1
M
404
110
67
34
26

B


19
#1
#2
#13
#47
SRa
SRa
SRa
SRa
SRa
20 nts

The substrates used above did not provide conclusive evidence as to the specificity of vhs induced cleavage, and thus I obtained more plasmids that would generate substrate RNAs with completely different UTRs and open-reading frames. The important structural elements of these constructs are summarized in Table 3.3. The *in vitro* transcripts produced from these plasmids were cap labeled and added to lysate containing pretranslated vhs. The 20 % sequencing gel (Fig. 3.11), on which the purified RNA products were analyzed, reveals that two of the four substrates gave rise to different cleavage products than normally seen with cap labeled 19 RNA (in this experiment 19X RNA was used). In the presence of *in vitro* translated vhs, #36 RNA was decapitated to produce cap labeled oligos of about 50 and 55 nt. However, when the 5' leader sequence of #36 was replaced with the UTK leader , common to 19X (i.e. #35), the cap labeled oligos produced were identical in size to those generated from the degradation of 19X. One prominent oligo was produced from #155 RNA with an apparent molecular weight of 45 nt. The data obtained using these constructs indicate that the site preference for vhs-induced degradation is not merely a function of distance from the cap. As the sequence downstream of the cap changes, so does the cleavage site.

I attempted to determine if vhs induced cleavage had any simple sequence specificity. Using the sequences surrounding the cleavage sites, I performed sequence homology searches using GeneWorks . Although, a few substrates were cleaved close to an AGAA sequence, others were not and thus no simple sequence governing endonuclease specificity could be determined.

Using, RNADraw, a secondary structure modeling program, I tested whether the regions adjacent to the cleavage sites formed a recurring structure. No such similarities were seen between different substrates. Although no simple structural feature of the substrates could account for the site specificity of vhs induced cleavage, it may be that both sequence and secondary structure play a role in determining where an mRNA is targeted. Determining the exact nucleotide residue where endonuclease activity occurs may prove useful.

3.6 Oligos annealed over the cleavage sites can block vhs induced cleavage.

With the possible sequence or structure specificity influencing the site of cleavage induced by vhs, I asked whether the endonucleolytic event could be blocked if the proposed sites were no longer accessible. To address this question I had several oligos constructed that annealed to regions surrounding and overlaying the 30 to 40 nt. region downstream of the cap of 19 RNA. Table 3.4 summarizes the sequences of the oligos and the regions on 19 RNA where they hybridize. The

experiment was performed by cap labeling 19 and 19X RNAs and then incubating them with the various oligos in reticulocyte buffer (see Materials and Methods) at 60 °C to allow annealing. The RNA : oligo complexes were then used as substrates for *in vitro* translated vhs and the products were analyzed on a 20% sequencing gel. Figure 3.12 shows that cap labeled 19 RNA that was hybridized to AB4849 or AB5688 was cleaved to produce the same cap labeled oligos of 30 and 40 nt. that are seen in the absence of an annealed oligo. Both

Table 3.3 Substrates used with different 5' leaders and ORFs.

The following table lists the RNA substrates that were cap labelled and used to determine vhs site specificity. All plasmids were obtained from D. Andrews, McMaster University, Hamilton, Ontario. Leader designation refers to the type of untranslated region. ORFs are: SRPa; chimp alpha globin (a-GLOBIN) and hamster preprolactin (PPL). The sequences of the 5' leaders is as follows:

UTK -- GAATACAAGCTTGCTTGTTCTTTTTGCAGAAGCTCAGAATAAACGCTCACTTTGGCAGATCT

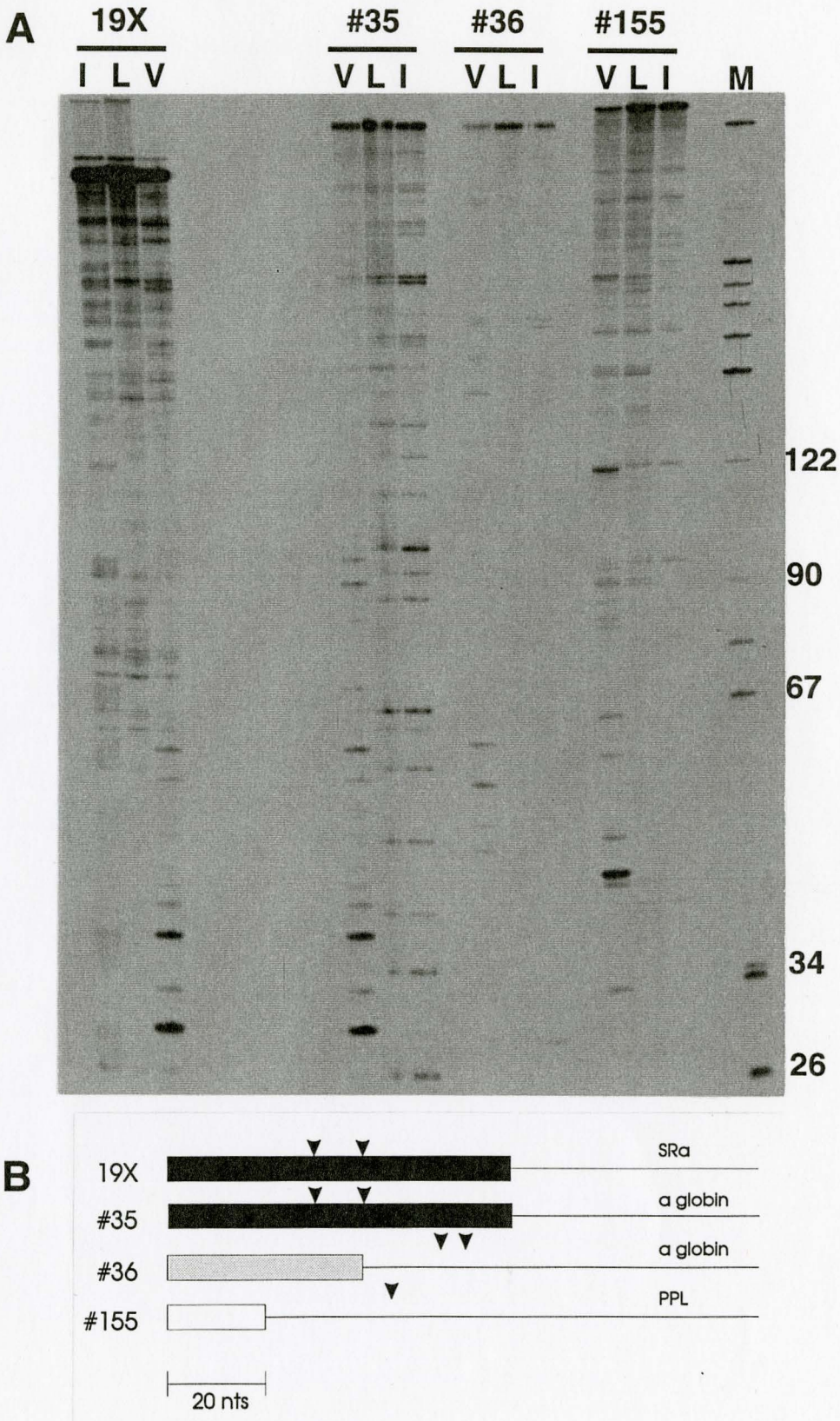
S -- GAATACAAGCTTGCATGCCTGCAGGTC

KD -- GAATACAAGCTGATCT

RNA	PLASMID	5' LEADER DESIGNATION	ORF
19/ 19X	pSPSR19N	UTK	SRPa
#35	pGEMSVGI	UTK	a-GLOBIN
#36	pGEMGI	S	a-GLOBIN
#155	pGEMKBPI	KD	PPL

Figure 3.11 Cap labeled cleavage products generated from RNA substrates with dissimilar 5' leader sequences.

Substrates described in Table 3.3 were cap labeled and added to lysate alone and containing pretranslated vhs. (A) Products from the lysate were then analyzed on a 20% sequencing gel. (B) The proposed cleavage sites were then mapped (arrows) to the known sequences of the RNAs. Shaded boxes with different colours represent 5' leader sequences of different sequence.
I: input cap labeled RNA; L: products from lysate alone; V: from vhs; SRa: ORF of signal recognition particle alpha; a globin: ORF of chimp alpha globin; PPL: ORF of hamster preprolactin. Marker sizes indicated in nt.


A
19X
#35
#36
#155
I L V
V L I
V L I
V L I
M
122
90
67
34
26
B
19X
#35
#36
#155
SRa
a globin
a globin
PPL
20 nts

AB4849 and AB5688 anneal to regions of 19 RNA that are downstream of the proposed cleavage sites. However, when an oligo is annealed over the cleavage sites (AB6257), the results show that the cleavage pattern is altered. The cap labeled products of 30 and 40 nt. (which accumulated over time) seemed to shift position by migrating one base faster . I was surprised, however, to see that the new cap labeled oligos of ca. 29 and 39 nt. were generated in reticulocyte lysate lacking vhs, since thus far this reaction required the presence of vhs. The presence of the oligo seemed to induce cleavage of the substrate 19 RNA when incubated with naive rabbit reticulocyte lysate. The only new product that was unique to vhs lanes was one of 60 nt., and therefore must be due to the presence of vhs. These results suggest that the presence of AB6257 prevents vhs induced cleavage at 30 and 40 nt. and causes the cleavage event to shift to approximately 60nt downstream of the cap. At a closer look 19 RNA annealed to AB4849 and AB5688 also gave rise to new bands that did not require the presence of vhs. These new bands seem to be due to endonucleolytic events occurring at the site at which the oligos anneal to 19 RNA which seems to be induceed by an RNase H-like activity inherent to the lysate (Cazenave et al., 1993; Walder and Walder, 1988).

The experiment was repeated using cap labeled 19X as the substrate RNA. In this experiment I used oligos AB5688 and AB6257 in the same manner as described above. The results obtained with these two oligos were identical with those of the experiment above (Fig 3.13). AB5688 had no effect on the cap labeled oligos induced by vhs and AB6257 caused the cleavage event to shift to 60 nt. In this experiment, I included an oligo (AB6586) which annealed to the

Table 3.4 Oligos used for oligo blocking.

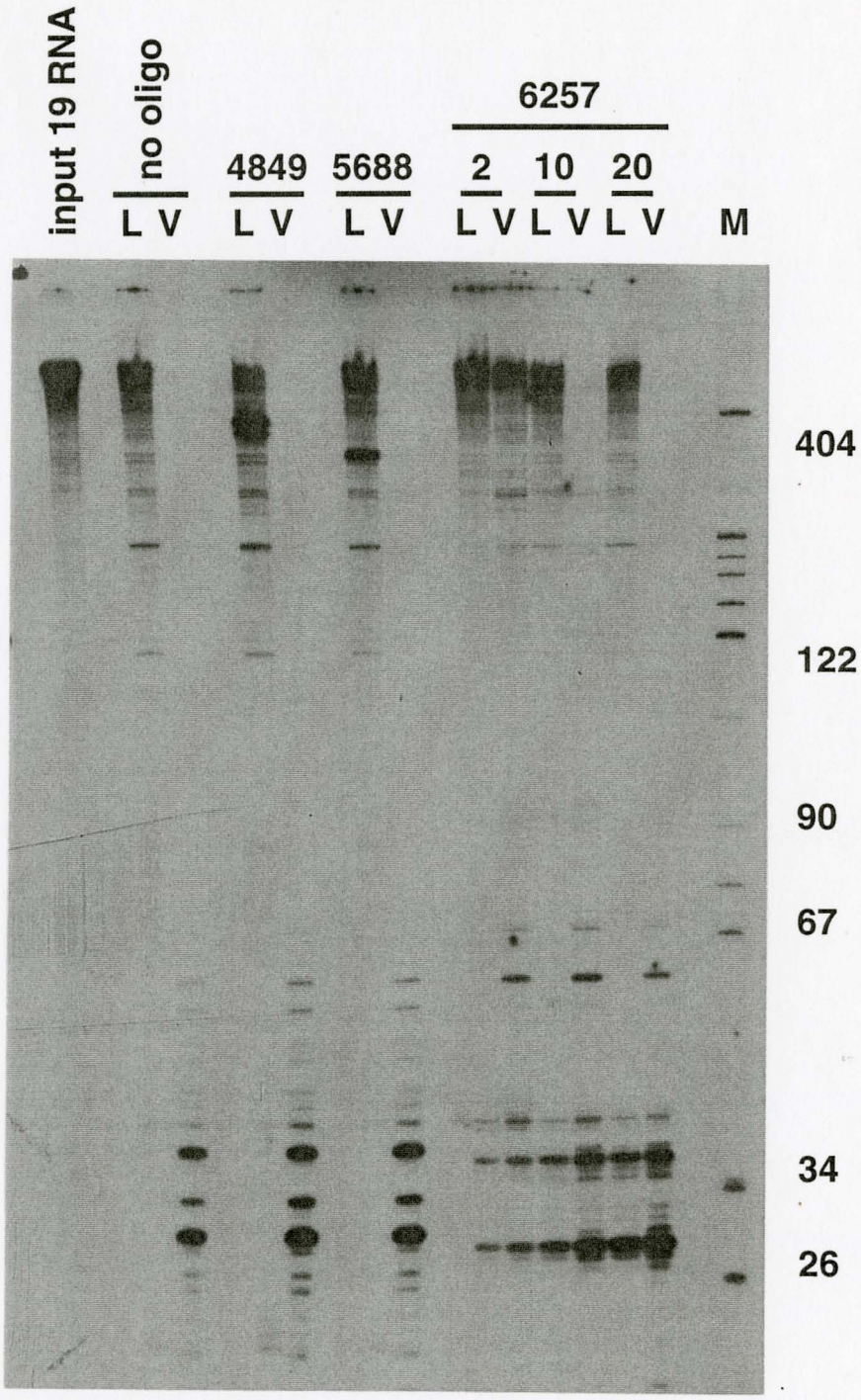
Cap labelled 19 and 19X RNA were annealed with the following oligos as described section 3.6. The sequence of the oligos are shown with dNTPs represented by G, C, A, T. The annealing site refers to the site downstream of the 5' end, in nt.

OLIGO	SEQUENCE	ANNEALING SITE
AB4849	GAGCTCTCCTTCTTGGCCCC	510 - 519
AB5688	CCCCCTGAACATTCTGGAAATCAAA	395 - 402
AB6257	GCGTTTATTCTGAGCTTCTGCAAAA	22 - 46
AB6586	GAACAAGCAAGCTTGTATTC	1 - 20

Figure 3.12 Alteration of vhs induced cleavage sites by annealed DNA oligos I.

Cap labeled 19 RNA was incubated separately with the DNA oligos described in Table 3.4 and allowed to anneal at 60 °C for 45 minutes. The annealing took place in Retic buffer which has the same ionic composition as rabbit reticulocyte lysate. The RNA: oligo complex was then added to lysate and lysate containing vhs. Samples were taken at the 2, 10 and 20 minutes for oligo AB6257 and at 20 minutes for the other oligos. The products were analyzed on a 20% sequencing gel. Marker sizes indicated in nt.

L: lysate alone; V: with pretranslated vhs.


input 19 RNA
no oligo
4849
5688
6257
2
10
20
L V L V L V L V L V L V
M
404
122
90
67
34
26

first 20 nt. of 19X RNA (1 through 19). The RNA : oligo complex, incubated with *in vitro* translated vhs, is cleaved to produce the same cap labeled oligos as with no oligo, demonstrating that the presence of this oligo has no effect on vhs induced cleavage. The results I obtained in this section indicate that vhs induced cleavage at preferred sites can be blocked by annealing a DNA oligo over the proposed sites. However, decapitation is not prevented but merely shifts to other preferable sites.

3.7 A capped mRNA substrate is preferentially targeted by vhs

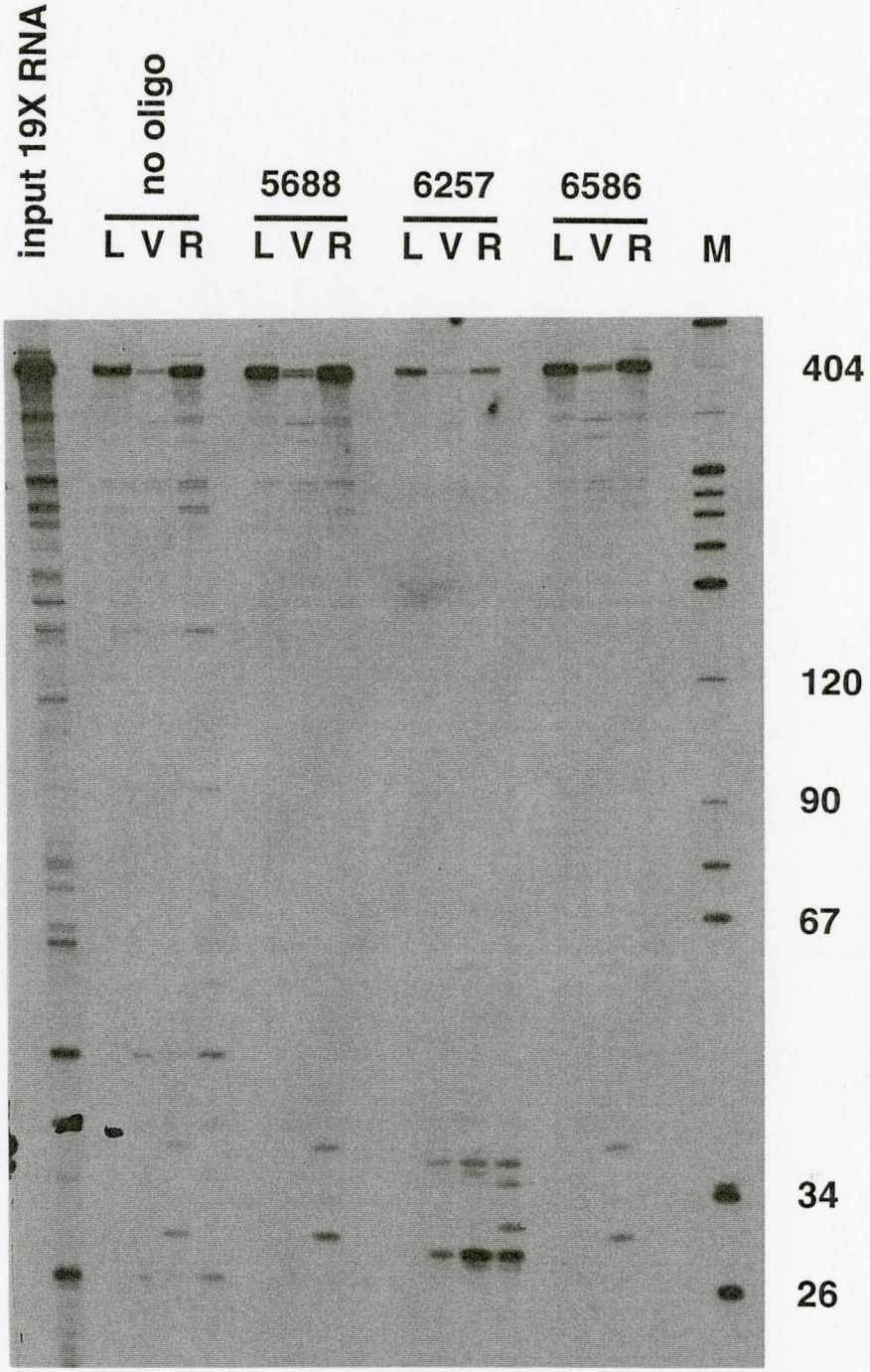
Given that vhs induced degradation selectively targets mRNAs *in vivo*, we hypothesized that the m^7G cap structure is the distinguishing feature which causes this specificity. Although the poly A tail is also a unique feature to mRNAs, our studies have used poly A minus substrates, which are actively degraded in the presence of vhs. This observation together with the vhs induced decapitation prompted us to investigate if mRNAs are targeted through the cap. To test our hypothesis, I initially asked if vhs activity, that is, the production of the cap labeled oligos, could be inhibited by the addition of cap analogs, such as m^7GTP. If vhs-induced degradation involves recognition of the cap structure then it may be possible to compete away mRNA targeting by increasing the concentration of the

inhibitor. In this experiment, vhs was pretranslated *in vitro* as usual for twenty minutes. Aliquots of the vhs reticulocyte lysate reaction were added to equal amounts of cap labeled 19X RNA containing various concentrations of either m^7GTP or GTP. The RNA was incubated in the presence of vhs for 20 minutes,

Figure 3.13 Alteration of vhs induced cleavage sites by annealed DNA oligos II.

Cap labeled 19X RNA (404 nt) was incubated with oligos AB5688, AB6257 and AB6536 (see Table 3.4) in Retic buffer at 60 °C for 45 minutes. The complexes were then added to lysate alone, lysate containing vhs and lysate containing recombinant RNaseH. The samples were analyzed on a 20% sequencing gel.

L: lysate alone; V: lysate containing vhs; R: lysate with RNaseH. Marker sizes indicated in nt.

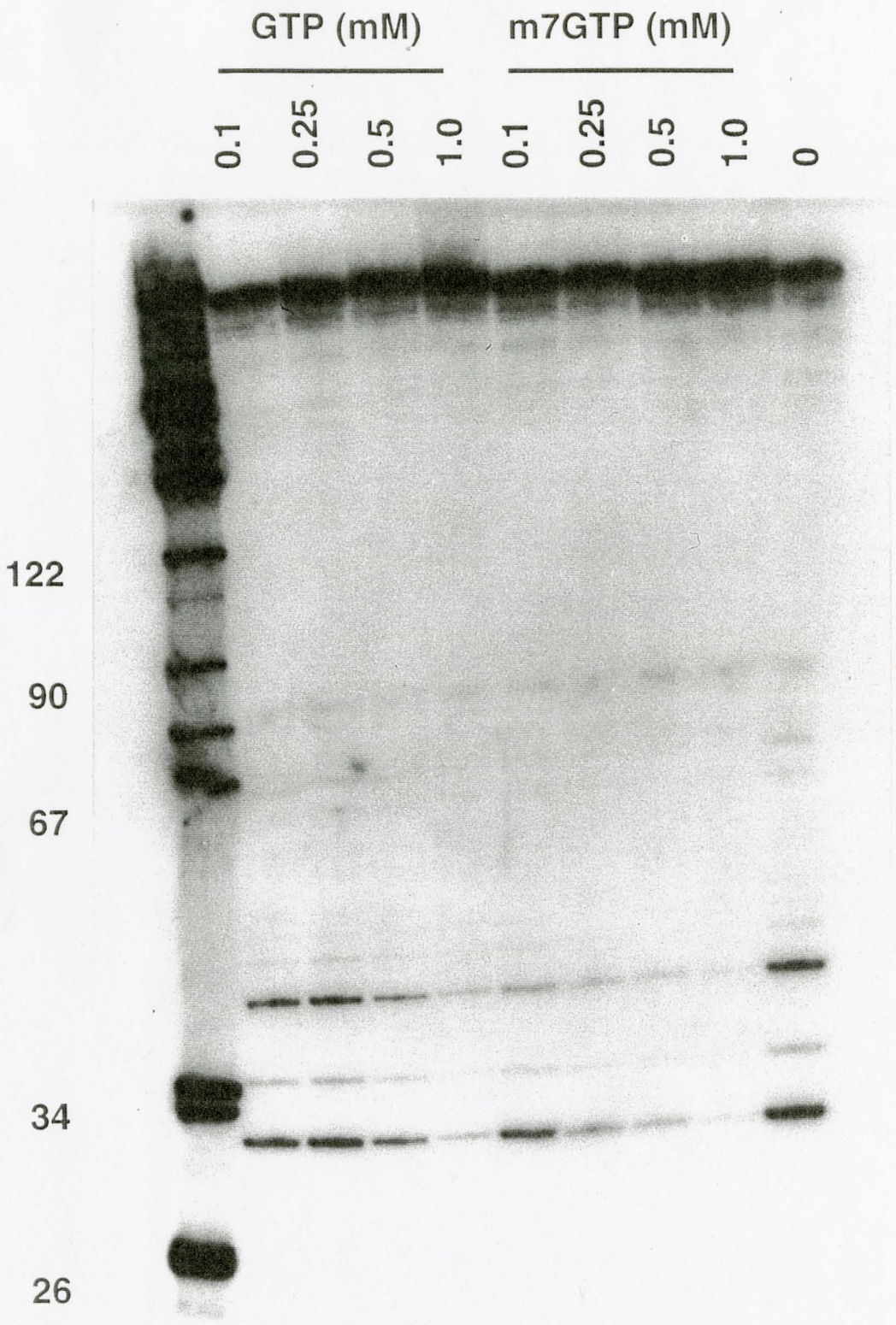

input 19X RNA
no oligo
5688
6257
6586
L V R
L V R
L V R
L V R
M
404
120
90
67
34
26

then purified and analyzed on a 20% sequencing gel. As illustrated in figure 3.14, increasing the concentration of m^7GTP caused a decrease in the abundance of the cap labeled oligos of 30 and 40 nt. A noticeable inhibitory effect is seen with the addition of m^7GTP and the effect increases with increasing concentration. I also tested to see if GTP would have any effect on the production of cap labeled oligos. It seems from the gel that GTP is inhibitory and its negative effect increases with concentration. However, GTP is not as effective at inhibiting vhs-induced degradation as m^7GTP since at equal concentrations the latter has a greater effect.

The fact that m^7GTP had a greater inhibitory effect than GTP does not conclusively demonstrate that mRNAs are targeted through the cap structure by vhs induced degradation. A better way of addressing this possibility is to ask whether a capped mRNA is preferentially targeted for vhs induced cleavage over an uncapped mRNA. If the cap structure of mRNAs is a source of recognition by the vhs system, then an RNA which lacks an m^7G cap should not be cleaved as efficiently, if at all. In order to eliminate the possibilities of different reactions conditions and substrate concentrations I decided to examine the degradation products of both capped and uncapped RNAs within the same reaction vessel. For this, I needed to use two different substrate mRNAs that gave rise to different cap
labeled cleavage products. This was necessary to track both sets of products within the one reaction.

Figure 3.14 Effect on vhs induced cleavage by the addition of cap analogs.

19X RNA was cap labeled and added to reticulocyte lysate aliquots containing equal amounts of pretranslated vhs and varying concentrations of GTP and m^7GTP (indicated in millimolar). Samples from the aliquots were then analyzed on a 20% sequencing gel. Marker sizes indicated in nt.


GTP (mM)
m7GTP (mM)
0.1
0.25
0.5
1.0
0.1
0.25
0.5
1.0
0
122
90
67
34
26

The two substrates I chose to use were 19X RNA and #47 RNA. Recall, that #47 RNA when tested in the sequence specificity experiments (see sect. 3.5), gave rise to cap labeled oligos of 65 and 75 nt. when incubated with *in vitro* translated vhs. For the initial experiment I decided to cap label #47 RNA. Capless 19X was generated by *in vitro* transcribing pSPSR19N (linearized with Xmn1) in the absence of the cap primer m^7GpppG (see Materials and Methods), resulting in an RNA possessing a 5' triphosphate end, rather than a cap. To label the capless 19X RNA, $\gamma\,^{32}$P-GTP was included in the transcription reaction. Since, the only nucleotide that retains the gamma phosphate is the penultimate 5' guanine residue, the 5' end is the only location that is radiolabeled. I termed this "gamma labeled". Therefore, the only difference between cap labeled #47 and 19X is the presence of the m^7G group in the former; the same phosphates are radiolabeled.

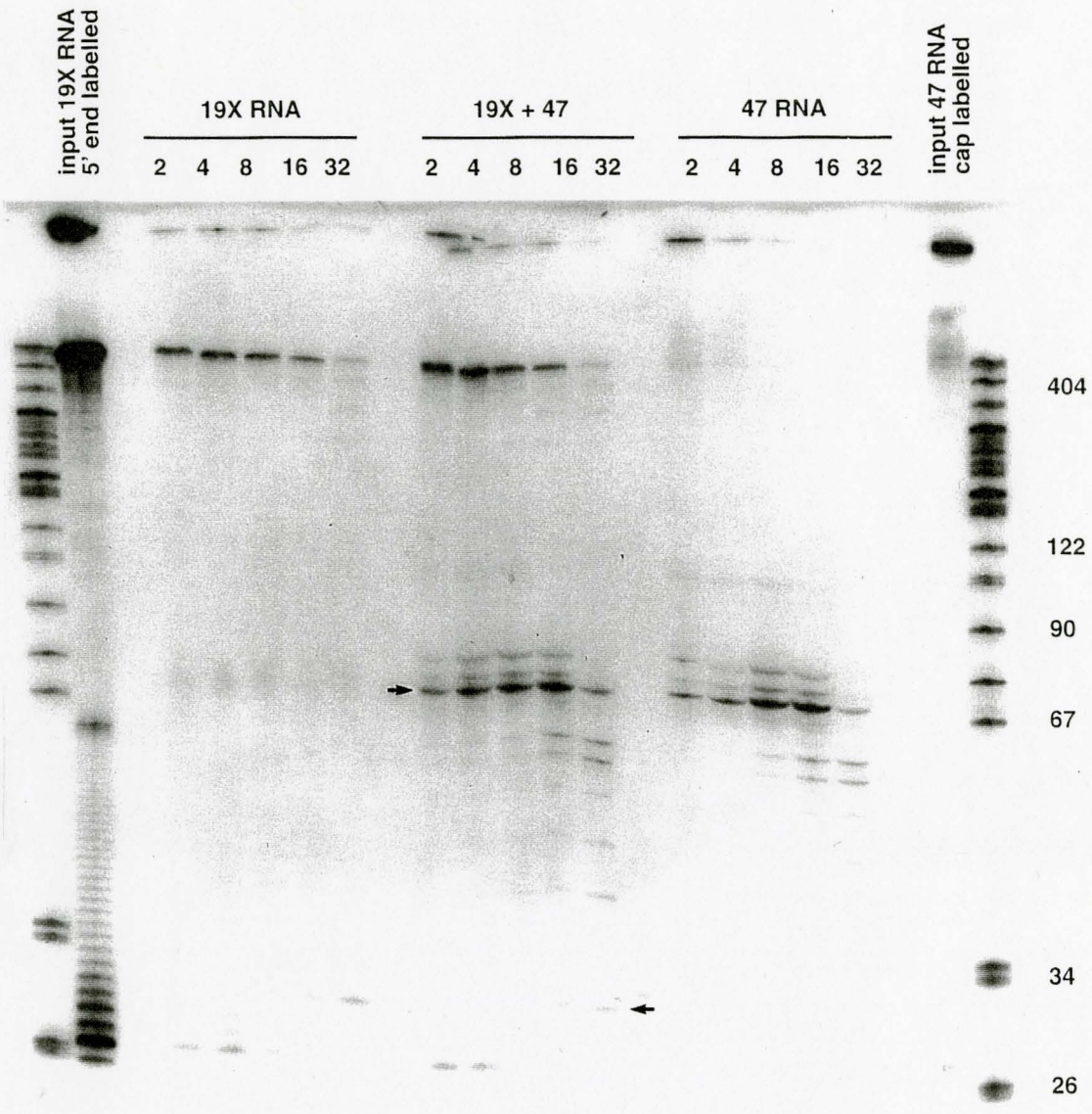
Cap labeled #47 RNA and gamma labeled, capless 19X RNA were used as substrates for reticulocyte lysates containing pretranslated vhs. The RNAs were incubated in the lysates separately or together in the same reaction. Samples were taken at various time points and the purified RNA was analyzed on a 20% sequencing gel (see Fig. 3.15). Cap labeled #47 RNA, when incubated separately from 19X, gave rise to the expected cleavage products of 65 and 75 nt. These products were apparent at the earliest timepoints and increased in abundance as the intact signal decreased. Capless 19X RNA, incubated alone, remained intact for much longer than #47 RNA and for longer than previously seen when cap labeled. At late times (see 32 min.), one labeled

degradation product seemed to appear. The apparent molecular weight of this product generated form capless 19X was 30 nt. When both RNAs were incubated together in the same reaction, completely analogous results were obtained. Again, cap labeled #47 RNA is cleaved to produce cap labeled oligos of 65 and 75 nt., seen at the earliest time points and capless 19X gives rise to a late appearing product of about 30 nt. These data suggest that the capped RNA (#47) is cleaved at a much faster rate than an uncapped RNA. However, the fact that a product is generated from 19X suggests that the vhs degradation system is preferential to capped substrates but not restricted to them. Although not present on this gel (Fig 3.15) the product generated from capless 19X is only seen in reactions containing active vhs. In order to determine the nature of the product generated from uncapped 19X, a separate experiment was performed in which, pretranslated vhs was incubated with cap labeled 19X and gamma labeled 19X independent of each other. The products from the reactions were run side by side on a 20% sequencing gel (Fig 3.16). The results of this experiment revealed that the product generated from capless 19X RNA migrated one base more rapidly than the 30 nt. cap labeled oligo produced in reactions containing cap labeled 19X. Although other products are present in the gamma labeled lanes, they also exist in the input and lysate alone samples. The 29 nt. product is the only one specific to vhs.

An interesting observation was made from the degradation pattern of cap labeled #47 RNA (fig 3.15). At later time points it seems that the cap labeled

Figure 3.15 Comparison of the degradation products of capped versus uncapped RNAs I.

5' labeled uncapped 19X RNA and cap labeled #47 RNA (see Table 3.2) were added separately and in combination to lysate containing pretranslated vhs. Samples were taken at the times indicated (in minutes) and analyzed on a 20% sequencing gel. The arrows indicate the vhs cleavage products from each RNA. Marker sizes indicated in nt.


input 19X RNA
5' end labelled
19X RNA
2 4 8 16 32
19X + 47
2 4 8 16 32
47 RNA
2 4 8 16 32
input 47 RNA
cap labelled
404
122
90
67
34
26

oligos of 65 and 75 nt. are themselves undergoing further degradation. This is most apparent at 32 minutes when the 65 and 75 nt. oligos become less abundant as new, smaller products begin to appear. This ongoing decay of the cleavage products has not been seen using cap labeled 19 RNA as a substrate.

Although the above data strongly suggests that capped RNAs are preferred, it may be possible that #47 was inherently a better substrate than 19X and is therefore, cleaved faster, independent of the presence of the cap. To test this possibility the experiment was repeated, however, the RNAs were labeled opposite to the previous experiment. 19X was cap labeled and #47 RNA was gamma labeled. In this case (Fig. 3.17), cap labeled 19X RNA gave rise to the expected products of 30 and 40 nt., whereas, no products (specific to vhs) seemed to be generated from the gamma labeled #47. With both RNAs in the same vhs reaction, identical results were obtained. Furthermore, when the experiment was repeated with both RNAs capped, the expected cap cleavage products (30 and 40 for 19X and 64 and 75 for #47) were generated from both substrates at roughly equivalent rates (Fig. 3.18). The data gathered from the above experiments using two substrate RNAs clearly demonstrates that RNAs possessing an m^7G cap structure are preferentially targeted for degradation over capless RNAs.

Figure 3.16 Side by side analysis of cleavage products of capped and uncapped 19X RNA.

Cap labeled and 5' end labeled (uncapped) 19X RNA were added separately to pretranslated vhs. Samples were taken at the indicated times (in minutes) and analyzed on a 20% sequencing gel.

C: cap labeled 19X incubated with vhs; F: 5' end labeled 19X RNA incubated with vhs; RRL: samples from lysate alone. Marker sizes are indicated in nt.

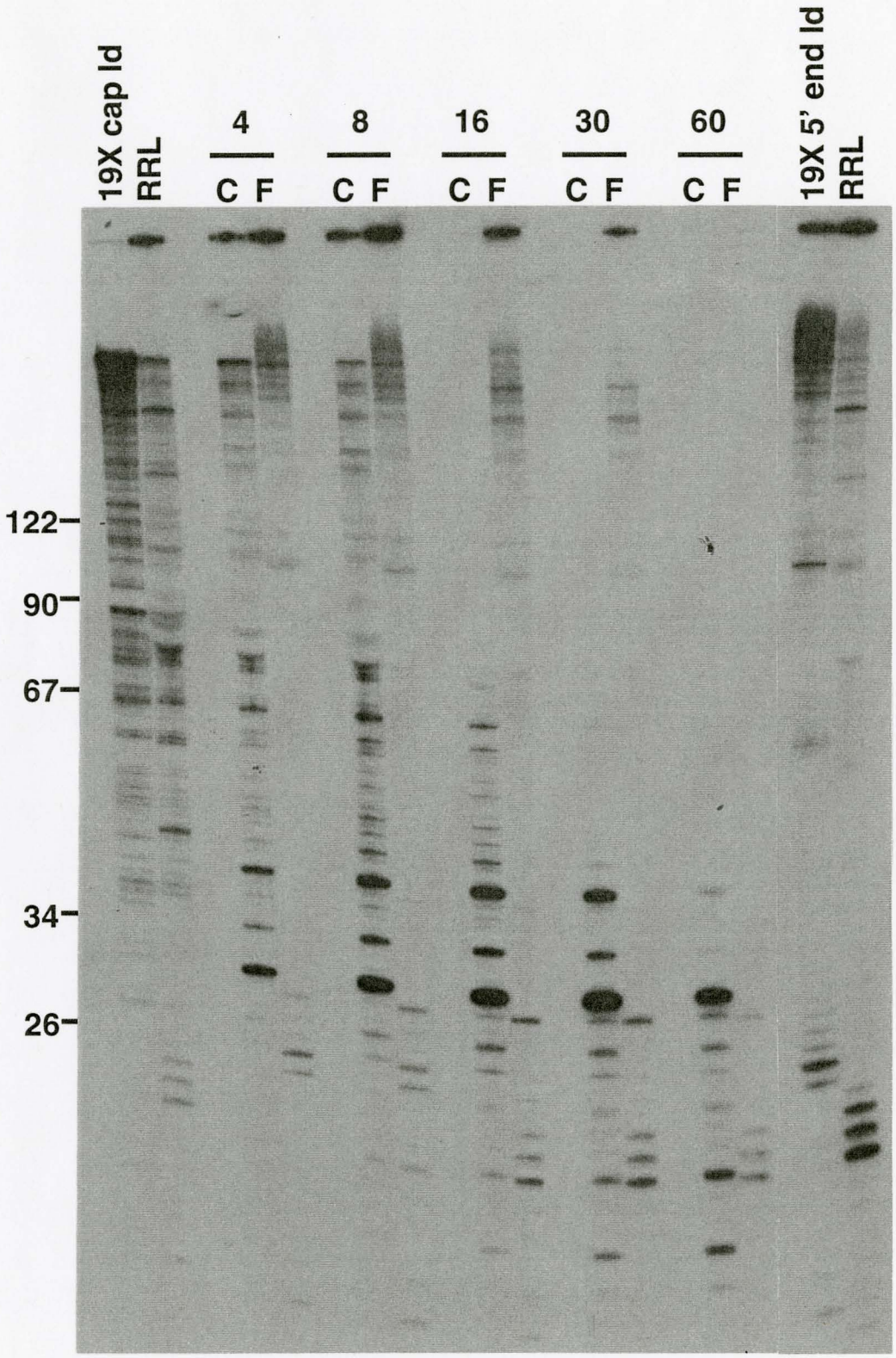

19X cap ld
RRL
4
8
16
30
60
C F
C F
C F
C F
C F
19X 5' end ld
RRL
122
90
67
34
26

Figure 3.17 Comparison of the degradation products of capped versus uncapped RNAs II.

Cap labeled 19X and 5' end labeled #47 RNA (opposite to figure 3.15) were added separately and in combination, to lysate containing pretranslated vhs. Samples were taken at the times indicated (in minutes) and analyzed on a 20% sequencing gel. RRL: samples from retic alone. Marker sizes indicated in nt.

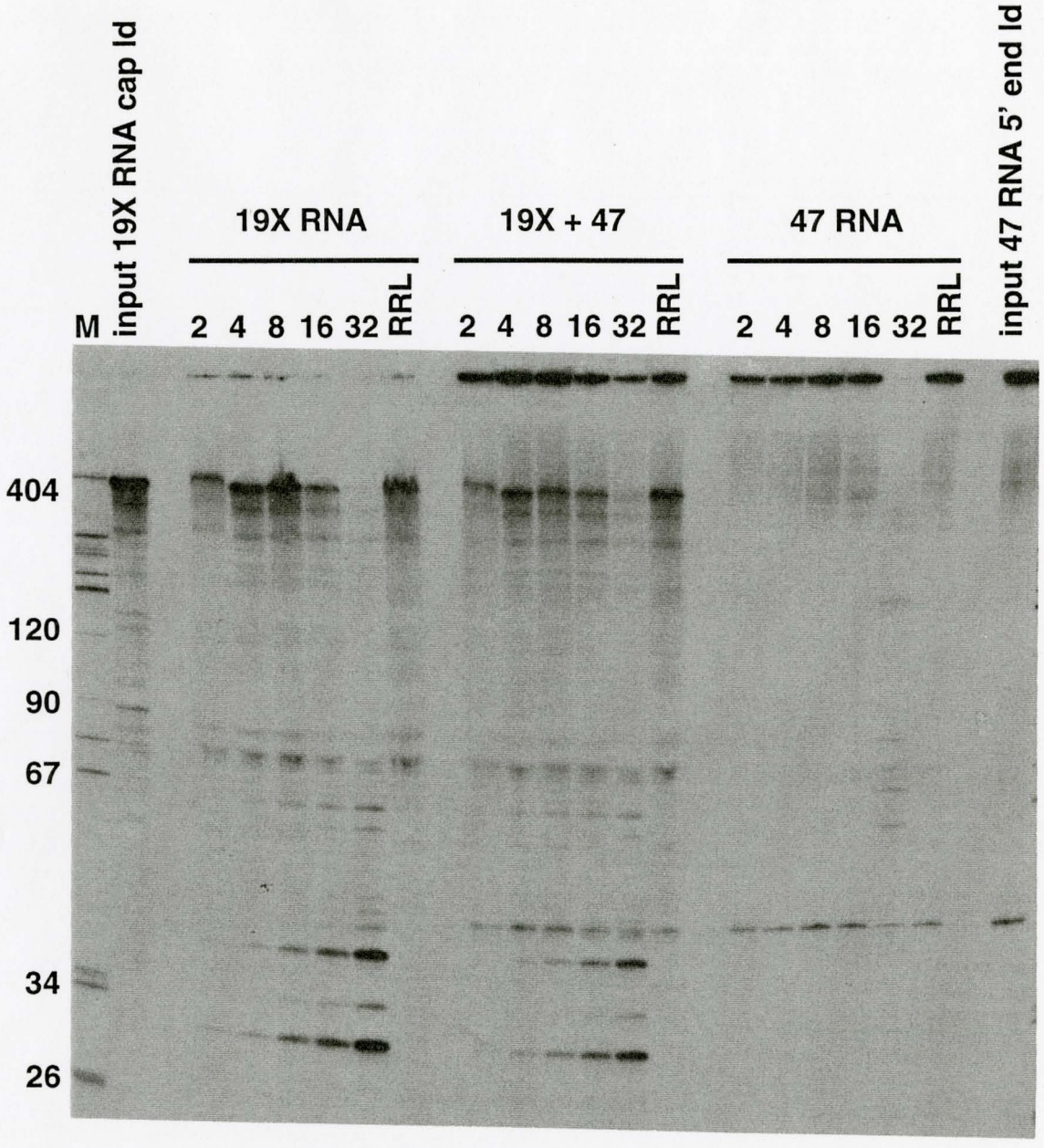
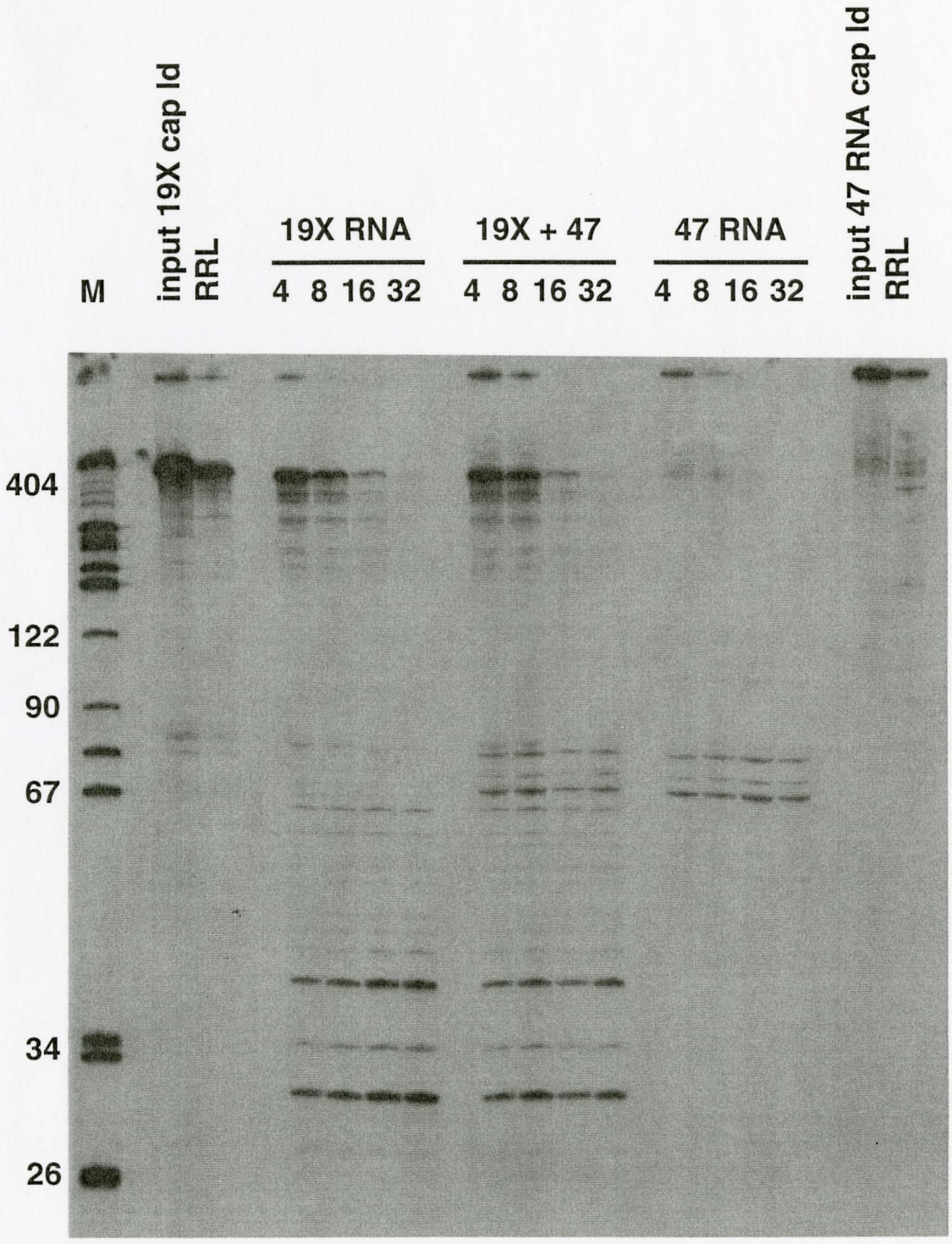

M
input 19X RNA cap ld
19X RNA
19X + 47
47 RNA
2 4 8 16 32 RRL
2 4 8 16 32 RRL
2 4 8 16 32 RRL
input 47 RNA 5' end ld
404
120
90
67
34
26

Figure 3.18 Comparison of the degradation products of two different capped substrates.

Cap labeled 19X and #47 RNA were added separately and in combination to pretranslated vhs. Samples were taken at the indicated times (in minutes) and analyzed on a 20% sequencing gel. RRL: samples from lysate alone. Marker sizes indicated in nt.


M
input 19X cap Id RRL
19X RNA
4 8 16 32
19X + 47
4 8 16 32
47 RNA
4 8 16 32
input 47 RNA cap Id RRL
404
122
90
67
34
26

IV. DISCUSSION

4.1 vhs induced mRNA degradation *in vivo*

In the initial section of my project, I examined the degradation of beta globin mRNA in MEL cells infected with HSV. Purified RNA samples were analyzed by gel electrophoresis followed by Northern hybridization using a radiolabeled DNA probe complementary to beta globin mRNA. In all cases the results demonstrated that infection with HSV caused a decrease in the levels of intact beta globin mRNA as time progressed. These results agree with previous observations describing the decay of mRNA in MEL cells (Nishioka and Silverstein, 1977; Nishioka and Silverstein, 1978; Smibert and Smiley, 1990). In my experiments, globin mRNA degradation was evident immediately following infection with HSV-2 333, with a half-life of approximately 15 minutes. Degradation was not seen when MEL cells were infected with the vhs mutant strain, vhsB, which demonstrates the requirement for a functional UL41 ORF for virion-associated shutoff (Fenwick and Everett, 1990; Read and Frenkel, 1983). Similar results were obtained with HSV-1 pAAR5 infected cells. Again beta globin mRNA decreased over time, with a half-life of approximately 60 minutes; a slower rate than that observed with HSV-2 infection. This can be attributed to the stronger shutoff effect seen with type 2 strains of HSV (Fenwick and Everett, 1990; Schek and Bachenheimer, 1985).

The cause for the different rates between HSV-1 and HSV-2 is not clear. An easy interpretation would be that vhs of HSV-2 possesses a stronger ability to induce degradation of mRNAs. This has been shown to be true through experiments in which the HSV-2 (G) analogous UL41 gene was placed into the genome of HSV-1 strain F, under the control of the its own promoter (Fenwick and Everett, 1990). In this case, the stronger shutoff phenotype was dominant. However, when HSV-1 (F) and HSV-2 (G) undergo a mixed infection, the weak shutoff is dominant (Fenwick and Everett, 1990). If vhs were simply an RNase, it would be hard to imagine why a mixed infection would not mimic the phenotype of the stronger RNase, or at least an intermediate phenotype. This may suggest that vhs functions as part of a complex or pathway involving other viral and/ or cellular factors. Evidence to support this hypothesis has come from experiments performed in our laboratory by Mabrouk Elgadi. Using *in vitro* translated vhs from HSV-1 and HSV-2, the rate of mRNA degradation seems to be equal between the two, suggesting that other viral factors modulate the difference between HSV-1 and HSV-2.

The results I obtained from these initial experiments demonstrate that degradation of beta globin mRNA occurs rapidly in HSV infected cells. However, in no case were degradation intermediates detected. The results indicate that the mRNA is either present in its intact form or too small for detection using Northern hybridization. In order to detect small changes in the beta-globin signal, I attempted to focus on different regions of the mRNA molecule. This was done using DNA oligos that hybridized to various beta globin mRNA

sequences, and RNase H to cleave the mRNA / oligo into two segments. The aim of this line of experiments was to determine if any polarity existed with vhs induced decay, that is, was degradation 5' to 3' or 3' to 5'. Small changes in the size of the segments would indicate such a polarity. However, although many attempts were made, the technique was never perfected and the results obtained seemed to suggest that no difference in the decay rates existed between the two RNA segments.

From these and the above results we concluded that vhs induced degradation of globin mRNA in MEL cells was too rapid to detect intermediates using Northern hybridization. We suggest that once an mRNA is targeted for degradation, it is rapidly destroyed through an unknown process. The difference between HSV-1 and HSV-2 seemed to be the rate at which mRNAs were targeted, which may represent the rate-limiting step. In retrospect, globin mRNA may have not been the ideal mRNA to analyze. Globin mRNA is very stable in its intact form (Lowenhaupt and Lingrel, 1978), however, it has been reported that modification to the transcript (such as the insertion of nonsense codons) causes immediate and rapid degradation of the mRNA (Maquat et al., 1981). Thus the cell may be posed to rid itself of aberrant transcripts that might cause deleterious effects. Perhaps a better way of tracking the decay of mRNA during HSV infection would be to use transcripts that possess regions that are resistant to nucleolytic attack. This approach has been used in several mRNA turnover studies as a method of trapping degradation intermediates (Decker and Parker, 1993; Muhlrad et al., 1994; Muhlrad and Parker, 1994).

4.2 *In vitro* Degradation Assay for vhs

The development of an *in vitro* assay that demonstrated the ability of vhs to induce mRNA degradation was crucial to my project. Using this rabbit reticulocyte system and various RNA substrates, I was able to show that the presence of functional vhs causes degradation of mRNAs. This observation is consistent with other studies in that vhs is the only viral protein required for RNA decay (Jones et al., 1995; Pak et al., 1995; Zelus et al., 1996). Agarose-formaldehyde gel electrophoresis of cap labeled 19 RNA compared with reprobed or internally labeled 19 RNA, showed that vhs induced rapid decay at, or near, the 5' end before the body of the RNA underwent complete destruction. The loss of the intact signal of cap labeled 19 and 19X RNA coincided with the production of two major cap labeled oligos ca. 30 and 40 nt. in length, that remained stable over the course of the experiments. These oligos were the only degradation products detected using 19X RNA indicating that their generation results from an early or initial decay event. This event is not likely mediated by a 5' to 3' exonuclease since this would result in release of the labeled cap structure in the form of a mono- or dinucleotide.

The cap labeled oligos could be generated in two ways: either through the action of a 3' to 5' exonuclease that pauses at sites 30 and 40 nt. downstream of the cap; or by endonucleolytic cleavage at these sites. Primer extension demonstrated that the latter is true. Using a DNA primer that annealed to 19 RNA (84 bases downstream of the cap), three prominent extension products were observed. These represented 19 RNA with an intact 5' end (i.e.

84 nt. long) and two other products of 54 and 44 nt.. The generation of these new ends was consistent with cleavage events occurring downstream from the cap. From these results we proposed the term "decapitation" to describe the activity induced by *in vitro* translated vhs. Decapitation was also seen using other cap labelled RNA substrates. Changing the sequence of the 5' UTR altered the sites where the cleavage occurred within 20 to 80 nt. from the 5' end. The selection of the cleavage site(s) did not seem to be simple sequence or secondary structure specific, however, it could be a more complex combination of the two. Interesting results were obtained using DNA oligos to block the proposed cleavage sites of 19 RNA. It appears that the annealing of an oligo over the area of cleavage does not prevent decapitation but merely shifts the location to preferred sites adjacent to the DNA oligo. Taken in combination, these results indicate that the cleavage sites are not dictated by a fixed distance from the 5' end of the mRNA.

Through the use of cap analogs and uncapped RNA substrates, my results indicate that vhs induced degradation shows a strong, but not absolute, preference for m^7G capped mRNAs. Whenever an RNA possessed a cap structure it was rapidly cleaved, whereas a capless RNA was degraded at a much slower rate. The apparent preference for capped RNAs provides a simple explanation for the specificity for mRNAs, and not rRNA or tRNA, during *in vivo* infection. Thus, the m^7G cap may serve as a target for vhs induced degradation of mRNAs. This seems plausible considering that the degradation of viral mRNAs (which are capped) is also induced by vhs (Kwong and Frenkel, 1987;

Oroskar and Read, 1989; Read and Frenkel, 1983; Strom and Frenkel, 1987). Despite the observed preference for capped mRNAs, our laboratory has demonstrated that the vhs protein does not bind to m^7GTP sepharose, nor is the cellular CBP eIF4E required for decapitation (data not shown). This implies that vhs neither possesses a cap binding domain nor uses the CBP as a targeting factor. Furthermore, vhs is still as active in post-ribosomal supernatant (Elgadi et al., in preparation) indicating that ongoing translation is not required for mRNA destabilization and that vhs is not closely associated with ribosomes. Perhaps mRNA recognition involves more than the m^7G moiety as sequences downstream of the cap may be influential. It is also possible that vhs may function through other components of the cap recognition complex (such as p220, eIF4B) utilizing their mRNA association capabilities.

Recent studies in our laboratory have yielded results that are in apparent conflict with the conclusion drawn from my cap preference experiments (data not shown). Using agarose-formaldehyde gel electrophoresis to compare vhs induced degradation of internally labeled capped 19 RNA and its uncapped equivalent, Mabrouk Elgadi found that the decay rates and patterns for the two substrates are similar. This conflicts with the cap vs. gamma labeled experiments in that my results demonstrate that the uncapped RNA does not give rise to the expected cleavage products obtained from cap labelled RNA. It may be possible that the 5' end labeled oligos are generated just as rapidly from an uncapped RNA but are unstable and are themselves degraded. However, this seems unlikely since my results also indicate that the uncapped substrate

remains intact much longer than the capped RNA and is therefore not consumed at the same rate. A possible reason for this difference is that, in the absence of a cap, vhs causes destruction of the RNA by another mechanism than the one that I have described. This could involve 3' to 5' degradation or endonucleolytic decay closer to the 3' end. These events could go undetected using 5' end labeled substrates and polyacrylamide gel electrophoresis. Zelus et al. (Zelus et al., 1996) have recently shown that vhs from wild-type virion extracts was capable of inducing 3' end directed cleavage of uncapped and capped RNAs at adenosine rich regions. Thus, without a preferred target vhs may initiate a more random process of mRNA decay. Experiments are currently being performed by Mabrouk Elgadi in an attempt to further clarify the above confusion.

How does the activity of vhs compare with other viral systems? Decapitation is similar to the mRNA cleavage ability of the influenza virus RNA polymerase. The polymerase possesses a cap-dependent endonuclease that cleaves mRNAs approximately 13 nt downstream of the cap and uses the capped RNA pieces as primers (Plotch et al., 1981). The vhs protein differs in this respect as cleavage does not occur at a fixed distance from the 5' end. Furthermore, vhs activity is dispensable for viral growth (Read and Frenkel, 1983), whereas influenza requires its endonuclease for transcription of viral genes. The degradation of mRNAs induced by vhs is perhaps more analogous to the activity seen with the L-A virus of *Saccharomyces cerevisiae*. This RNA virus removes the caps from the yeast transcripts in order to trigger the cell to degrade its own mRNAs via the Xrn1p exonuclease; a component of the cell's

RNA turnover pathway (Masison et al., 1995). This partially frees the L-A viruses capless RNA genome from decay allowing viral expression. Similarly, vhs triggers decapitation which is followed by destruction of the entire transcript. Although, this also results in the decreased stability of viral mRNAs, the rapid expression of HSV genes may allow the viral transcripts to escape degradation and thus be translated into proteins.

The fate of the body of an mRNA, following decapitation, was not determined in this project. However, the use of Northern hybridization and internally labeled substrate mRNAs indicates that, in the presence of vhs, the entire transcript becomes unstable and is eventually reduced to low molecular weight products. Furthermore my primer extension data reveals that the RNA body, following decapitation, is unstable as the primer extension products corresponding to the body's 5' end becomes less abundant over time. In fact, new, smaller primer extension products appear. This suggests that destruction of the RNA body occurs after decapitation in a process that at least involves decay at the 5' end of the decapped product. Determining the form in which these products exist (e.g. oligo or mononucleotides) will provide some insight into the decay pathway that ensues the initial cleavage(s) and whether vhs plays an active role in the subsequent events. An interesting model would involve pre-existing cellular mRNA decay pathways. The presence of premature nonsense codons in eukaryotic mRNAs results in rapid decapping followed by degradation of the transcript; a process known as mRNA surveillance (reviewed in Beelman and Parker, 1995) . In yeast decapping results in decay via the Xrn1p 5'

exonuclease and similar 5' end decay has been seen in mammalian cells (Lim et al., 1992; Muhlrad and Parker, 1994). Perhaps vhs induced decapitation mimics the decapping event in cells and thus by-passes the initial step in the mRNA surveillance pathway. Alternatively, vhs may actively continue degradation following decapitation. Whether this involves a processive endonuclease activity or a random decay (exo or endo), remains to be determined.

For several reasons the vhs induced decapitation model provides a plausible mechanism for HSV mediated host shutoff *in vivo.* First, several studies have shown that the cessation of translation induced by HSV infection, precedes detectable loss of mRNA (Nishioka and Silverstein, 1978; Schek and Bachenheimer, 1985). The loss of 20 to 60 bases (for example) from the 5' end of an mRNA, as suggested by our decapitation model, may possibly go undetected using conventional Northern analysis, however, it would result in an immediate loss of translational ability due to the absence of the cap. Translation inactivation of mRNA by HSV has also been shown by Fenwick and McMenamin (1984). Second, a recent study by Kaar and Read (1996, in press) demonstrates that degradation of mRNAs induced by vhs, involves a general 5' to 3' polarity *in vivo.* Thus a cleavage event downstream of the cap could initiate such decay with an activity similar to that of the Xrn1 exonuclease following. Third, experiments performed in our laboratory by Evan LLewelyn using the vhs homologue of pseudorabies virus indicate that this protein induces degradation of mRNAs *in vitro* by a similar decapping event. None of the *in vitro* experiments that I performed used RNA substrates with polyA tails. This suggests that the

polyA tail is not required for vhs induced decay. Similar conclusions were made from *in vivo* studies showing that vhs does not alter the overall length of polyA tails (Kaar and Read, in press). mRNA surveillance does not require prior deadenylation for decapping to occur (Muhlrad and Parker, 1994), providing further support for vhs as a by-pass step in the deadenylation-independent decay pathway. It would be interesting to see if HSV infected cell lysates to which cap labelled mRNA substrates were added, generates cap labelled oligos similar to those observed *in vitro*. My *in vivo* experiments were not stringent enough to be able to detect such small products, however, this could be overcome is radioactive substrates could be used.

How does our proposed model of vhs activity fit into a typical HSV infection? VP16 and vhs are believed to be in a complex in the tegument (Smibert et al., 1994). Following viral penetration, the vhs/ VP16 complex dissociates in the cytoplasm as VP16 translocates to the nucleus. Active vhs then engages in the destruction of pre-existing cellular mRNAs by decapitation. This immediately induces translational arrest that frees up cellular components for viral protein synthesis. My data provides evidence that vhs targets mRNAs through their cap structure. Whether this is direct or indirect is still unknown. It is possible that vhs acts through cellular components associated with the mRNP complex. Later during infection new VP16 synthesis may downregulate the activity of vhs and enable its packaging into virions (Lam et al., 1996).

There are still many questions that need answering in order to fully understand vhs induced shutoff. A major question asks whether vhs itself possesses the RNase activity required for decapitation, or does it act as an effector that recruits other cellular proteins to perform this task? Recent data, providing support for vhs as an mRNase, has come from studies in Jeffrey Ross' lab (Zelus et al., 1996). Using vhs from crude virion extracts, they were able to demonstrate that vhs was capable of degrading homopolymeric RNA substrates in the absence of cellular proteins. Furthermore, incubating the extract with an antibody to vhs caused inhibition of degradation. Since these experiments used crude sources of vhs it cannot be unequivocally concluded that vhs itself possesses an mRNase function. Does vhs recognize the cap structure or is this an indirect effect of vhs' association with a translation initiation factor or other mRNP component? The isolation and purification of biologically active vhs, currently being attempted by Mabrouk Elgadi, will shed much light on these questions. Determining the fate of the body of the mRNA and what role vhs plays in this is also critical to our further understanding of vhs. Although my *in vivo* studies did not provide much information about vhs' activity during infection, further investigation using different mRNAs and techniques that may be able to trap degradation intermediates, may yield results that directly correlate with our *in vitro* data. In any case, the results presented in this report provides novel material about the function of a viral trans-acting regulator of mRNA stability.

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