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STUDIES OF THE VIRION HOST SHUTOFF FUNCTION OF HERPES SIMPLEX VIRUS

by

Annette Schmidt Pak

A Dissertation Submitted to the Faculty of the Graduate School

of Loyola University Chicago in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

January

1992

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INTRODUCTION

Upon infection with herpes simplex virus, cellular protein synthesis is rapidly suppressed. This phenomenon, called the virion host shutoff (vhs) function (Read and Frenkel 1983), is brought about by a virion protein which induces the degradation of cellular mRNAs (Oroskar and Read 1987; Kwong and Frenkel 1987). Interestingly, wild-type HSV virions have also been found to decrease the half-lives of HSV mRNAs (Oroskar and Read 1989). Consequently, the virion host shutoff activity of HSV is believed to nonspecifically induce the degradation of most mRNAs.

By mapping the mutation in the host shutoff mutant virus *vhs* 1 (Kwong *et al*. 1987), the *vhs* gene was found to be coded by the UL41 open-reading frame of herpes simplex virus (McGeoch *et al*. 1988). Using wild-type and *vhs* deletion mutant viruses, the *vhs* polypeptide has also been identified within purified virions and infected cells (Read *et al*., unpublished data). Although these studies have clearly demonstrated that the *vhs* gene is required for the induction of mRNA turnover, it could not be discerned whether the *vhs* gene product is the only viral protein required for this function.

There exist two possibilities in which viral proteins may

be involved in the host shutoff function. Either the UL41 (vhs) gene product acts in conjunction with one or more viral polypeptides to induce the degradation of mRNAs, or the vhs gene product is the only viral polypeptide required for this function. To determine which of these alternatives, in fact, occurs, we sought to develop an assay which could limit the number of viral genes expressed and assay the viral gene products for vhs activity.

Our assay involved the transfection of the vhs gene either alone or with a defined number of other viral genes. After establishing that this assay does, in fact, reflect the host shutoff activity of HSV, we were able to conclude that the vhs gene is the only viral gene required for the HSV host shutoff activity. The expediency and efficiency of this assay also allowed us to use it to screen in vitro generated mutants of the vhs gene for their effect on host shutoff function. Similarly, in vitro constructed recombinants of vhs genes from HSV types 1 and 2 were screened for host shutoff activity to gain a further understanding of the structure and function of the gene.

LITERATURE REVIEW

The family herpesviridae.

Herpesvirus infections are widespread in nature. Indeed, herpesviruses have been isolated not only from humans but also from most vertebrates. Nearly 100 viruses have been classified into the family herpesviridae. At least six distinct human herpesviruses have been isolated, namely, herpes simplex viruses (HSVs) types 1 and 2, cytomegalovirus, varicella zoster virus, Epstein Barr virus and, most recently, human herpes virus 6. Classification of herpesviruses has been largely based on the structure of the virion. There are four structural components that belong to all herpesvirions; 1) a toroidal core which contains the double stranded linear DNA genome, 2) an icosahedral capsid enclosing the core, 3) an amorphous structure surrounding the capsid called the tegument, 4) a lipid bilayer envelope serving as the outer covering of the herpesvirus. Members of the family herpesviridae vary considerably in their biological properties, however, one unique property belonging to all herpesviruses is their ability to remain latent in their natural host.

The herpesviridae family has been classified into three

subfamilies based on their biological properties. The properties include host range, duration of the reproductive cycle, cytopathology, and characteristics of the latent infection. The first subfamily, alphaherpesvirinae, have a variable host range, a short reproductive cycle, are highly cytopathic in cultured cells, and establish latent infections in the sensory ganglia. The human herpesviruses belonging to this subfamily are herpes simplex viruses types 1 and 2 and varicella zoster virus. The next subfamily, betaherpesvirinae, have a narrow host range and a relatively long replication cycle. They are less cytopathic and the host cells frequently become enlarged (cytomegalia) both *in vivo* and *in vitro*. The betaherpesviruses may establish latent infections and repeatedly be reactivated under certain stimuli. It has not, however, been fully established which cells may become latently infected. Cytomegalovirus is the human herpesvirus belonging to this subfamily. Members of the last subfamily, the gammaherpesvirinae, have a narrow host range with a predilection for lymphoblastoid cells. The infection is often arrested in the prelytic or lytic stage and does not produce infectious virus. Latent gammaherpesviruses are often found in lymphoid tissue and like the other members of herpesviridae, may be spontaneously reactivated. The human herpesvirus belonging to this subfamily is the Epstein-Barr virus.

Herpes simplex virus.

Records of diseases resembling herpes simplex virus infections date back to ancient Greek times. Today, HSVs have been reported worldwide in both developed and underdeveloped countries. HSVs cause a variety of infections in humans and are a major cause of venereal disease. Both HSVs types 1 and 2 have also been demonstrated to transform cells in tissue culture. In the past, HSV-2 has been associated with cervical squamous cell carcinoma, however, a definite link between HSV-2 and oncogenic activity has not been established. Aside from the various clinical aspects, HSV has intrigued numerous investigators because of its unique biological properties including the ability to remain latent throughout the life of its natural host. With the developments in molecular biology, our knowledge of HSV has increased dramatically over the past 15 years. HSV is now often used as a model for the study of synaptic connections in the nervous system, gene regulation, and translocation of proteins in viruses as well as eukaryotic cells.

HSV virion structure.

As described above, all members of the family herpes-viridae contain four basic structural elements. A more detailed description of the HSV virion follows:

1) The core, appears as a fibrillar spool with an outer diameter of 75 nm and an inner diameter of 18nm. The linear double stranded genomic DNA is found

wrapped around this torus shaped core (Furlong et al. 1972; Schrag et al. 1989).

2) The icosahedral capsid is 100 nm in diameter and is made of 150 hexameric and 12 pentameric capsomers (Wildy and Watson 1963; Steven et al. 1986).

3) The tegument is an electron dense section of the virion between the capsid and the envelope. It appears to be made of globular protein and its thickness varies depending on the location of the virion in the cell (Roizman and Furlong 1974; Fong et al. 1973).

4) The envelope is composed mainly of host specific phospholipid from the nuclear membrane (Ben-Porat and Kaplan 1971). The envelope contains numerous glycoprotein spikes which are about 8 nm in length (Wildy and Watson 1963; Stannard et al. 1987).

The diameter of the herpes virion has been reported to vary from 120-280 nm (Roizman and Furlong 1974). This is partly due to the variable size of the tegument. The diameter of the virion is also affected by the state of the envelope. Virions with damaged envelopes often have a much larger diameter than

an intact virion.

HSV genome.

As mentioned above, the genomes of HSVs types 1 and 2 are linear double-stranded molecules packaged in the form of a toroid. HSV DNAs, as well as other herpesvirus DNAs, have been found to contain nicks and gaps (Wadsworth et al. 1976). The HSV genome is approximately 150 kb in length and the G-C content for HSV-1 and HSV-2 are 68% and 69% respectively (Becker et al. 1968; Keiff et al. 1971). Recently, DNA nucleotide sequence the entire genome of HSV-1 strain 17 was published and was found to be 152,260 bp in length and has a G-C content of 68.3% (McGeoch et al. 1988). Intertypic and intratypic variations in DNA structure will be discussed below.

An interesting feature of the HSV DNAs is their sequence arrangements. HSV DNA is usually described as consisting of two covalently linked components. The components have been designated L (long) and S (short) and comprise 82% and 18% of the genome respectively. The two components contain largely unique sequences (U_L and U_S) and both are flanked by inverted repeats (Sheldrick and Berthelot 1975). There are reiterated sequences bordering both the L and S components. The sequences bordering the L component are designated ab and b'a' and the sequences bordering the S component are designated ca and a'c' where the primed letters are the inverted repeat sequences. A schematic representation of the genome is shown in Figure 1.

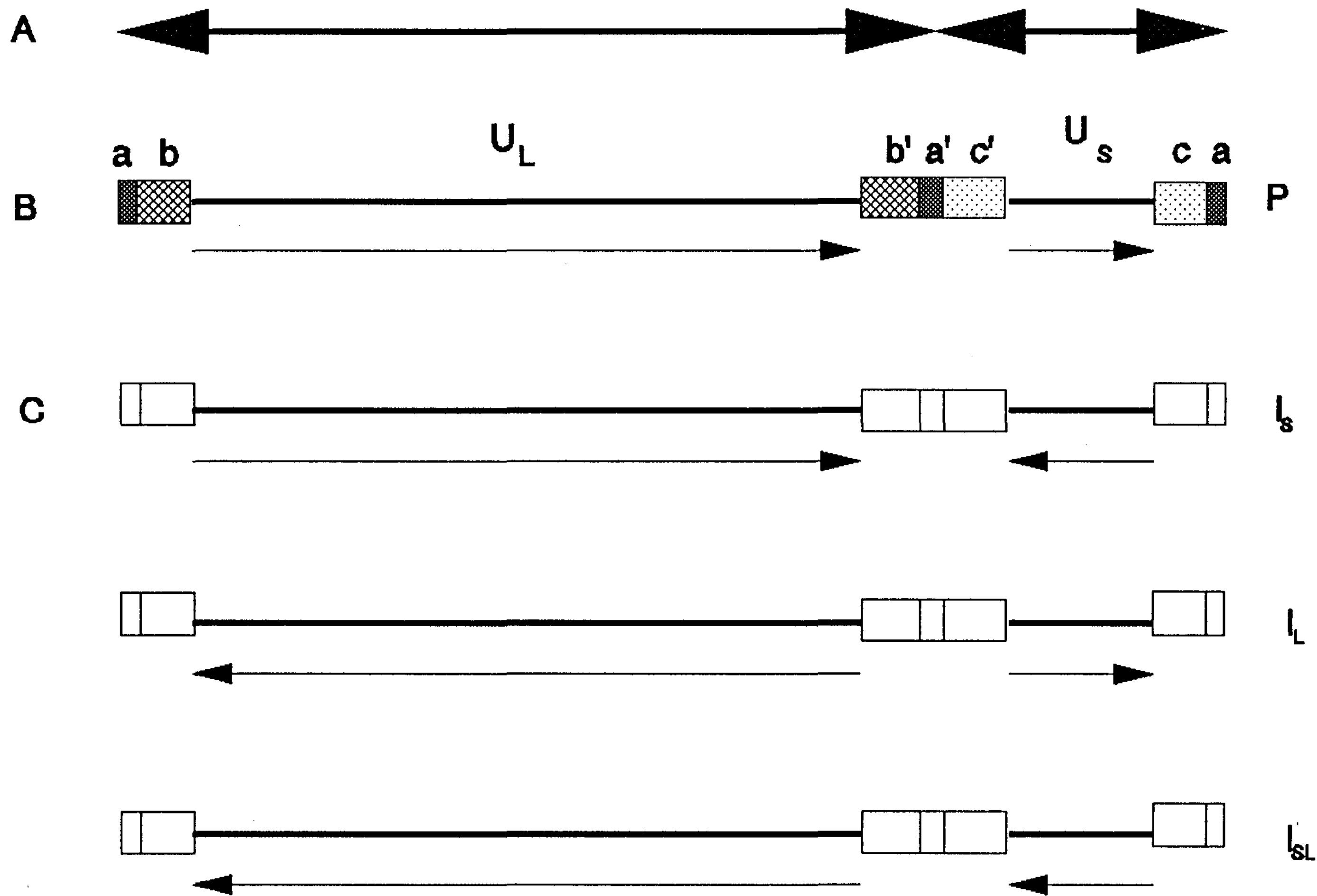
The two terminal a sequences are designated a_L and a_S, and each of them has been found to have a 3' overhanging nucleotide at its end. (Mokarski and Roizman 1982). A variable number of additional a sequences may be found at the L terminus (a_n where n=0 to many). Similarly, multiple a sequences may be found directly repeated at the LS junction (a_m, where m=1 to many)(Davison and Wilkie 1981; Wagner and Summers 1978). It was hypothesized that the reiterated sequences could be used in a recombination event which might lead to the inversion of the L or S component. Hayward et al. (1975) established that the components do indeed invert relative to one another.

HSV DNA actually exists as four isomers differing only in the orientation of the L and S components. The four isomers are found in equimolar amounts upon analysis of wild-type HSV DNAs. The four isomers have been designated as follows (Roizman et al. 1974; Hayward et al. 1975):

1) P - prototype
2) I_S- inversion of S component
3) I_L- inversion of L component
4) I_{SL}-inversion of both components

The internal reiterated sequences required for the isomerization of the viral genome are not essential. Mutants which have had most of the internal repeat deleted from the wild type genome have been isolated in all four arrangements. Although these mutants are unable to invert to form different isomers, they are still viable in cell culture (Poffenberger

Figure 1 Structure of the herpes simplex virus genome. **A.**The herpes simplex genome consists of two components, L and S, which are designated by the arrows. **B.**Each component consists of a unique region (U_L or U_S) flanked by inverted repeats designated by the open boxes. The inverted repeats are subdivided into sequences designated a, a' etc. and represent inverted forms of the same sequence. **C.**During the course of infection, the L and S components can invert relative to one another leading to four isomers of the HSV genome. After infection, progeny DNA consists of equimolar amounts of the four isomers which are designated: P-prototype, I_S-inversion of the S segment, I_L-inversion of the L segment, and I_{SL}-inversion of both L and S segments


A
a b
UL
b' a' c'
Us
c a
B
P
C
Is
IL
ISL

and Roizman 1985; Jenkins *et al*. 1986).

Intratypic variability of HSV DNA.

Variability in HSV DNA structure can be found in the number of reiterated sequences at the L terminus and at the L-S junction. It has been shown that the number of *a* sequences at these sites can vary not only between strains of virus, but even among progeny of plaque purified virus (Locker and Frenkel 1979; Mocarski and Roizman 1981). Although the number of repeats found in a given strain is variable, the restriction enzyme cleavage sites are relatively stable (Hayward *et al*. 1975). In fact, restriction enzyme patterns of HSV-1 strain KOS were demonstrated to be identical when the virus was reisolated from the same individual 12 years after the original isolation (Buchman *et al*. 1978). Similarly, identical restriction patterns were found in viruses isolated from epidemiologically related patients (Buchman *et al*. 1979). Conversely, no two epidemiologically unrelated isolates have been shown to be identical (Roizman and Buchman 1979: Buchman *et al*. 1980).

Intertypic variation of HSV DNA.

The genomes of HSV-1 and HSV-2 were originally demonstrated to be 47% to 50% homologous under relatively stringent hybridization conditions (Keiff *et al*. 1972). And as stated above, the two types also vary in their G-C content. Analyses of restriction endonuclease cleavage sites of HSV-1 and HSV-2 have given us a better understanding of the

similarities and differences between the two DNAs. In fact, the gene organization of the two virus types has been found to be collinear. Because the whole genome of HSV-1 strain 17 has been sequenced, the selected genes of HSV-2 which have been sequenced may be directly compared with those in HSV-1.

HSV virion proteins.

HSV-1 virions contain at least 33 polypeptides that have been designated virion polypeptides (VPs)(Heine et al. 1974). It has been difficult to identify a precise number of proteins due to the difficulty resolving the large number of polypeptides electrophoretically. Also, detection of the minor polypeptides is often difficult in the presence of the more abundant proteins. Of the approximately 33 proteins, eight are glycosylated. These proteins are all believed to be integral membrane proteins and are the only proteins found on the surface of the virion. The glycoproteins play an important role in mediating adsorption and penetration of the host cell as well as the envelopment of the virus.

Approximately seven virion polypeptides are believed to make up the capsid (Gibson and Roizman 1972; 1983). Besides their obvious structural function, some capsid proteins may be involved in the incorporation of viral DNA into the core. The rest of the virion proteins are probably constituents of the tegument. The proteins identified in the tegument appear to have a variety of functions. Examples of tegument proteins are the alpha trans-inducing factor (alpha-TIF)(Batterson and

Roizman 1983) and a protein associated with a complex that binds to the a sequence of viral DNA (VP1) (Roizman and Sears 1990). There is also preliminary evidence that the virion host shutoff (vhs) protein is found in the tegument of HSV (Read and Knight, unpublished observations).

HSV replication.

The initial step of HSV replication is the attachment of the virion to host cell surface receptors. It is most likely that envelope glycoproteins mediate viral adsorption because these are the proteins exposed on the surface of the virion and are available for interaction with cell surface molecules. One host cell molecule which has been identified as a surface receptor for HSV is heparin sulfate proteoglycan (Wudunn and Spear 1989). However, cultured cells lacking heparin sulfate proteoglycans have not been found. Because only humans and chimpanzees are naturally infected by HSV, surface proteins other than heparin sulfate proteoglycan must be required for this species specificity.

If the infection is to proceed after attachment to the cell surface, the virion must penetrate the cell. For HSVs, penetration involves the fusion of the cell membrane with the viral envelope (Campadelli-Fiume et al. 1988). The HSV glycoproteins gB and gD have been determined to play major roles in this fusion process (Manservigi et al. 1977; Johnson and Ligus 1988). After penetration, the capsid is released into the cytoplasm of the host cell and transported to the

nuclear pores (Tognon et al. 1981; Batterson et al. 1983). At the nuclear pores, a virus specific function causes the HSV DNA to be release into the nucleus (Batterson et al. 1983).

HSV gene expression.

HSV proteins expressed in cells were initially divided into three groups based on their kinetics and requirements for synthesis. These groups, namely alpha, beta, and gamma (immediate early, early, and late) are expressed in a cascade pattern originally described by Honess and Roizman (1974). Since then, the beta group has been divided into two subclasses $beta_1$ and $beta_2$; and the gamma proteins have been divided into $gamma_1$ and $gamma_2$ subclasses.

The appearance of HSV mRNA parallels the rate of synthesis of the corresponding viral polypeptides and thus the messages are classified in the same manner. The viral messages, like host cell messages, are transcribed in the nucleus by RNA polymerase II (Costanzo et al. 1977). The viral and cellular messages are also similar in that they are capped at the 5' end, polyadenylated at the 3' end and are internally methylated. One significant difference between HSV and host cell messages is that most HSV mRNAs are not spliced.

The first group of genes transcribed after infection are the alpha genes. The alpha genes have been defined as those genes that do not require de novo viral protein synthesis for their expression (Pereira et al. 1977). There are only five alpha genes, all of which are transcriptionally activated by

the tegument protein alpha-TIF (Kristie and Roizman 1986). After virion penetration of the cell, alpha-TIF is transported to the nucleus independent of the viral DNA (Batterson and Roizman 1983). In the nucleus, alpha-TIF forms a complex with host proteins and binds to cis-acting sites found upstream of all the alpha genes (Gerster and Roeder 1988; McKnight et al. 1987).

The appearance of alpha proteins begins about 1 h after infection and they reach a peak rate of synthesis at 2-4 h after infection (Honess and Roizman 1974). The alpha proteins all appear to be regulatory proteins and are required for the synthesis of subsequent beta and gamma polypeptides. The five alpha genes have been designated infected cell protein (ICP) 0, ICP4, ICP22, ICP27 and ICP47.

ICP4 is the major trans-activator of HSV genes and is required continuously for the synthesis of all the subsequent classes of RNA (Watson and Clements 1980). Moreover, ICP4 is autoregulatory. It has been found to be capable of binding DNA and repressing its own transcription along with that of other alpha genes (Dixon and Schaffer 1980; Kristie and Roizman 1986). Another alpha gene, ICP0 has been characterized using transient expression systems. ICP0 appears to be a promiscuous trans-activator when cotransfected with various target genes in cell culture (Everett 1987). Cotransfection experiments with ICP0 and ICP4 suggest that ICP0 may be a trans-activator of the ICP4 gene (O'Hare and

Hayward 1984; Everett 1984). HSV mutants in which the ICP0 gene has been deleted are viable in cell culture but their growth rate is significantly decreased (Sacks and Schaffer 1987).

As the rate of alpha protein synthesis begins to decline, beta genes begin to be expressed. The peak rate of protein synthesis for beta polypeptides occurs 5-7 h post-infection (Honess and Roizman 1974). Proteins belonging to the beta group may be defined as those polypeptides which require prior synthesis of viral proteins (alpha proteins) but do not require DNA synthesis for their expression. The beta polypeptide group has been divided into two subgroups, $beta_1$ and $beta_2$. The $beta_1$ proteins are detected very early in infection -- so early that they have been mistaken for alpha proteins (Clements _et al_. 1977). The $beta_1$ proteins are distinguished from the alpha proteins by the requirement for ICP4 for their expression. Examples of $beta_1$ proteins are the major DNA binding protein (Conley _et al_. 1981), and the large component of the viral ribonucleotide reductase (Huszar and Bacchetti,1981). Examples of proteins found in the $beta_2$ group are HSV thymidine kinase and DNA polymerase. Generally, the proteins belonging to the beta group are involved in DNA synthesis.

Concomitant with a decline in beta protein synthesis, the final temporally regulated class of proteins appears. The gamma proteins are generally structural proteins and their

peak rates of synthesis vary. The gamma polypeptide group is distinguished from the beta polypeptides by its requirement for viral DNA synthesis for maximal gene expression. The gamma group is also divided into two subgroups, $gamma_1$ and $gamma_2$. $Gamma_1$ genes are expressed in small amounts before the onset of viral DNA synthesis, however, they are not expressed in appreciable amounts until viral DNA synthesis occurs. $Gamma_2$ genes are expressed late in infection and have an absolute requirement for DNA synthesis before they can be expressed.

HSV DNA replication.

Replication of the viral DNA occurs in the nucleus of the infected cell and it requires a number of viral encoded proteins. Viral DNA synthesis may be detected as early as 3 h post infection and proceeds for another 9 to 12 h (Roizman and Roane 1964;Roizman *et al*. 1965). The overall mechanism of DNA replication is not yet well characterized but several important features have been uncovered.

Three origins of replication have been identified in the viral genome. These *cis*-acting elements were initially inferred from the structure of defective viral genomes (Frenkel *et al*. 1976; Spaete and Frenkel 1982). The origins of replication have been operationally defined as those sequences required in *cis* to allow transfected DNA to be replicated when it is introduced into HSV infected cells (Mocarski and Roizman 1982; Vlazny and Frenkel 1981). Two

identical origins of replication are found in the inverted repeats of the S component of the genome and the third origin of replication is located in the unique portion of the L component (Mocarski and Roizman 1982; Deb and Doelberg 1988).

The isolation of the origins of replication aided in experiments designed to identify the viral genes required for DNA replication. In these experiments, (Challberg 1988), cells were cotransfected with a plasmid containing an origin of replication and various fragments of the HSV genome. This complementation assay could be used to determine the gene products needed in *trans* for DNA replication as well as any other genes needed for their expression. Replicated plasmid DNA was differentiated from the input DNA by digesting with *Dpn*I. The transfected plasmid DNA is methylated by the *Escherichia coli dam* methylation system and is sensitive to *Dpn*I whereas the replicated DNA is not methylated and therefore resistant to *Dpn*I. Results from this assay indicated that at least seven genes are essential for viral-origin-dependent DNA replication.

Interestingly, only a small fraction of the parental DNA of HSV is replicated (Jacob and Roizman, 1977). Pulse labeling of DNA does not detect free ends, indicating that the DNA conformation is circular of concatomeic (Jacob *et al.* 1979; Jacob and Roizman 1977). At early times in DNA synthesis parental DNA, circles, and linear branched forms of DNA are detected. A rolling circle mechanism of DNA

replication would explain the appearance of linear branched forms and the absence of a theta structure. Later in infection, the three DNA forms are replaced by large bodies of tangled DNA. These DNA concatomers are packaged into empty capsids by a 'head full' mechanism (Deiss et al. 1986).

Effects of HSV infection on host-cell structure.

The first structural change observed in the host cell due to a HSV infection is in the nucleolus (Jacob and Roizman 1969; Roizman and Furlong 1974). The nucleolus becomes enlarged and is moved toward the nuclear membrane. At the same time, host cell chromosomes become marginated and the nucleus becomes distorted and multilobed. Late in infection thick patches are observed on the nuclear and plasma membranes (Ni et al. 1968; Roizman and Spear 1971). The plasma membrane is further altered during infection when new cell-surface antigens are acquired and viral glycoproteins are found in the plasma membranes (Heine et al. 1972; Roizman and Spear 1971). Interactions between cell membranes are also altered in that intercellular adhesiveness is increased and cell fusion is observed (Ruyechan 1979).

Effects of HSV infection on host-cell macromolecular metabolism.

A characteristic of HSVs is the suppression of most of the host cell's metabolic activity. Upon infection, the synthesis of host-cell ribosomal RNA and also the processing of large molecules to form ribosomal RNA are reduced (Fenwick

and Walker 1978; Wagner and Roizman 1969). Host DNA synthesis and host polypeptide synthesis are also rapidly shut off(Roizman and Roane 1964; Roizman et al. 1965; Read and Frenkel 1983); The most extensive research has been involved with investigating the mechanism of host-cell polypeptide shutoff.

One of the first results of an HSV infection is the suppression of host cell protein synthesis. Early studies by Sydiskis and Roizman (1966;1967) examined the polysomal profiles of infected cells by centrifuging cell lysates through sucrose gradients. It was discovered that early after infection with HSV-1, cellular polyribosomes were dispersed into monoribosomes. Similar experiments by Silverstein and Engelhardt (1979) later established that after the initial disaggregation of host polysomes, the ribosomal subunits were recruited for viral protein synthesis. The dissociation of host cell polyribosomes is most likely responsible, in part, for the shutoff of cellular protein synthesis. There are, however, other important factors which will be discussed below.

Fenwick and Walker (1978) observed the rate of protein synthesis after infection with HSV-2 by measuring the incorporation of ^{14}C amino acids into acid precipitable counts. Only one hour after infection, protein synthesis had declined to 20% of the rate in mock-infected cells. Similar results were obtained when cells were infected in the presence of the

transcriptional inhibitor actinomycin-D or with U.V.-irradiated viruses (Fenwick and Walker 1978). These results indicated that the shutoff of host polypeptide synthesis occurred without the synthesis of any viral proteins and that a virion component(s) was responsible for host cell protein shutoff. Further evidence that viral protein synthesis is not required for host cell shutoff involved HSV-2 infection of enucleated cells. A decline in cellular protein synthesis was again observed in the cytoplasts (Fenwick and Walker 1978), suggesting that shutoff does not involve the nucleus and must therefore be a cytoplasmic event.

Further studies of the virion associated host shutoff were directed at the analysis of cellular mRNAs. In order to prevent the expression of viral proteins in their analysis, Fenwick and McManamin (1984) used irradiated HSV-2 as the infecting virus or the infections were carried out in the presence of actinomycin-D. Total cytoplasmic RNA was extracted from cells infected with irradiated HSV-2 or from mock-infected cells. In vitro translations indicated that the HSV-2 infected cells underwent a rapid loss of functional mRNAs. Similarly, cellular message extracted from HSV infected cells was substantially less than mock infected cells. Whether the loss of functional mRNA coincides with the disaggregation of polysomes or occurs afterward will be discussed below.

Characterization of the virion associated host shutoff

mechanism was aided by the isolation of mutants for this function. Read and Frenkel (1983) isolated six mutants from HSV-1 strain KOS by the method of bromodeoxyuridine mutagenesis. These mutants have been designated virion host shutoff (vhs) mutants 1 through 6. These mutants are unable to shut off host cell protein synthesis as measured by the incorporation of ^{14}C amino acids into acid precipitable counts and also by SDS polyacrylamide gel electrophoresis of the proteins. In general, protein gels of cells infected with wild type virus showed a decline in all cellular proteins whereas infection with vhs mutants did not shut off protein synthesis. These experiments were all conducted in the presence of actinomycin D to insure that the shutoff was being carried out by a virion component and not by a viral protein expressed after infection. An unexpected finding was that the alpha proteins were overproduced in vhs mutant infected cells.

To investigate the mechanism underlying the overproduction of alpha proteins, cycloheximide reversal experiments were conducted. Vero cells were first infected with either wild-type virus or the mutant virus vhs1 in the presence of cycloheximide which allowed the synthesis of only cellular and alpha mRNA transcripts. Five hours after infection, the cycloheximide was replaced with actinomycin D and the cells were incubated for various times. This procedure prevented the synthesis of beta and gamma proteins

while allowing the remaining alpha transcripts to be translated into the corresponding proteins. As expected, polyacrylamide gels of proteins showed host shutoff in the wt infected cells and normal cellular protein synthesis in the vhs1 infected cells. Synthesis of alpha proteins in wt infected cells occurred immediately after cycloheximide reversal but decreased rapidly thereafter and was undetectable in the mutant infected cells at to 10 h after the reversal. However, synthesis of alpha proteins in cells infected with the mutant vhs1 continued until at least 10 h after the cycloheximide reversal. Since the cells were maintained in actinomycin-D after the removal of cycloheximide the levels of alpha and host protein synthesis reflected a difference in the functional stability of the message and not alterations in levels of transcription.

Schek and Bachenheimer (1985) investigated the accumulation of mRNA in cells infected with HSV-1 and HSV-2. Northern blot hybridizations were used to detect the levels of specific cellular mRNAs during the HSV infections. Total cytoplasmic RNA was probed with plasmids containing genomic DNA fragments of the histone H3 and H4 genes and with cDNA clones of beta-tubulin, beta-actin, and gamma actin. The abundance of these cytoplasmic mRNAs all declined, albeit at different rates. In contrast, cells infected with the mutant vhs1 did not show any reduction in the levels of the same messages (Strom and Frenkel 1987). These experiments were

also performed in the presence of actinomycin D, demonstrating: 1) a virion component is responsible for the decline in mRNA; and 2) the stability of the messages and not changes in transcription rates is responsible for the decline of mRNA levels. Experiments infecting cells with the vhs1 mutant showed no alteration in cellular mRNA abundance supporting the notion that the virion component responsible for host cell protein shutoff is also responsible for the physical degradation of mRNAs (Strom and Frenkel 1987).

From the above mentioned observations it seemed plausible that cellular mRNA could be destabilized by the initial disaggregation of host polyribosomes. Strom and Frenkel (1987) used a variety of translational inhibitors to examine the relationship between polyribosomal disaggregation of polyribosomes and mRNA degradation. Both cycloheximide and emitine inhibit ribosomal translocation and thus prevent the disaggregation of polyribosomes. Neither of these drugs prevented or reduced the mRNA degradation in wt infected cells, indicating that polyribosomal RNA is not protected from degradation. Other drugs such as puromycin, sodium fluoride and pactamycin are known to cause the disaggregation of polyribosomes. Treatment of wt infected cells with these drugs did not decrease the stability of the mRNA nor did it aide in stabilizing the message in vhs1 infected cells. Together, these experiments provide indirect evidence that the state of polyribosomal aggregation does not affect the mRNA

stability in HSV infected cells.

Since the physical degradation of mRNAs is the primary event responsible for the shutoff of host-cell protein synthesis, it could be predicted that a similar mechanism is responsible for the prolonged synthesis of alpha proteins in vhs1 infected cells. Cycloheximide reversal experiments were used to examine the functional and physical stability of alpha mRNAs (Oroskar and Read 1987). Vero cells were infected with wt or vhs1 virus in the presence of cycloheximide. After messages had accumulated for 5 h, the cycloheximide was removed and protein synthesis was allowed in the presence of actinomycin D. Cytoplasmic RNA was extracted 0 and 5 h after cycloheximide reversal and was subsequently translated *in vitro* or electrophoresed for Northern blot hybridizations. *In vitro* translation of mRNA from wt infected cells showed the synthesis of functional alpha polypeptides at the 0 h time point but 5 h after cycloheximide reversal, alpha polypeptide synthesis was barely detectable. In contrast, vhs1 infected cells showed a higher level of alpha mRNAs at 0 h and this level was maintained for at least 5 h. Northern blots revealed that the increased functional stability of alpha messages in vhs1 mutant infections was due to an increase in their physical stability.

In the aforementioned experiments the mutation in vhs1 appears to increase the stability of both cellular and viral alpha messages. To determine whether the vhs function induced

nonspecific destabilization of messages, the functional stabilities of beta and gamma mRNAs were analyzed in wt and vhs1 infected cells (Kwong and Frenkel 1987). Vero cells were infected with either wt or vhs1 virus. In order to allow the expression of beta and gamma genes the infection was allowed to proceed in the absence of inhibitors. At 3, 5, 10, and 16 h after infection, actinomycin-D was added to inhibit further mRNA synthesis. The rate of protein synthesis was then measured by labeling cells immediately after the addition of actinomycin-D or 6 h later. In wt infected cells, beta protein synthesis peaked 10 h after infection and had declined by 16 h. In contrast, in vhs1 infected cells, beta protein synthesis was relatively high by 3 h after infection and declined only slightly at 16 h. This suggested that the stability of beta messages might also be increased by the vhs1 mutation. Direct examination of beta and gamma messages using Northern blot hybridizations has confirmed that the increase in the physical stabilities of beta and gamma mRNAs is due to the vhs1 mutation (Oroskar and Read 1989).

HSV-2 strains generally inhibit host protein synthesis more rapidly than HSV-1 strains (Pereira et al. 1977; Powell and Courtney 1975). This information was utilized to map the host shutoff function. HSV-1 x HSV-2 intertypic recombinants and parental strains were examined for their rate of inhibition of protein synthesis and the genetic locus responsible for the more rapid HSV-2 host shutoff was found to

lie between 0.52 and 0.59 map units on the prototype arrangement (Morse et al. 1977). The shutoff function was localized more precisely by Kwong et al. (1986) by mapping the vhs1 mutation to a 265 base pair (bp) restriction fragment which spans map coordinates 0.604 to 0.606 of the HSV viral genome. Experiments cotransfecting total vhs1 DNA and the 265 bp NruI-XmaII restriction fragment from wild-type virus showed that the host shutoff defect could be rescued by the wild-type restriction fragment. McGeoch and coworkers (1986) have designated the open reading frame in this region UL41. UL41 encodes a protein with a predicted molecular weight of 54,900 and an apparent molecular weight on SDS PAGE of 58,000. A single 1.6 kb mRNA is transcribed from this region and is expressed as a $gamma_1$ gene (Frink et al. 1981). Although the mapping of transcripts (Frink et al. 1981) and mutational analysis of the shutoff function were obtained from HSV-1 (Read and Frenkel 1983), the collinearity of HSV-1 and HSV-2 make it possible to predict that the gene responsible for the virion associated host shutoff function of HSV-2 is contained in the BglII N fragment of HSV-2 (Keiff et al. 1972). Jenkins and Howett (1984) mapped the locations of nine messages in the Bgl N fragment. The BglII N fragment of HSV-2 corresponds to a segment of DNA overlapping the HindIII K and L fragments of HSV-1 (Anderson et al. 1981; Frink et al. 1981) Comparison of the transcripts showed that the positions and sizes of mRNAs mapped by Jenkins and Howett are similar to those mapped to

the corresponding portion of the HSV-1 genome. A 1.9 kb transcript has been mapped between 0.599 and 0.590 map units and is predicted to encode a 61 kilodalton (kd) protein. This transcript is oriented in the same direction as the 1.9 kb transcript of the virion host shutoff gene of HSV-1.

Fenwick and Everett (1990) removed a 3.6 kb *SstI* subfragment from the *BglII* N fragment of HSV-2 (G) which was predicted to contain the *vhs* gene. The *SstI* fragment was inserted it into the thymidine kinase gene of HSV-1(17$^+$). HSV-1(17)$^+$ has a weak shutoff phenotype, however,the recombinant virus possessed the strong shutoff phenotype of HSV-2. The strong shutoff phenotype in the recombinant virus indicates that the *vhs* gene was indeed contained in the 3.6 kb *SstI* fragment. Interestingly mixed infections with equal multiplicities of HSV-1 strain 17$^+$ and HSV-2 strain G showed the weaker *vhs* phenotype of HSV-1(17$^+$) to be dominant (Fenwick and Everett 1990). The reason for these different results when two copies of the *vhs* gene are in the same of different viruses is at present unclear.

In addition to the study mentioned above, Everett and Fenwick (1990) have compared the DNA sequences of the HSV-1(17$^+$) and HSV-2(G) *vhs* genes. The sequences of these genes were 86% homologous and many of the regions which were not homologous encoded conservative amino acid changes. A *vhs* gene from a third strain of virus, HSV-2 strain HG52 was also sequenced. HSV-2(HG52) is unusual in that it is a type 2

strain of HSV but it has a defective host shutoff function. The first 342 amino acids of the HG52 vhs are identical to those of the strain G protein. However, a frameshift mutation in strain HG52 causes the carboxyl third of the protein to diverge from the strain G amino acid sequence. This implies that the carboxyl third of the vhs protein in HSV-2(G) is essential for host shutoff activity.

Host shutoff in other eukaryotic viruses .

Host shutoff is not common to all viruses. For instance, retroviruses, parvoviruses and paramyxoviruses do not exhibit any suppression of host cell protein synthesis. Notwithstanding, many viruses do exhibit host cell shutoff and at nearly every level of macromolecular metabolism. The first level of metabolism which may be exploited is host cell transcription. Inhibition of transcription is often accomplished simply by the virus competing for cellular transcription factors and RNA polymerase II. There is, however, a limited amount of information describing other modes by which viruses inhibit host cell metabolism at the transcriptional level.

There is evidence that vesicular stomatitis virus (VSV) has a unique mechanism for blocking RNA polymerase III transcription (Weck and Wagner, 1979). A small RNA encoded by VSV accumulates in the nucleus early in infection (Kurilla et al. 1982). This RNA has been found to associate with a host cell factor (La protein) that is required for efficient

transcription (Kurilla and Keene, 1983). There is indirect evidence that binding of the RNA prevents binding of the La protein to the cellular polymerase III promoters and thus inhibits cellular transcription (Grinell and Wagner 1985).

If cellular transcription proceeds unimpaired by viral infection, the transcript is processed and transported to the cytoplasm. Adenovirus has been found to inhibit the transport of cellular message to the cytoplasm (Beltz and Flint 1979). It is not understood how host transcripts are distinguished from viral transcripts, only that the adenovirus proteins E1B-55K and E4-34K are involved in the process (Babiss et al. 1985).

The host cell transcripts which are transported to the cytoplasm intact are subject to virus induced degradation. This mode of host cell shutoff has been discussed in detail for HSVs, however it is not unique to these viruses. This mode of inhibition is also present in vaccinia virus and influenza virus (Inglis 1982; Rice and Roberts 1982). As described above, HSV destabilizes viral mRNA as well as host cellular mRNA. The increased turnover of viral message may impart an advantage for HSV in that it promotes the rapid transition from one regulatory class to the next.

If the cellular message remains sufficiently stable in the cytoplasm, translation proceeds. Here, there is another opportunity to invoke host cell shutoff by interfering with the translation of cellular transcripts. Just as viruses can

compete with the host cell for RNA polymerase II and transcription factors, so too can it compete for ribosomes and cellular translation factors. An example of this occurs during VSV infections when an abundance of viral mRNA out-competes the cellular message for a limited number of ribosomes (Schnitzlein et al. 1983). VSV message also has a higher affinity for ribosomes than the cellular message (Nuss and Koch 1976). The preferential binding of ribosomes to viral mRNA is accomplished in part by the inactivation of the translational initiation factor eIF2.

Another way host cell translation may be affected by viral infection is by changing the specificity of the host translational machinery. This mode of regulation may be exemplified by poliovirus. Host protein synthesis is shut off by inactivating an initiation factor that mediates the translation of capped mRNAs (Etchison et al. 1982). More specifically, poliovirus cleaves one of the constituent proteins of the cap binding complex (CBP). CBP mediates the efficient initiation of translation of the host cell's capped messages. Poliovirus has the ability to translate messages that are not capped and thus efficiently translates mRNA without the CBP complex (Ambros et al. 1978; Pelletier and Sonenberg 1988).

Adenovirus, like poliovirus, exploits the cellular requirement for CBP complex to inhibit cellular protein synthesis. Adenovirus mRNAs possess a 5' noncoding region

called the tripartite leader which permits mRNA translation without the presence of a cap or the need for the CBP complex. Upon infection, adenovirus inactivates the CBP complex, thus inhibiting translation of cellular messages. However, instead of being proteolytically degraded as is seen upon infection with poliovirus, the CBP complex is functionally inactivated by causing and underphosphorylation of one of the constituents of this complex (Huang and Schneider, 1991).

<u>mRNA decay in eukaryotic cells</u>.

The <u>vhs</u> gene product has been shown to destabilize both viral and cellular messages. The overall decrease in cellular mRNA confers an advantage to HSV on two levels. First, there is less mRNA competing for a limited number of ribosomes. Another advantage is that the decreased stability of viral message augments HSV transcriptional controls which allow a rapid transition from one regulatory class of viral proteins to the next. But the regulation of mRNA stability is not only beneficial to HSVs, it is also a significant control mechanism in eukaryotic gene regulation.

Steady-state levels of mRNA are often attributed to their rate of transcription. Nevertheless, mRNA levels are frequently determined by their turnover rate. Carneiro and Schibler (1984) measured the transcription rates and mRNA levels of ten constitutively expressed housekeeping genes in mouse L cell fibroblasts. Genes transcribed at higher rates often had low levels of message whereas the messages

transcribed at low rated could be fairly abundant. In all cases, the steady state levels of the mRNAs correlated with their stability and not their transcription rate. Over the past ten years, these experiments and others have given credence to the notion that mRNA decay is a major control point of gene expression. Because, in most cases, the rate of protein synthesis is proportional to the levels of mRNA, the regulation of mRNA stability is an important element in regulating cellular protein abundance.

Despite the understanding that mRNA turnover is a major control mechanism in gene expression, the question of how this regulation is achieved has not been answered. Several factors have been shown to be involved in mRNA stability. The decay process is affected by ribonucleases acting in concert with hormones and cellular factors. However, the primary structure of a mRNA molecule is ultimately responsible for its turnover rate. Any segment of an mRNA molecule can affect its turnover. Examples of these control regions are described below.

Experiments with the proto-oncogene c-*myc* mRNA provide an example of the 5' noncoding region of a transcript affecting its decay rate. The half-life of c-*myc* is normally about 10 min (Dani *et* *al*. 1984). This extreme instability first suggested to investigators that post-transcriptional mechanisms might contribute to the regulation of the gene. Piechaczyk and colleagues (1985) found that the c-*myc* messages

obtained from plasmacytomas had half-lives of several hours. The stable c-*myc* messages were truncated but still gave rise to full-length proteins. It was discovered that these transcripts had deletions in the first exon which contains most of the 5'-noncoding region and these deletions were responsible for the dramatic increase in half-life. Although the 5' noncoding region of the c-*myc* mRNA was shown to affect mRNA turnover, whether it is due to changes in secondary structure, the alteration of binding sites for cellular factors, or some other mechanism remains to be determined. The 3'-untranslated region of c-*myc* mRNAs has also been shown to contain a destabilizing element and will be discussed below.

The coding regions of some transcripts have also been shown to influence mRNA turnover. Nonsense mutations introduced into histone genes were found to decrease the half-lives of these messages (Capasso *et al*. 1987). Moreover, mutations which changed the normal termination site of histone genes and allowed the ribosome to translate up to the 3' terminus increased the mRNA turnover rate (Graves *et al*. 1987). It has been suggested that these mutations affect the overall polysome structure which may decrease the stability of the message by rendering it a better substrate for nucleases. Nonsense mutations have also been shown to destabilize yeast *ura*3 mRNA (Losson and Lacroote 1979) and immunoglobulin mRNA in B lymphocytes (Bauman *et al*. 1985). In both cases, nonsense mutations inserted at the 5'-end of the gene resulted

in shorter mRNA half-lives than did mutations at the 3'-end. Additional experiments support a link between polysome structure and mRNA turnover. Inhibition of protein synthesis using drugs which inhibit the elongation of translation increase the half-lives of many cellular messages (Ernest 1982; Dani *et al*. 1984; Stimac *et al*. 1984). Perhaps this freezes the ribosomes on the mRNA in such a way that ribonucleases are excluded.

The 3'-untranslated region of an mRNA molecule probably contains the most important sequences involved in mRNA stability. Indirect evidence showing the importance of this region was first shown in comparisons between beta globin and delta globin genes. The messages of these genes are homologous in the 5' noncoding and coding regions of their genes but differ more than 50% in their 3' untranslated regions. The turnover rate of the delta globin message is four to six times faster than beta globin mRNA. The simplest conclusion from these observation is that the 3' untranslated region of the delta globin message makes it more susceptible to ribonucleases than the corresponding sequences on the beta globin message.

Direct evidence of the involvement of the 3'-untranslated region in mRNA stability was obtained from experiments using chimeric genes. The two genes used were c-*fos* which has a very labile message and beta globin which has a relatively stable message. When the 5'- and coding-regions of c-*fos* were

linked to the 3'-globin region, the resulting message was stable. Conversely, when the 5'-regions were encoded by beta globin sequences and the 3'-regions were from c-fos, the transcripts were unstable (Treisman 1985). A 60 nucleotide sequence, which is believed to confer this instability, has recently been identified in c-fos and other mRNAs involved in cell growth (Caput et al. 1986; Shaw and Kamen 1986; Fort et al. 1987).

The 3' untranslated region of the c-myc gene has been implicated in regulating the stability of its message. Shaw and Kamen (1986) originally identified adenosine and uracil (AU) rich sequences in the 3' nondcoding region of c-myc and c-fos mRNAs as well as human granulocyte-macrophage colony-stimulating factor (GMCSF) mRNAs which have been demonstrated to play an important role in their degradation. More recently, Vakalopoulou et al. (1991) identified a 32 kD polypeptide which interacts with "AUUA" sequences within the "AU" rich region. Binding of this protein was also shown to correlate with a decrease in steady state levels of lymphokine and proto-oncogenic mRNAs.

Interestingly, Schuler and Cole (1988) have described a monocytic cell line in which GMCSF transcripts were stabilized while c-myc and c-fos mRNAs retained their short half-lives. These results seem to indicate that trans-acting factors may differentiate between "AU" rich regions of lymphokine and proto-oncogenic mRNAs. Recently, Bohjanen et al. (1991) have

reported a sequence-specific factor which binds to "AUUA" multimers within the "AU" rich region of lymphokine but not proto-oncogenic mRNAs. This factor (AU-B) is believed to be a lymphokine specific regulator of mRNA metabolism.

The last region of an mRNA molecule to be discussed, the polyadenylated (poly(A)) tail, has been shown definitively to affect mRNA turnover. Many of the experiments examining the role of poly(A) tails in mRNA stability use histone messages which are usually not polyadenylated. Histone messages in Xenopus oocytes, however, are polyadenylated and can remain stable for months. After fertilization, the message is de-adenylated and is rapidly degraded (Levenson and Marco 1976; Ruderman et al. 1979). Although this result does not prove that de-adenylated message is more susceptible to degradation, it has led to further analysis of the adenylated state of histone messages. When histone messages from HeLa cells are microinjected into oocytes, they are extremely labile, but if the same message is modified by adding a poly(A) tail before microinjection, it becomes stable (Huez et al. 1978). Similarly, deadenylated rabbit globin mRNA has been found to be less stable in HeLa cells than its native form (Huez et al. 1981). Ross and coworkers have been using a cell free system to investigate the pathway and mechanism of histone mRNA degradation. They have found that mRNA modified by adding a poly(A) tail is ten to twenty times more stable than its native non-adenylated form. In this same cell free system,

the unmodified histone mRNA has been found to be degraded by an exonuclease in a 3' to 5' direction (Ross and Kobs 1986; Peltz et al. 1987).

As described above, the addition of a poly(A) tail to a nonadenylated mRNA molecule appears to stabilize mRNA, whereas the removal of poly(A) from an adenylated message seems to render it susceptible to ribonucleases. These observations suggest that poly(A) metabolism is linked to mRNA turnover. First, it has been demonstrated that newly synthesized metallothionein mRNA has a poly(A) tail of 150-200 residues. Five to ten hours after synthesis, the poly(A) tail shortens to 30 residues after which the steady state levels of the message rapidly decrease (Mercer and Wake 1985). Full length deadenylated mRNA is not observed, indicating that after the poly(A) tract has been reduced to 30 nucleotides, the message quickly becomes degraded. Secondly, immediately preceding mRNA degradation of the proto-oncogenes c-myc and c-fos, the poly(A) tail is shortened and finally removed (Swartout and Kinniburgh 1989; Wilson and Treisman 1989).

It has been demonstrated by several investigators that after the poly(A) tail has been shortened to 30-40 nucleotides the remainder of the adenosines and the rest of the mRNA molecules are quickly degraded. Examples of this occurrence may be observed in oocytes where mRNAs with poly(A) tracts of less than 32 residues have the same half-life as the completely deadenylated message (Nudel et al. 1976).

Bernstein and Ross (1989) explain these observations by the fact that the poly(A) tract is organized into a "nucleosome-like ribonucleoprotein". The poly(A) binding protein (PABP), which binds to segments of 27 A residues along the poly(A) tracts is believed to be responsible for the organization and folding of the poly(A) tail (Baer and Kornberg 1983; Bernstein and Ross 1989). The binding of PABP to the poly(A) tract is thought by some investigators to protect mRNAs from cellular nucleases. This idea was first supported by the observation that a poly(A) tract which had had PABP removed was less stable than mRNA associated with PABP (Bergman and Braweman 1977). More recently, the long half-life of globin mRNA has been shown to be dependent on PABP (Bernstein _et al_. 1989).

The question remains, how can two polyadenylated messages such as beta globin with a half-life of about 30 h and c-_myc_ which has an approximately 30 min half-life be differentially regulated by PABP. Ross suggests that c-_myc_ (and other short lived messages) possess instability sequences that interfere with the ability of PABP to protect the poly(A) tract (Bernstein and Ross (1989). These regions may include the specific "AU"-rich sequences in the 3' untranslated portion of c-_myc_, c-_fos_ and GMCSF mRNAs described above.

Aside from ribonucleases, a number of other factors can direct the degradation of specific mRNA molecules. Some hormones have been shown to regulate mRNA turnover. For example, estrogen increases the steady-state level of vitel-

logenin mRNA (Brock and Shapiro 1983). This is accomplished by an increase in the transcription rate and an increase in the stability of vitellogenin mRNA. Other hormones which have been shown to regulate mRNA stability include prolactin, which increases the stability of casein mRNA about 20 fold (Guyette et al. 1979), and cortisone which increases the stability of human growth hormone message (Paek and Axel 1987).

In some cases, a protein has been shown to influence the level of its own message. For instance, histone mRNA is degraded at its characteristic rate during the S phase of the cell cycle when large amounts of histones are bound to the newly synthesized DNA. When DNA synthesis stops and the level of free histones in the cytoplasm rises, an increase in the degradation of histone messages is induced (Stein and Stein 1984; Wu and Bonner 1985). Using an in vitro mRNA degradation system, Peltz and Ross (1987) confirmed that the increased degradation of message was due to a rise in the level of histone proteins. They found that the rate of degradation of histone message quadrupled when histones were added to polyribosomes isolated from cells.

Alpha and beta tubulins, the principle constituents of microtubules, have also been found to regulate the degradation of their own messages. Early studies using inhibitors of microtubule polymerization initially led investigators to believe that tubulin synthesis was autoregulated. It was

observed that when cells were exposed to colchicine, which induces rapid microtubule disassembly and raises the levels of free tubulin, the rate of alpha and beta tubulin synthesis was lowered 5-10 fold (Ben Ze'ev et al. 1979).

When cells were treated with vinblastine, microtubule disassembly was induced, the drug also caused the precipitation of tubulin, thus lowering the cytoplasmic tubulin concentration. Cells treated with vinblastin increase tubulin synthesis. In order to be certain that these results were not due to some other action of the drugs, experiments were conducted which directly microinjected purified tubulin into cells. The addition of free tubulin rapidly suppressed new tubulin synthesis (Cleveland et al. 1983).

Using-tubulin specific cDNA probes, Cleveland et al. (1981) demonstrated that the changes in the rate of tubulin synthesis paralleled changes in the levels of tubulin mRNA. The correlation between tubulin mRNA levels and the rate of tubulin synthesis initially led investigators to believe that transcriptional control was responsible. However, this hypothesis was dismissed when the same mechanism of regulation of tubulin synthesis could be found in enucleated cells (Pittenger and Cleveland 1985); Caron et al. 1985).

In some recent transfection experiments, hybrid genes encoding portions of tubulin genes were inserted into reporter genes. It was discovered that only the first thirteen nucleotides of the coding region of tubulin mRNA could confer

the auto-regulated instability of tubulin mRNA (Yen _et_ _al_. 1988). Point mutations were introduced into these thirteen nucleotides of the tubulin gene. Mutants which had the third base of a codon changed but retained the same amino acid sequence also retained tubulin autoregulation. However, point mutations which changed the amino acid sequence lost tubulin auto-regulation. Similar transfection experiments which led to a frameshift mutation in the thirteen nucleotide region abrogated tubulin auto-regulation (Yen _et_ _al_. 1988).

The observations described above have led to the proposal that the first four amino acids, and not necessarily the first thirteen nucleotides in the coding region of tubulin mRNA are responsible for its auto-regulation. A model for the instability of tubulin mRNA has been proposed by Yen _et_ _al_.(1988). As the levels of free tubulin in the cell increase, there is an interaction between an auto-regulatory factor (presumably free tubulin) and the nascent amino terminal tetrapeptide. This protein-protein interaction then somehow signals for the degradation of the tubulin mRNAs presently being translated. Two possibilities of how degradation is induced have been proposed. The protein-protein interaction may induce a cellular ribonuclease or the interaction may cause the ribosome to stall, which leaves the message in an 'exposed state' allowing ribonuclease degradation. This mechanism of mRNA degradation for the control of tubulin synthesis demonstrates an important link

between mRNA stability and ongoing protein synthesis.

The HSV vhs protein is, of course, another example of an extrinsic factor that affects cellular mRNA stability. This mechanism is different from those described above in that the degradation of message is non-specific. The vhs gene product may be a ribonuclease or, more likely, it may induces a cellular ribonuclease.

MATERIALS AND METHODS

<u>Cells</u>. Vero cells were purchased from the American Type Culture Collection (ATCC Rockville, Maryland). Cells were maintained in Eagle's minimum essential medium (MEM, Gibco, Grand Island, NY) supplemented with 10% calf serum, 7.5% $NaHCO_3$ (to adjust pH to 7.2), and 1 mM L-glutamine. Antibiotic and antimycotic agents also added to the medium consisted of 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. Confluent monolayers were passaged 1:5, twice a week. Cell stocks were stored in MEM + 10% calf serum and 15% dimethyl sulfoxide at -90°C.

<u>Plasmids</u>. pSV3CAT and pKX2-P4 were kindly provided by Neal DeLuca, Harvard Medical School, Boston, Massachusetts. pSV3CAT contains the bacterial chloramphenicol acetyl transferase (CAT) gene inserted into the eucaryotic vector pSV2 (DeLuca and Schaffer, 1985). pKX2-P4 contains the promoter and structural portion of the ICP4 gene from HSV-1 strain KOS (DeLuca <u>et</u> <u>al</u>., 1985). pSG1, provided by Myron Levine, Univ. of Michigan, Ann Arbor, Michigan, contains the <u>Eco</u>R1 J-K fragment of wild type HSV-1, strain KOS (Goldin <u>et</u> <u>al</u>., 1981). pHS consists of a <u>Hin</u>dIII-<u>Sal</u>I fragment from HSV-1 strain KOS (map coordinates 0.592-0.614) which contains the entire <u>vhs</u> gene inserted between the <u>Hin</u>dIII and <u>Sal</u>I site of

Bluescript KS (Read and Knight, unpublished data; Stratagene, San Diego, CA). pHSΔSMA was constructed by removing a 588 bp SmaI fragment from the center of the vhs gene in pHS (Read and Knight, unpublished data). The plasmid pKC7BglIIN, kindly provided by Denise Galloway, Fred Hutchinson Cancer Research Center, Seattle, Washington, contains the BglIIN fragment of HSV-2 strain 333 (map coordinates 0.58-0.625) inserted into the BglII site of pKC7. The plasmid pX1r11, kindly provided by Jeff Doering, Loyola University Chicago, Chicago, Illinois, contains a 4.6 kilobase (kb) Xenopus laevis-DNA fragment cloned into the EcoRI site of Colicin E1. The plasmid pHcGAP contains a 1.2 kb cDNA fragment encoding part of the human glyceraldhyde-3-phophate dehydrogenase gene and was obtained from the ATCC. The plasmids pSG1, pHcGAP and pX1r11 were maintained in E. coli strain HB101, all other plasmids were maintained in E. coli strain DH5α (BRL, Bethesda Research Laboratories, Rockville, Maryland.

Virus. The recombinant virus vhsΔSma has a 588 bp SmaI fragment deleted from the center of the vhs gene. (Read and Knight,unpublished data).

Plasmid DNA Isolation. Plasmids used for cloning and transfections were prepared by the alkaline lysis method for large scale plasmid preparation as described by Sambrook (Sambrook et al., 1982). Plasmids used for transfections, however, were purified through two rounds of cesium chloride density-gradient centrifugation. A rapid, small-scale

plasmid preparation method(Morelle 1989) was used for restriction-enzyme analysis of recombinant plasmids.

DNA manipulation used for cloning. Plasmid DNA was digested with restriction enzymes purchased from New England Biolabs (Beverly, MA)and used as recommended by the manufacturer. Digested DNA samples were size fractionated on 1.0% agarose gels. The agarose gels were stained with ethidium bromide, and the DNA fragments were visualized using ultraviolet light. The appropriate fragments were cut out with a scalpel and recovered from the agarose using a Geneclean kit (Bio 101 Inc., La Jolla, CA). Ligation reactions were carried out overnight at 15°C using T4 DNA ligase (New England Biolabs). Recombinant plasmids were transformed into *E. coli* strain DH5α from Bethesda Research Laboratories (BRL) using a calcium chloride procedure for making competent cells (Sambrook *et al.*, 1982). The vector used for all recombinants was Bluescript KS. When transformed into DH5α, Bluescript KS allows blue-white color selection. Colonies containing plasmids with no inserts are blue, while those containing inserts are white when grown on Luria Broth agar plates containing 100μg/ml ampicillin, 40 μg/ml X-Gal, and 5mM IPTG.

Construction of recombinant plasmids. pKS:*Bgl*IIN was constructed by digesting pKC7*Bgl*IIN with the restriction enzyme *Bgl*II N. The 7.3 kb *Bgl*IIN fragment was then gel purified and inserted into the *Bam*HI site of Bluescript KS.

pKS:Bam was constructed by cutting pKC7BglIIN with BamHI. The 3.4 kb BamHI fragment containing the vhs open reading frame was inserted into the BamHI site of Bluescript KS.

HSV-1/HSV-2 recombinant plasmids. Recombinant vhs genes were constructed using the SacI or PvuI restriction sites. There are unique SacI sites in analogous positions in the vhs genes of HSV-1 and HSV-2, two thirds of the way down the coding region of the gene. The plasmid pHSV2-1:Sac was constructed by first digesting pKS:Bam to completion with BamHI and SacI. A 1725 bp SacI-BamHI fragment containing the first two thirds of the HSV-2 vhs gene was inserted between the SacI and BamHI sites of Bluescript KS. This intermediate clone was called pKS:Bam-Sac. The plasmid pHS was then digested to completion with SacI and a 1380 bp SacI fragment containing the last one third of the HSV-1 vhs gene was inserted into the SacI site of pKS:Bam-Sac. Digestion of pHSV2-1:Sac with various restriction enzymes verified that the SacI fragment was in the correct orientation. The plasmid pHSV1-2:Sac is the inverse of pHSV2-1, that is, the first two thirds of the vhs gene is from HSV-1 and the last third is from HSV-2. pHSV1-2:Sac was constructed by first cutting pHSI to completion with SacI and PstI. A 1676 bp fragment containing the first two thifds of the HSV-1 vhs gene was inserted between the PstI and SacI sites of Bluescript KS. This intermediate clone was called pKS:Pst-Sac. pKS:BAM was then cut with SacI and a 1441 SacI fragment containing the

last thifd of the HSV-2 _vhs_ gene was inserted into the _Sac_I site of pKS:Pst-Sac. The orientation of the _Sac_I fragment was verified by cutting with various restriction enzymes.

There are unique _Pvu_I sites in analogous positions in the _vhs_ genes of HSV-1 and HSV-2, one third of the way down the coding region of the gene. pHSV1-2:Pvu was constructed in the following manner. The plasmid pHSI was digested with _Sal_I and treated with calf intestinal phosphatase. Linear pHSI was then digested with _Pvu_I and a 1533 bp fragment containing the first third of the _vhs_ gene was recovered. pKS:BAM was cut with _Bam_HI, treated with calf intestinal phosphatase, followed by digestion with _Pvu_I and a 2524 bp fragment containing the last two thirds of the _vhs_ gene was recovered. Bluescript KS was cut with _Sal_I and _Bam_HI to completion. The three fragments were added in approximately equimolar amounts in a ligation reaction and the recombinant pHSVI-2:_Pvu_ was recovered and tested for the proper orientation of inserts. pHSV2-1:Pvu was constructed in a similar manner. pKS:BAM was cut with _Bam_HI, treated with calf intestinal phosphatase, followed by digestion with _Pvu_I. An 866 bp fragment was recovered from the various fragments. pHSI was digested with _Hin_dIII, followed by treatment with calf intestinal phosphatase. The DNA was then cut with _Pvu_I and a 2246 kb fragment containing the last two thirds of the HSV-1 _vhs_ gene was recovered. Bluescript KS was digested to completion with _Bam_HI and _Hin_dIII and subsequently combined in a ligation

reaction with the 2246bp and 866 bp fragments. The ligation product, pHSV2-1:Pvu was confirmed by restriction enzyme analysis.

Transfections. Transfections were performed using the calcium phosphate precipitation method of Graham and Van der Ebb (1973). Approximately 2 x 10^6 vero cells were plated onto 100 mm petri dishes the day before transfection. Three hours before the addition of precipitate, the medium was removed and 10 ml of fresh MEM containing 10% calf serum were added to each dish. Three-milliliter volumes of DNA-calcium phosphate coprecipitates were prepared by adding plasmid DNAs to HEPES buffered saline (HBS). HBS contained 25 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid), 140mM NaCl, 0.75mM Na_2HPO_4, 5 mM KCl and 6 mM glucose. Five micrograms of pSV3CAT, 10 μg each of pSG1 and PKX2-P4 and 10 μg of a plasmid containing the wild type or mutant vhs gene were used in each precipitation. DNA was always brought up to a final concentration of 33 μg/ml using salmon sperm DNA as a carrier. $CaCl_2$ was added to the solution last at a final concentration of 120 mM. The precipitates were allowed to form for 30 min at room temperature and were then added to each of the petri dishes. The cells were incubated with the precipitate for 1 h at 37°C followed by the addition of 7 ml MEM supplemented with 10% calf serum and three additional hours of incubation. The medium was aspirated and cells were subjected to a glycerol shock as described by (Parker and Stark, 1979). MEM

containing 15% glycerol was added to each petri dish for 1 min. Cells were washed two times with warm phosphate-buffered saline (PBS) and incubated at 37°C with fresh MEM + 10% calf serum.

CAT assays. Cell harvesting and CAT assays were carried out as described by Gorman *et al.*(1982). Forty-to-forty-eight hours after transfection, cells were washed once with cold PBS, scraped into 15 ml conical tubes with 5 ml cold PBS and pelleted by low-speed centrifugation at 4°C. The pellet was resuspended in 100 μl 0.25M Tris (pH 7.8) and transferred to microfuge tubes. Cell extracts were made by three freeze-thaw cycles followed by centrifugation at 15,000 rpm at 4°C in a microcentrifuge. Supernatant fluids were transferred to a second microfuge tube and assayed for protein concentration using the Bio-Rad (Richmond, CA) protein microassay with bovine serum albumin as a protein standard. At this point of the experiment, extracts containing an equal concentration of protein were assayed for CAT activity or stored at -20°C. The reaction mixture consisted of 0.25 v*M* Tris, 3 m*M* acetyl CoA (Sigma, St. Louis, MO), 0.2 μCi ^{14}C-chloramphenicol (New England Nuclear, Boston, Massachusetts), 5-25 μg cellular protein (depending on the experiment) in a final volume of 100 μl. Reactions were carried out at 37°C for 45 min. Chloramphenicol and its acetylated derivatives were extracted two times with 0.5 ml ethyl acetate. The organic phase was dried down, resuspended in 25 μl ethyl acetate, and spotted on

a plastic-backed-silica-gel, thin-layer-chromatography plate. The acetylated products were separated by ascending chromatography for 45 min using 19:1 (v/v) chloroform-methanol as the solvent. The dried plate was then exposed to XAR-5 X-ray film overnight to visualize the acetylated and unacetylated forms of chloramphenicol. For quantification, spots were cut out and ^{14}C content was determined by liquid scintillation spectroscopy.

Cytoplasmic RNA extraction. Cytoplasmic RNA was extracted using a modification of the procedure described by Berk and Sharp (1977). Vero cells were washed twice with cold PBS and scraped into microfuge tubes with 1 ml PBS. Cells were pelleted at high speed in a microcentrifuge for 30 sec. The cells were resuspended in 190 μl of a solution containing 10 mM Tris (pH7.9), 0.15 M NaCL, 1.5 mM $MgCl_2$. Cells were then lysed by adding 10 μl of a solution containing 10 mM tris (pH7.9), 0.15 M NaCL, 1.5 mM $MgCl_2$ and 13% of the detergent NP40 and incubating on ice for 10 min. The cell lysate was centrifuged at 4°C for 3 min in order to pellet the nuclei. The supernatant fluid was added to a microcentrifuge tube already containing 200 μl urea buffer (10 m M Tris (pH7.9), 7 M urea, 0.35 M NaCl, 10 mM EDTA and 1% SDS) and 300 μl phenol. This mixture was vortexed and 300 μl chloroform was added and vortexed. The aqueous and organic phases were separated by a three minutes of high-speed centrifugation in a table-top microcentrifuge. The organic phase was extracted again using

200μl of urea buffer. The aqueous phases were combined and phenol-chloroform extracted a second time followed by two more chloroform extractions. The aqueous phase was then split into two microfuge tubes and the RNA was ethanol precipitated with 2.5 volumes of ethanol at -20°C overnight. A cytoplasmic RNA pellet was recovered by centrifugation at 15,000 rpm for 10 min at 4°C in a microcentrifuge. This pellet was resuspended in 50 μl TE and stored at -90°C.

Labeling of DNA probes. Radioactive probes were prepared by nick translation. The plasmids pX1r11 and pSV3CAT were labeled using the nick translation kit and protocol supplied by BRL. One μ g of uncut plasmid was added to a mixture containing 125 μCi α[^{32}P]-dCTP, 5 μl of a 0.2 mM solution of the four deoxyribonucloside triphosphates, 5 μl of a solution containing DNaseI and DNA polymerase I in a total volume of 50 μl. The labeling reaction was incubated at 15°C for 1 h and then stopped by the addition of 5 μl of of a 5 mM EDTA solution. Unincorporated nucleotides were removed by Sephadex G-50 chromatography using the spun column procedure described by Sambrook et al.(1989).

Slot blots. Total cytoplasmic RNA from transfected Vero cells was first denatured by treatment with glyoxal. The RNA was incubated in 1 M glyoxal, 10 mM sodium phosphate buffer (pH 6.8) at 50°C for 1 h. RNA was cooled on ice and diluted so each sample could be loaded in 5 μg, 2.5 μg, and 1 μg quantities. Diluted samples were blotted onto a Zeta-Probe

(BioRad) membrane using a Bio-Dot SF blotting apparatus and protocol supplied by BioRad. Glyoxal was removed from zeta-probe membrane by placing the membrane in a vacuum oven at 80°C for 1 h.

Hybridization of slot blots. The membrane was placed in a zip-lock bag and prehybridized in a solution of 1% bovine serum albumin (BSA), 1 mM EDTA, 7% SDS, and 0.5 M $NaPo_4$ (pH7.2) for 15 min at 65°C. The solution was poured out of the bag and replaced with the same solution containing the denatured probe. Hybridization was carried out overnight at 65°C. After hybridization, the blots were rinsed in 2 x SSC followed by three 15 min washes at room temperature. The first wash was in 2 x SSC containing 0.1% SDS. The second wash was in 0.5 x SSC containing 0.1% SDS followed by a final wash in 0.1 x SSC containing 0.1% SDS. The blot was removed, placed in a new zip lock bag, and exposed to XAR-5 X-ray film for 2-12 h.

RNA gel electrophoresis. Cytoplasmic RNA was denatured by treatment with glyoxal (Thomas,1980). Here, the glyoxal reaction consisted of 10 µg RNA, 1 M deionized glyoxal, 50% dimethylsulfoxide in 10 mM $NaPO_4$ buffer (pH 6.8) and was incubated at 50°C for 1 h. The RNA was immediately put on ice and 4 µl of tracking dye (1% bromophenol blue and 1% xylene cyanol) was added. Half of the sample of RNA was frozen for future use, and the other half was run on a 1% agarose gel in

10m$\underline{M}$ NaH_2PO_4 (pH7.0) for 3 h. at 115V with constant buffer recirculation.

Northern blots. The fractionated RNA was transferred by capillary blotting onto Nytran modified Nylon-66 membrane (Schleicher and Schuell. Petersburo, New Hampshire). Capillary blotting was performed overnight according to the manufacturers instructions using 10 x SSPE. SSPE contains 0.18 $\underline{M}$ NaCl, 10 m$\underline{M}$ $NaPO_4$, and 1 m$\underline{M}$ EDTA. After blotting was complete, the Nytran membrane was baked at 80°C for 1 h in order to immobilize the RNA.

Hybridization of Northern blots. The membrane was first prehybridized by placing it in a zip-lock bag with 0.25 ml prehybridization buffer for each square centimeter of membrane. The prehybridization buffer consisted of 50% deionized formamide, 1 x Denhardt's reagent (0.02% Ficoll, 0.02% polyvinylpyrollidone, 0.02 % BSA), 0.1 % SDS, 5 X SSPE, and denatured salmon sperm DNA (200 μg/ml). The bag was placed at 42^0C for 2 h with agitation. The prehybridization buffer was poured from the bag, and the hybridization buffer (which was the same as the prehybridization buffer but contained 2.5 x Denhardts reagent) was added to the same bag. The radioactive DNA probe was denatured by boiling for 5 min and put immediately on ice. Once chilled the probe was added to the hybridization buffer and hybridization was carried out at 42°C overnight. After hybridization, the blot was washed twice in 2 x SSPE containing 4% SDS for 45 min at room

temperature with constant shaking. The blot was then washed twice with 0.1 x SSPE at 60°C in a shaking water bath for 15 min. After washing, the blot was placed in a new zip lock bag and exposed to XAR-5 X-ray film between intensifying screens for 4-24 h. The bands on the autoradiogram were scanned in a Gilford Response spectrophotometer.

RESULTS

Basis for an in vitro assay of vhs function.

In the past, information regarding *vhs* gene function has been obtained from experiments using whole HSV viruses carrying either wild type or mutant *vhs* genes. Mutants of *vhs* function were generated in either of two ways. In the first method, *vhs* mutants were obtained by exposing virus stocks to bromodeoxyuridine, plaque purifying the survivors and screening them for virus defective in host shutoff activity (Read and Frenkel 1982). While mutants obtained from this procedure are certainly valuable in analyzing the host shutoff function, the sites of the mutations are random. It is also probable that multiple mutations in other portions of the viral genome were created.

A second method of mutagenesis eliminated these problems by taking advantage of the mapping of the *vhs* gene. One of the bromodeoxyuridine generated mutations, *vhs*1, was mapped to a 265 base pair fragment which subsequently allowed the HSV UL41 open reading frame to be identified as the *vhs* gene (Kwong *et* *al*. 1987; McGeoch *et* *al*. 1988). In addition, the *vhs* protein was recently identified in purified virions and infected cells using antibodies rased against *vhs*-

betagalactosidase fusion proteins (Read *et al.*, submitted for publication). Although these studies have clearly demonstrated that the UL41 (*vhs*) gene product is required for the HSV host shutoff function, it has not been discerned whether it is sufficient for this function. Our aim was to determine whether the *vhs* gene product is the only viral polypeptide required for the virion induced degradation of mRNAs, or whether this phenomonon requires one or more additional virion proteins. The problem was approached by developing an assay which could measure the *vhs* activity of genes transfected into tissue culture cells. With the ability to express only one or a select few viral genes, it should be possible to demonstrate whether any other viral genes are required in conjunction with UL41 for *vhs* activity.

The mapping of the the host shutoff function to the UL41 open-reading frame of HSV has also allowed further analysis of the structure and function of the *vhs* gene. The *vhs* gene of HSV-1 (KOS) was removed from the viral genome, and site-specific mutations were generated *in vitro*. The mutated genes were then reintroduced into the virus for *in vivo* examination of *vhs* function (Read and Knight unpublished data). While this method is the most direct means of characterizing the structure and function of the *vhs* gene, it does not allow quick screening of large numbers of mutants. Our *in vitro* assay could also be used to quickly and easily test *vhs* function on a number of *in vitro* generated mutants.

Strategy for an in vitro assay of vhs function.

A transient expression system was used to express the vhs gene and examine its activity. The assay involved cotransfecting Vero cells with the reporter gene pSV3CAT, a plasmid containing a wild type-or mutant vhs gene, and plasmids containing the HSV transcriptional activators ICP4 and ICP0. Approximately 40 h after the transfection, cells were harvested and analyzed for CAT activity. The rationale behind the correlation between CAT and vhs activities in this assay is based on the observation that the vhs protein nonselectively induces the degradation of viral and cellular mRNAs (Oroskar and Read 1987;1989). If a functional vhs gene is expressed in this system, it should induce accelerated degradation of cellular messages as well as messages of all the transfected genes. The degradation of CAT message would lead to a decrease in CAT protein synthesis and ultimately less CAT enzymatic activity. The genes encoding the alpha polypeptides, ICP4 and ICP0, were originally included in the co-transfection because the vhs gene belongs to the $beta_2$ class of HSV proteins and is thus induced during virus infection by alpha polypeptides (DeLuca and Schaffer 1985; O'Hare and Hayward 1984).

We chose to use the vhs gene from HSV-2 strain 333 for the initial testing of our assay because type II strains of HSV generally shut off host protein synthesis more efficiently than type 1 strains (Pereira et al. 1977; Powell and Courtney

1975). The high degree of host shutoff activity in HSV-2 strains would be more likely to be detected in the initial experiments. Since HSV-1 and HSV-2 are collinear, the location of the homologue of the UL41 (vhs) open reading frame of HSV-1 could be predicted to be contained in the 7.3 kb BglIIN fragment spanning map coordinates 0.580 to 0.620 of HSV-2 (figure 2). A plasmid containing the HSV-2 BglIIN fragment, pKC7BglN, was thus used in cotransfections to supply the HSV-2 vhs gene. In addition to the UL41 open reading frame, the BglIIN fragment contains the HSV-2 homologues of the HSV-1 UL40, UL42 and part of the UL39 open reading frames (Galloway et al. 1984).

As a control, pSV3CAT, Bluescript KS, and plasmids containing ICP4 and ICP0 were cotransfected into Vero cells in the absence of any sequences predicted to encode the vhs gene (figure 3). As expected, cell extracts from this transfection had considerable CAT activity. When pKC7BglN was included in the transfection, the level of CAT activity was reduced by more than five fold (figure 3). To ensure that the decrease in enzymatic activity was due to sequences within the BglIIN fragment and not to the pKC7 vector sequences, the BglIIN fragment was recloned into Bluescript KS. When the vhs gene was supplied by adding pKS:Bgl N to the transfection, the level of CAT activity was again reduced 5 fold compared to the control transfection (figure 3).

Figure 2 The *BglII* N fragment of HSV-2. The top two lines represent the HSV-2 genome while the third line shows the *BglII* N fragment of HSV-2 spanning map coordinates .580-.620. Below, the transcripts mapped to the *BglII* N fragment are designated by the arrows while the polypeptides they encode and their sizes are designated by the open boxes. The 1.5 kb mRNA located between the open-reading frames UL42 and UL44 presumably contains an open reading frame corresponding to UL43 of HSV-1. However, this part of the HSV-2 genome has not yet been sequenced. Therefore the 1.5 kb mRNA is shown without an open reading frame.

HSV-2

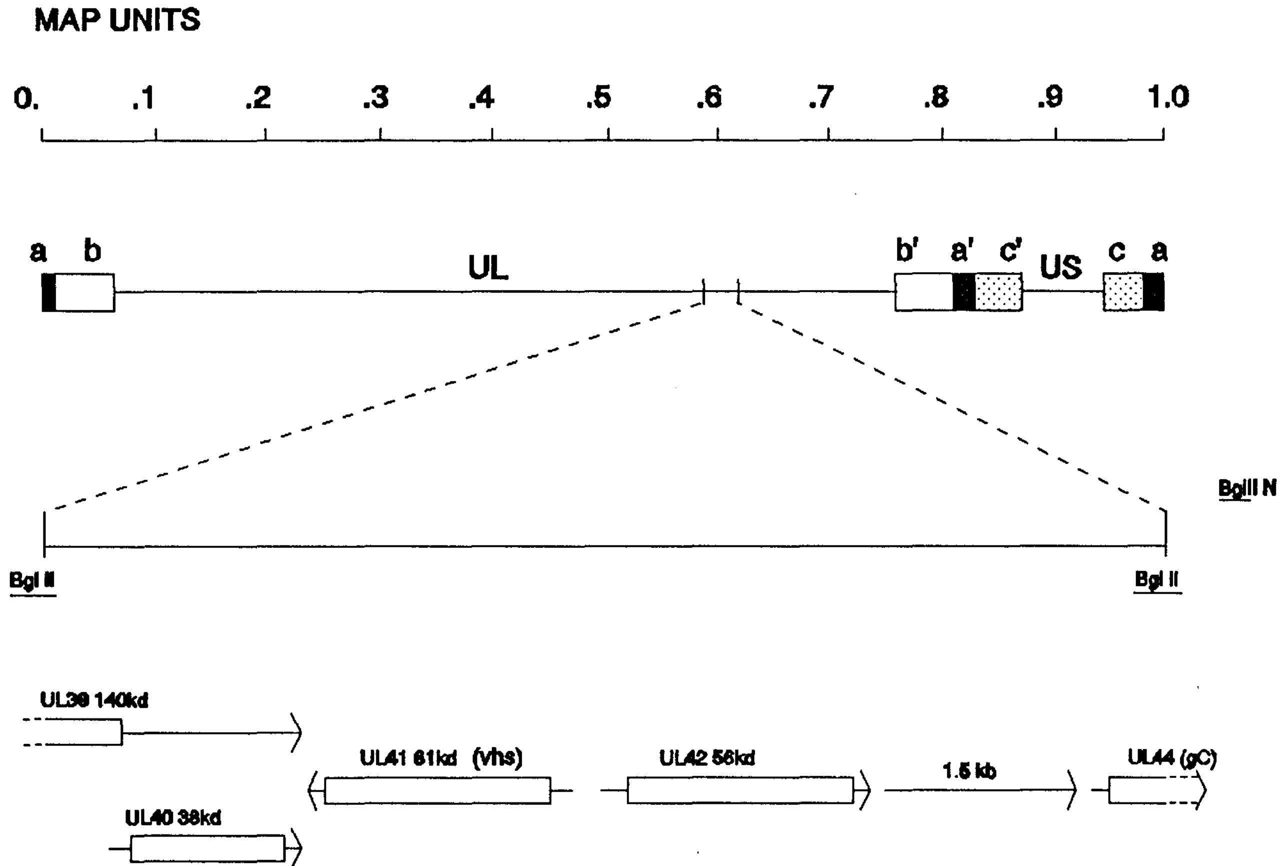
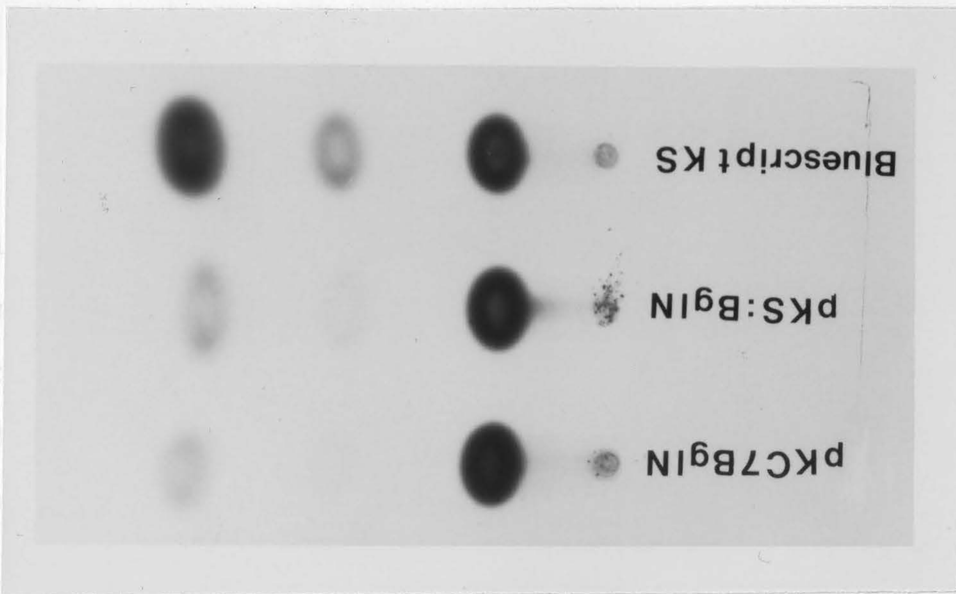
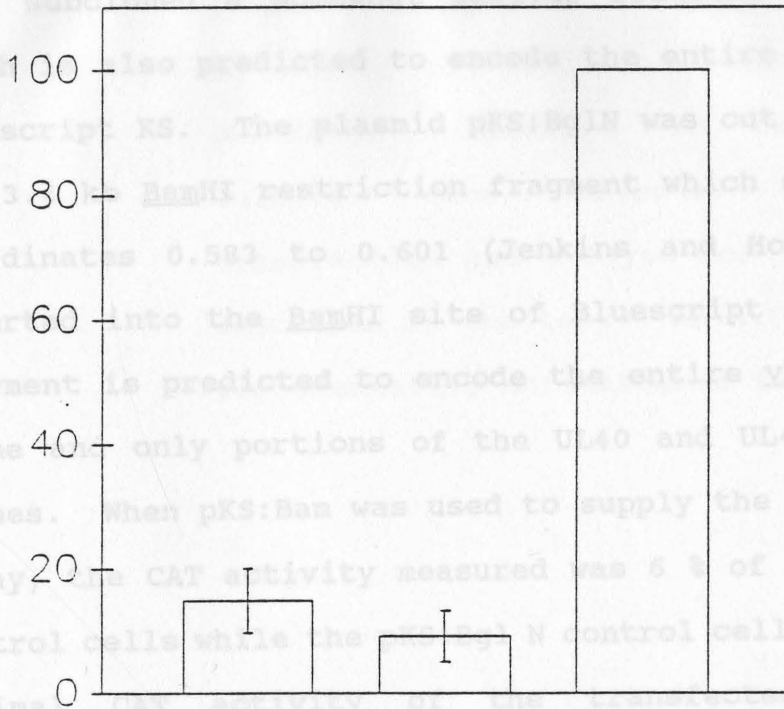


Figure 3 The plasmids containing the _BglII_ N fragment of HSV-2 inhibit CAT gene expression. Vero cells in 100 mm Petri dishes were transfected with mixtures containing 5 μg of pSV3CAT, 10 μg of pKX2P4, 10 μg of pSG1, 65 μg of salmon sperm DNA, and 10 μg of one of the following plasmids: pKC7BglN, pKS:BglN or Bluescript KS. Cultures were harvested 48 h after transfection and assayed for CAT activity as described in the text. **A.** The relative CAT activity is defined as the ratio of percent acetylation by the indicated plasmid to the percent acetylation by the control plasmid Bluescript KS. **B.** Autoradiogram of a representative chromatogram.


B
Bluescript KS
pKS:BglN
pKC7BglN

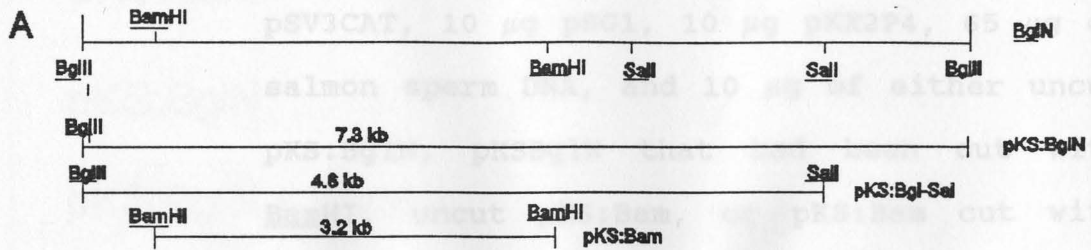

A
RELATIVE CAT ACTIVITY
0
20
40
60
80
100
pKC7Bgl N
pKS:Bgl N
Bluescript KS

To delimit the sequences responsible for the decrease in CAT activity, portions of the BglIIN fragment were subclonedinto Bluescript KS. First, a 4.6 kb BglII-SalI fragment spanning map coordinates 0.585 to 0.617 (Jenkins and Howett 1984) of the HSV-2 genome was inserted between the BamHI and SalI sites of Bluescript KS. As can be seen in Figure 4, when pKS:Bgl-Sal was cotransfected in our assay, the level of CAT activity was again significantly reduced. We next subcloned a somewhat smaller fragment from pKS:BglN, which is also predicted to encode the entire vhs gene, into Bluescript KS. The plasmid pKS:BglN was cut with BamHI and the 3.4 kb BamHI restriction fragment which spans HSV-2 map coordinates 0.583 to 0.601 (Jenkins and Howett 1984) was inserted into the BamHI site of Bluescript KS. The BamHI fragment is predicted to encode the entire vhs open reading frame and only portions of the UL40 and UL42 open reading frames. When pKS:Bam was used to supply the vhs gene in our assay, the CAT activity measured was 6 % of the activity in control cells while the pKS:Bgl N control cells had 5 % of the maximal CAT activity of the transfected cells. An autoradiogram of this experiment is shown in Figure 5. These values indicate that the Bam HI restriction fragment contains all of the sequence within the BglIIN fragment necessary for vhs activity.

In the next set of experiments, we used a second approach to define further the regions within the BglIIN fragment which

Figure 4 Subfragments of HSV-2 BglII N inhibit CAT gene expression. **A.** The BglII N fragment and relevant restriction enzymes sites are shown in the first line. The next three lines show the fragments of BglII N used to create the plasmids pKS:BglN, pKS:Bgl-Sal and pKS:Bam. **B.** Vero cells in 100 mm Petri dishes were transfected with mixtures containing 5 μg pSV3CAT, 10 μg pKX2P4, 10 μg pSG1, and 10 μg of one of the following plasmids: pKC7BglN, pKS:BglN, Bluescript KS or pKS:Bgl-Sal. Cultures were harvested 48 h. after transfection and assayed for CAT activity as described in the text. An autoradiogram of a representative chromatogram is shown.

A



B

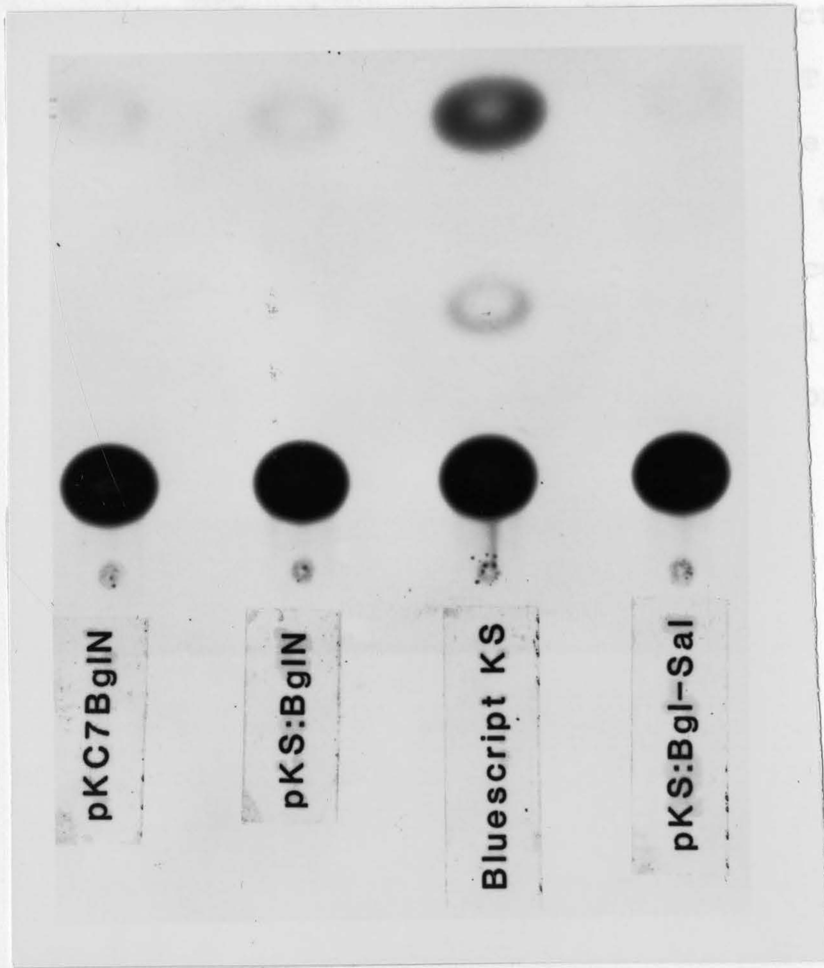
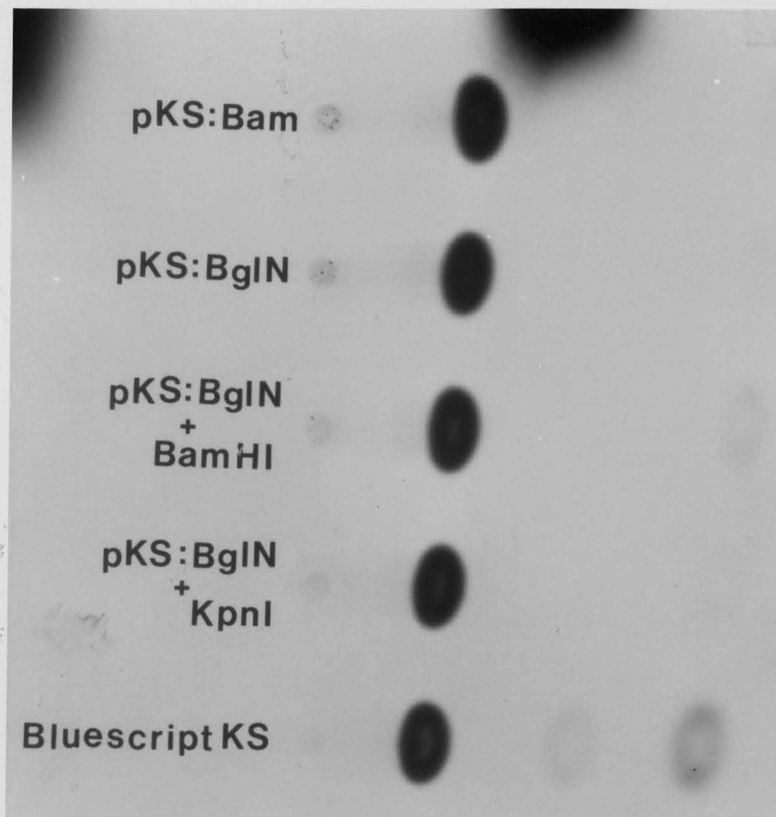


Figure 5 Effect of restriction enzyme cleavage on the ability of plasmids containing the vhs gene to inhibit CAT gene expression. Vero cells were transfected with mixtures containing 5 μg pSV3CAT, 10 μg pSG1, 10 μg pKX2P4, 65 μg of salmon sperm DNA, and 10 μg of either uncut pKS:BglN, pKSBglN that had been cut with BamHI, uncut pKS:Bam, or pKS:Bam cut with KpnI. Cultures were harvested 48 h. after transfection and assayed for CAT activity as described in the text. Relative CAT activity is defined as the ratio of the percent acetylation in the transfection with the indicated plasmid to the percent acetylation in the transfection with the control plasmid, Bluescript KS. Representative autoradiogram of chromatograph is shown.

Plasmid transfected	Relative CAT Activity
pKS:Bam	5.8
pKS:BglN	7.7
pKS:BglN + BamHI	18.6
pKS:BglN + KpnI	7.9
Bluescript KS	100



are required for vhs gene expression and activity. Either pKS:BglN or pKS:Bam was digested at restriction sites within the coding region or outside of the vhs gene. The linear fragments were cotransfected with both the CAT gene and HSV transcriptional activators and were subsequently assayed for vhs function. It was predicted that if the DNA was cleaved within the vhs gene or at control regions of the gene, vhs activity would be decreased or eliminated, while cutting the plasmid outside of UL41 would not affect vhs activity. Through these experiments, we hoped to correlate the expression of the vhs polypeptide with a decrease in CAT activity.

To first demonstrate that cutting pKS:BglN with a restriction enzyme in itself does not affect vhs function, pKS:BglN was cut with KpnI which has a single restriction site in the Bluescript vector sequence. As is shown in Figure 5, the restricted plasmid causes the same decrease in CAT activity as the intact pKS:BglN. In addition, pKS:BglN was cleaved with BamHI and the fragments were assayed for their effect on CAT activity. In this case, acetylation was slightly higher than in cells transfected with pKS:BglN or pKS:Bam; however, a five-fold decrease in CAT activity was still observed.

Having determined that linear fragments of DNA are suitable for assaying vhs function, pKS:Bam was cut with either BstEII, HindII, PstI, PvuI or XhoI. As shown by the

restriction map in Figure 6, each of these enzymes has a single restriction site within or near the vhs gene. As can be seen in Figure 7, cutting to the 3'side of the vhs gene with BstEII had no effect on vhs activity. However, cutting at the HindII site which is found nearer to the 3'-end of the vhs gene, showed a two-fold decrease in CAT activity. When pKS:Bam was cut with PstI, PvuI or XhoI, all of which lie within the central third of the vhs open reading frame, vhs activity was abolished. Control experiments which transfected pKS:BglN or pKS:Bam generated a 20 fold decrease in CAT activity.

Measurement of vhs function from HSV type 1

The preceding experiments were all carried out using the vhs gene from HSV-2 strain 333. Since type 1 strains of HSV have a somewhat lower level of host shutoff activity, we wanted to determine whether this level of vhs activity could be detected in our assay. For these experiments the vhs gene was produced by HSV-1 strain KOS. As is illustrated in Figure 8, a 3.78 kb HindIII-SalI fragment from HSV-1 strain KOS was inserted between the HindIII and SalI sites of Bluescript KS and this construct was designated pHS. The plasmid pHS was then used in our assay to determine whether the weaker host shutoff function of an HSV-1 strain KOS could be observed. The results shown in figure 8 indicate that the pHS transfected cells had a significant reduction in the level of

Figure 6 Detailed restriction map of the *BglII* N fragment. Cleavage sites for *PstI*, *PvuI*, and *XhoI* are located within the *vhs* gene. Cleavage sites for *HindII* and *BstEII* are found in the 3'-noncoding region of the *vhs* gene.

Restriction Map of HSV-2 BglII N

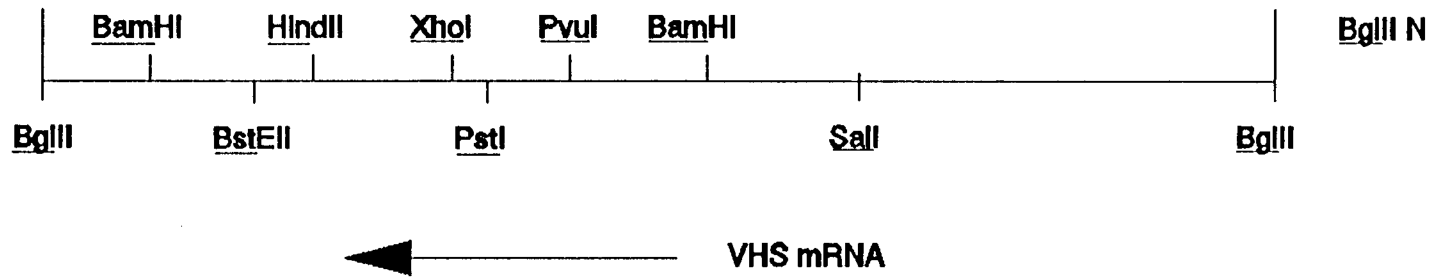


Figure 7 Effect of restriction enzyme cleavage of the *vhs* gene on CAT gene expression. Vero cells were transfected with mixtures containing 5 μg pSV3CAT, 10 μg pSG1, 10 μg pKX2P4, 65 μg of salmon sperm DNA, and 10 μg of one of the following plasmids: Bluescript KS, pKS:Bam, pKS:BglN, pKS:Bam cut with *Bst*EII, *Hin*dII, *Pst*I, *Pvu*I, or *Xho*I. Cultures were harvested 48 h after transfection and assayed for CAT activity as described in the text. Relative CAT activity is defined as the ratio of the percent acetylation by the indicated plasmid to the percent acetylation by the control plasmid, Bluescript KS. The values for CAT activity represent the means of multiple experiments and the error bars represent the standard errors of the mean.

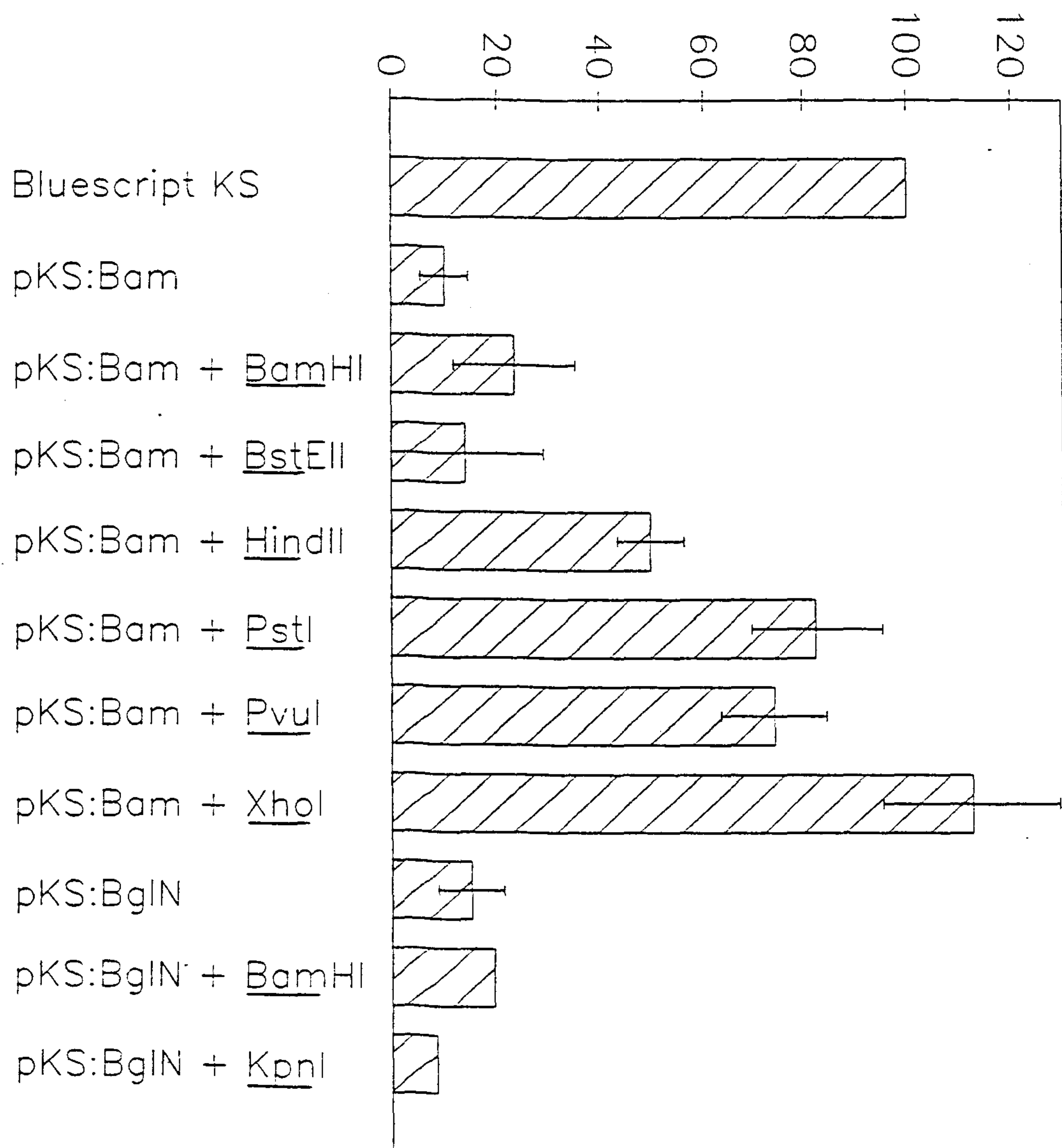
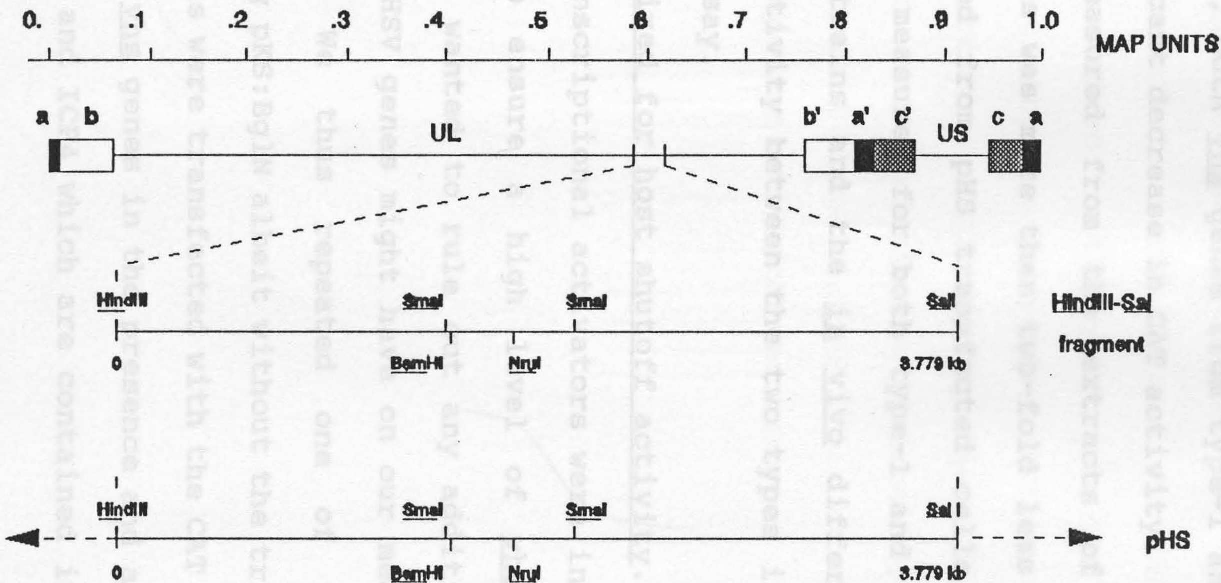
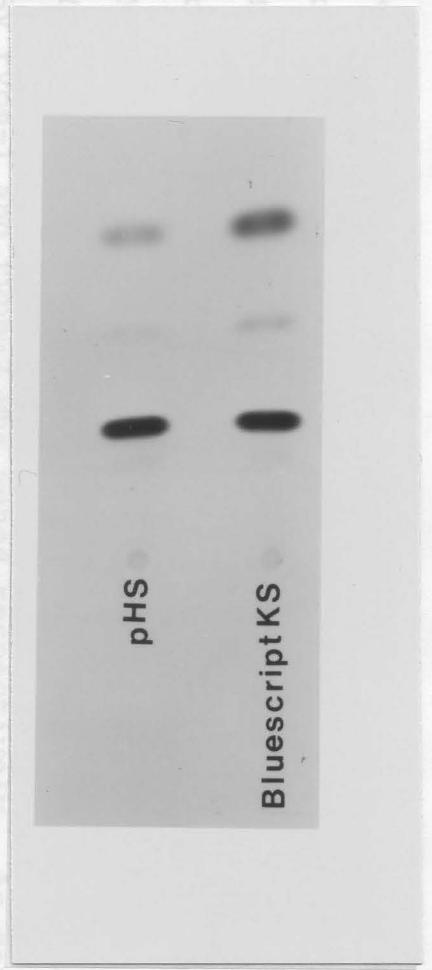

RELATIVE CAT ACTIVITY
0
20
40
60
80
100
120
Bluescript KS
pKS:Bam
pKS:Bam + BamHI
pKS:Bam + BstEII
pKS:Bam + HindII
pKS:Bam + PstI
pKS:Bam + PvuI
pKS:Bam + XhoI
pKS:BglN
pKS:BglN + BamHI
pKS:BglN + KpnI

Figure 8 Construction of pHS. **A.** A 4.779 kb HindIII-SalI fragment containing the vhs gene was removed from HSV-1 (KOS). The fragment was inserted into the HindIII and SalI sites of bluescript KS (dashed arrow) and designated pHS. **B.** Host shutoff function of HSV-1 strains can be observed using our transient expression-CAT assay. Vero cells were transfected with mixtures containing 5 μg pSV3CAT, 10 μg pSG1, 10 μg pKX2P4, 65 μg of salmon sperm DNA, and 10 μg of either pHS or Bluescript KS. Cultures were harvested 48 h. after transfection and assayed for CAT activity as described in the text.


A
Construction of pHS
0. .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0
MAP UNITS
a b UL b' a' c' US c a
HindIII SmaI SmaI SalI
HindIII-SalI fragment
0 BamHI NruI 3.779 kb
HindIII SmaI SmaI Sal I
pHS
0 BamHI NruI 3.779 kb


B
pHS
Bluescript KS

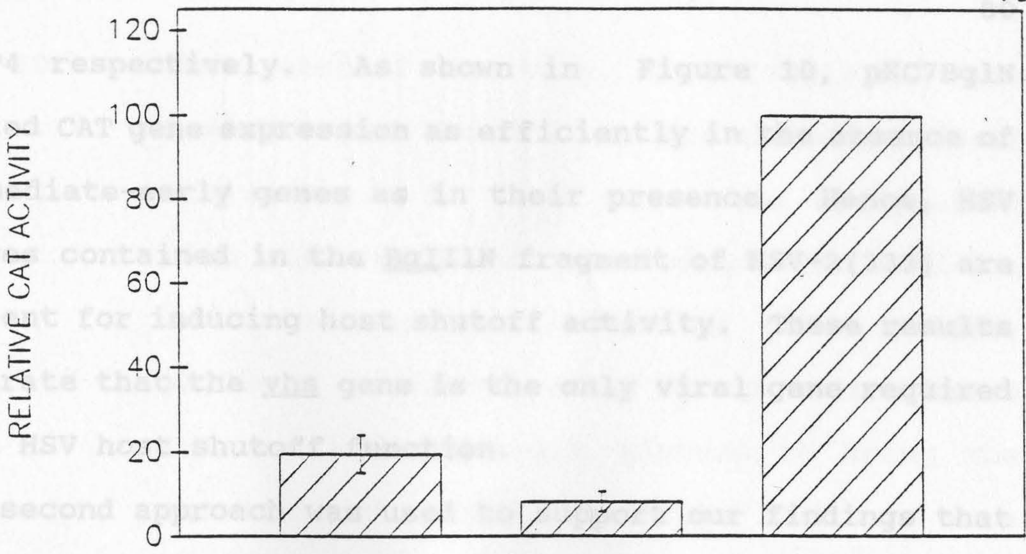
CAT activity as compared to the control transfection where Bluescript KS was transfected instead of vhs sequences.

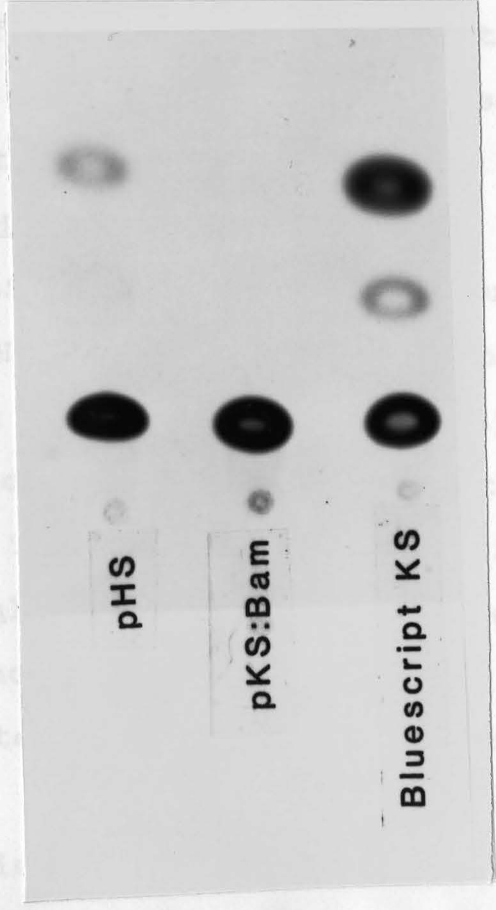
The next experiment was undertaken to observe whether a difference in levels of CAT activity from cells transfected with vhs genes from both HSV type 1 and type 2. Parallel transfections were performed with pHS and pKS:Bam which carry the HSV type-1 and type-2 vhs genes respectively. As can be seen in Figure 9, both vhs genes from type-1 and type-2 HSVs caused a significant decrease in CAT activity. Moreover, the CAT activity measured from the extracts of the pKS:Bam transfected cells was more than two-fold less than the CAT activity measured from pHS transfected cells. Thus, vhs activity can be measured for both type-1 and type-2 herpes simplex virus strains and the in vivo differences in the extent of vhs activity between the two types is retained in this in vitro assay.

Viral genes required for host shutoff activity.

The HSV transcriptional activators were included in our transfections to ensure a high level of vhs expression. Nevertheless, we wanted to rule out any additional effects these accessory HSV genes might have on our measurements of vhs function. We thus repeated one of the previous experiments using pKS:BglN albeit without the transcriptional activators. Cells were transfected with the CAT gene alone or both the CAT and vhs genes in the presence and absence of the alpha genes ICP0 and ICP4 which are contained in pSG1 and

Figure 9 The differences in extent of _vhs_ activity between HSV-1 and HSV-2 measured _in vivo_ can also be shown _in vitro_. Vero cells were transfected with mixtures containing 5 μg pSV3CAT, 10 μg pSG1, 10 μg pKX2P4, 65 μg of salmon sperm DNA, and 10 μg of either pHS (HSV-1), pKS:Bam (HSV-2) or Bluescript KS. Cultures were harvested 48 h. after transfection and assayed for CAT activity as described in the text. **A.** The relative CAT activity is defined as the ratio of the percent acetylation by the indicated plasmid to the percent acetylation by the control plasmid Bluescript KS. **B.** Autoradiogram of a representative chromatogram.


A
RELATIVE CAT ACTIVITY
120
100
80
60
40
20
0
pHS
(HSV-1, KOS)
pKS:BAM
(HSV-2, 333)
BLUESCRIPT KS


B
pHS
pKS:Bam
Bluescript KS

pKX2-P4 respectively. As shown in Figure 10, pKC7BglN inhibited CAT gene expression as efficiently in the absence of the immediate-early genes as in their presence. Hence, HSV sequences contained in the BglIlN fragment of HSV-2(333) are sufficient for inducing host shutoff activity. These results demonstrate that the vhs gene is the only viral gene required for the HSV host shutoff function.

A second approach was used to support our findings that vhs gene expression is indeed responsible for the inhibition of CAT activity in cotransfection experiments. In the following series of experiments, various in vitro generated mutants of the vhs gene were examined for their affect on CAT activity upon cotransfection with the CAT reporter gene. The mutant genes include a deletion mutant, four nonsense mutants, and four insertion mutants.

Construction of the pHSΔSma deletion mutant.

The plasmid pHSΔSma was constructed in our laboratory using the vhs gene of HSV-1 strain KOS which is carried in the plasmid pHS (Read and Knight, unpublished data). A 588 bp SmaI fragment was removed from the center of the UL41 open reading frame in pHS. The two ends of the linearized plasmid, each of which had a SmaI restriction site were then religated. This construction should allow the mutant gene to be translated without interrupting the proper reading frame of the vhs polypeptide. The coding region of the vhs gene contained in pHSΔSma is predicted to encode a shortened

Figure 10 Host shutoff activity of pKC7BglN in the presence and absence of HSV transcriptional activators. **A.** Vero cells were transfected with mixtures containing 5 μg pSV3CAT, and the indicated amounts (in μG) of pSG1, pKX2P4, pKC7BglN or pKS:Bgl-Sal. Enough salmon sperm DNA was added to each mixture to bring the total amount of DNA to 100 μg. Cultures were harvested 48 h. after transfection and assayed for CAT activity as described in the text. Relative CAT activity is defined as the ratio of the percent acetylation by the indicated plasmid to the percent acetylation by the control plasmid, Bluescript KS. **B.** Autoradiogram of chromatograph of experiments transfecting pSV3CAT or pSV3CAT + pKC7BglN.

pSV3CAT

pSV3CAT+pKC7BglN

protein in which the first 147 amino acids are joined to amino acids 344 through 489 (Figure 11A).

Effect of ΔSma mutation on vhs activity

The plasmid pHSΔSma was cotransfected with pSV3CAT, and the HSV-1 transcriptional activators. As can be seen from the autoradiogram in Figure 11B, CAT activity was similar to control transfections. This result may be interpreted as the complete elimination of *vhs* activity by removal of the center third of the *vhs* protein. These results were corroborated by an *in vivo* experiment in which the ΔSma deletion mutant was subcloned into the HSV-1 genome. Upon infection with the mutant virus, no virion associated host shutoff activity was observed (Read and Knight; unpublished data)

Construction of nonsense and insertion mutants in a vhs gene.

In collaboration with Kim Knight, eight mutants of the *vhs* gene of HSV-1 strain KOS were constructed. This was accomplished by the insertion of a 26 bp synthetic linker into four separate blunt end restriction sites in the coding region of the *vhs* gene. The sequence of the linker

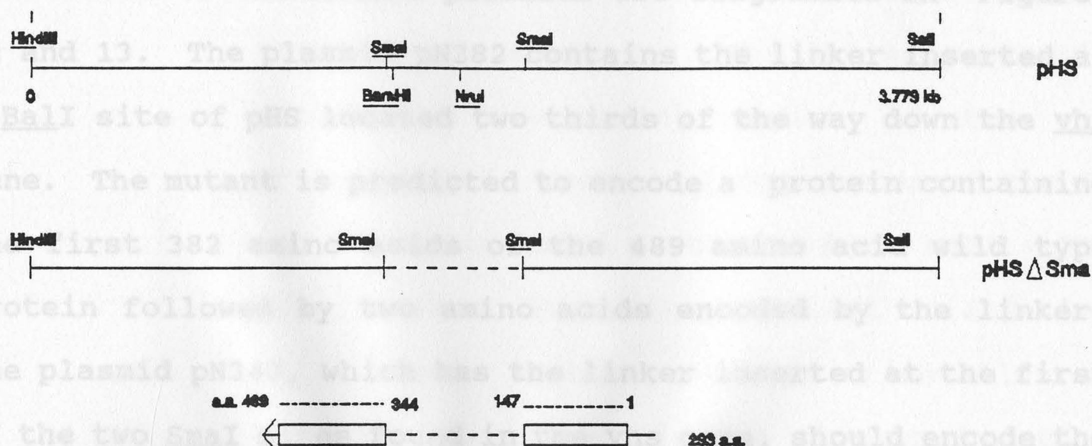
5' AAG CTT AAT TGA ATT CAA TTA AGC TT 3'
3' TTC GAA TTA ACT TAA GTT AAT TCG AA 5'

allows the construction of a nonsense mutant and a 6 bp insertion mutant at each of four blunt end restriction sites within the *vhs* gene. Encoded in the linker sequence are stop codons in all three reading frames and in both directions. This allows the construction of a nonsense mutation wherever the linker is inserted. In addition, the linker sequence

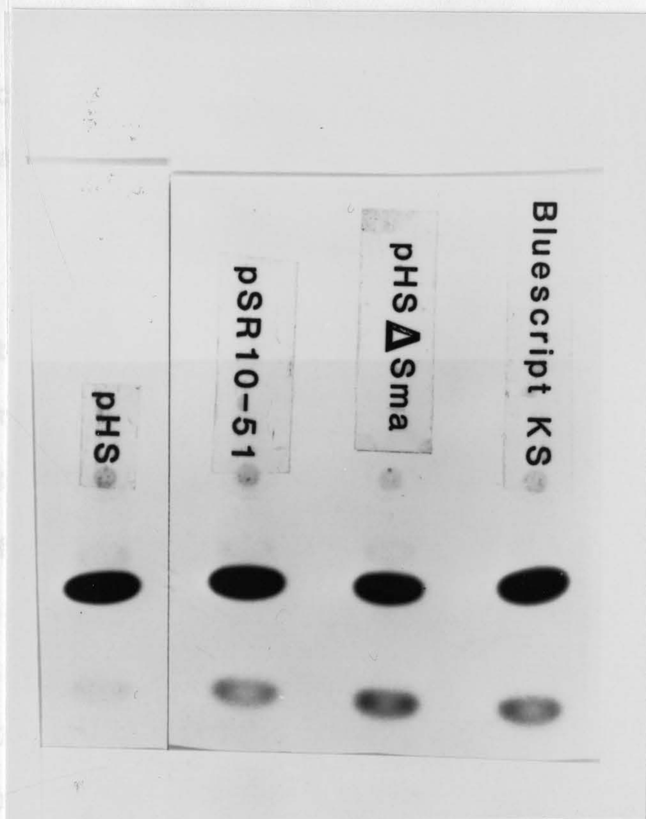
Figure 11. **A**.Construction of pHSΔSma. A 588 bp SmaI fragment was removed from pHS. The plasmid was then religated creating a deletion mutation in the vhs gene. The bottom line depicts the 293 amino acid predicted to be encoded by the mutant gene. **B**. Effect of ΔSma mutation on CAT gene expression. Vero cells were transfected with mixtures containing 5 μg pSV3CAT, 10 μg pSG1, 10 μg pKX2P4, 65 μg of salmon sperm DNA, and 10 μg of PHS, PHSΔSma, pSR10-51 or Bluescript KS. pSR10-51 was constructed by deleting a 1466 bp fragment from PHS containing the entire vhs gene and a portion of the UL40 open reading frame. Cultures were harvested 48 h. after transfection and assayed for CAT activity as described in the text. A representative autoradiogram of the chromatograph is shown.

Construction of pHS△SMA

A

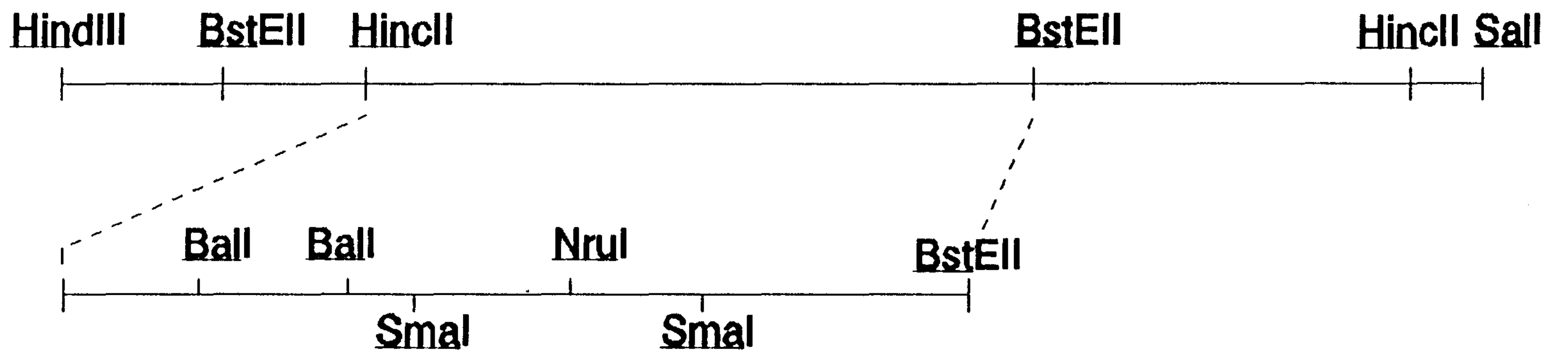


B



contains HindIII sites at each end of the molecule. After inserting the oligonucleotide into the vhs gene, the plasmid may be cut with HindIII and religated, thus constructing a 6 bp insertion mutant at the site of the insertion. The constructs of the mutant plasmids are diagrammed in Figures 12 and 13. The plasmid pN382 contains the linker inserted at a BalI site of pHS located two thirds of the way down the vhs gene. The mutant is predicted to encode a protein containing the first 382 amino acids of the 489 amino acid wild type protein followed by two amino acids encoded by the linker. The plasmid pN343, which has the linker inserted at the first of the two SmaI sites found in the vhs gene, should encode the first 343 amino acids of the vhs gene followed by two amino acids encoded by the linker. The plasmid pN237 was constructed by inserting the linker into the unique NruI site found in the vhs coding sequence. The plasmid pN237 is predicted to encode a protein containing the first 237 amino acids of the vhs gene followed by six amino acids encoded by the linker. pN147 has the linker inserted into the 5'-SmaI site of the gene and should encode the first 147 amino acids of the vhs gene plus two amino acids encoded by the linker. The four insertion mutations were constructed by cleaving each of the plasmids containing the nonsense mutants (pN382, pN343, pN237, pN147) with HindIII. The plasmid was then religated and the remaining HindIII site (AAGCTT) is the inserted sequence. The plasmids containing the insertion mutants have

Figure 12 Construction of nonsense mutations in the vhs gene of HSV-1(KOS). The first two lines represent a restriction map of the HindIII-SalI fragment of pHS. The location of the wild-type vhs gene within pHS is shown by the arrow and labeled pHS. The filled rectangle represents the coding region of the vhs protein, while the thin lines represent the 5' and 3' untranslated portions of the mRNA. The gene is shown in a right to left orientation as it exists in the prototype orientation of the HSV-1 genome. Lines 4 through 7 depict the structures of the four vhs nonsense mutants described in the text. In each case, the closed rectangle represents the portion of the wild type vhs protein that is predicted to be coded by the mutant gene. At the right of each line, the number of N-terminal amino acids from the wild type polypeptide that is contained within the mutant protein, followed by the number of C-terminal amino acids encoded by the synthetic oligonucleotide is indicated.


HindIII
BstEII
HincII
BstEII
HincII
SalI
BalI
BalI
NruI
BstEII
SmaI
SmaI

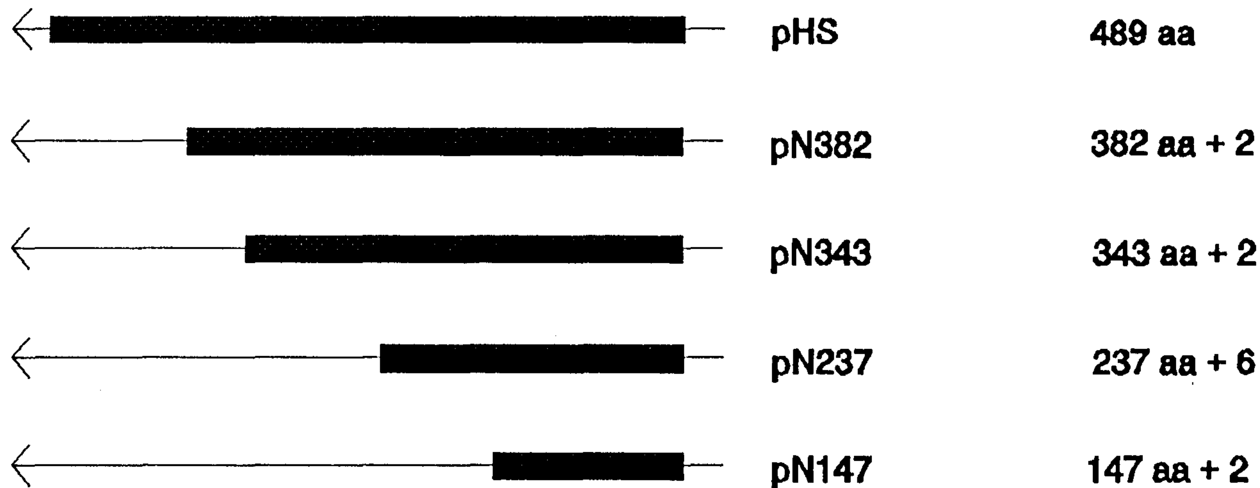
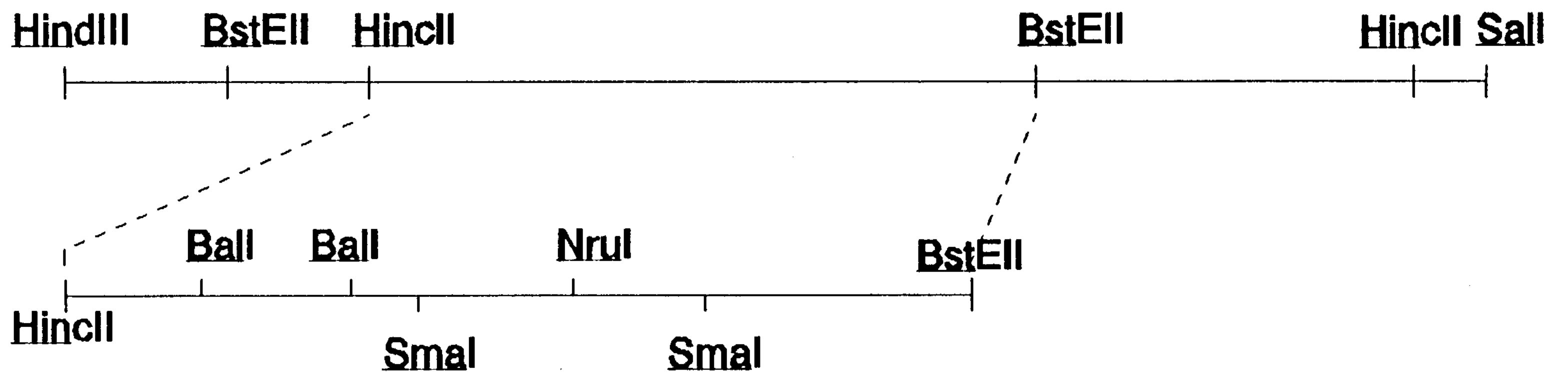
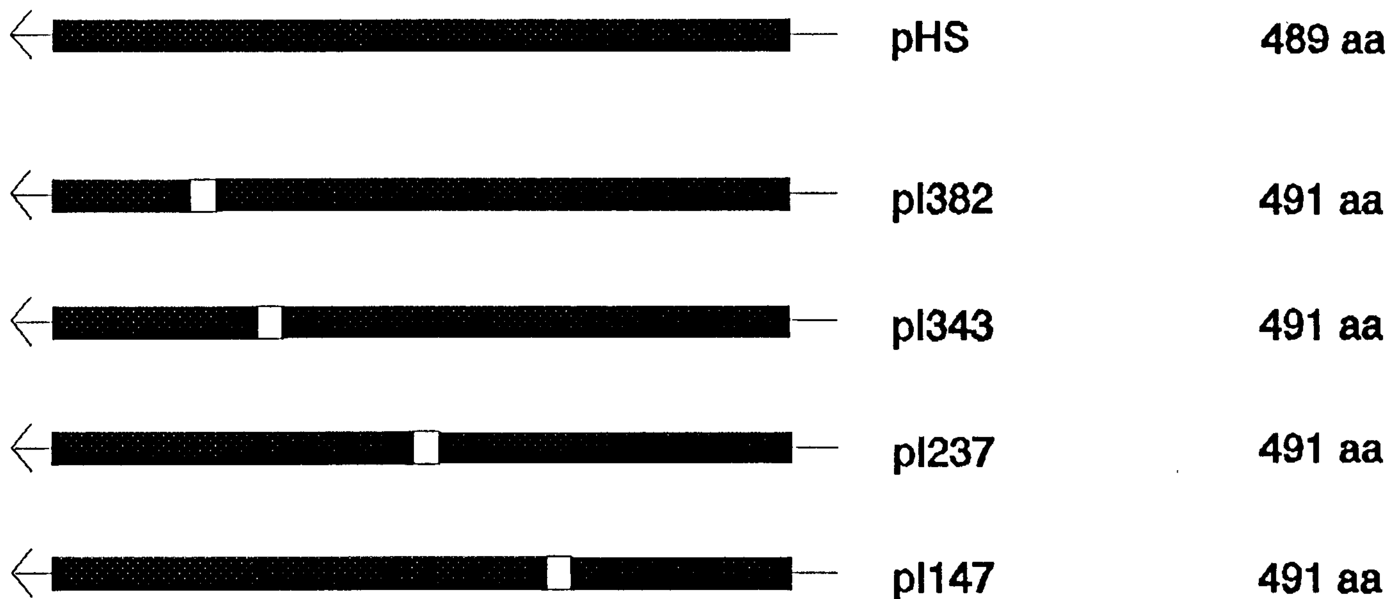

pHS
489 aa
pN382
382 aa + 2
pN343
343 aa + 2
pN237
237 aa + 6
pN147
147 aa + 2

Figure 13 Construction of insertion mutations in the *vhs* gene of HSV-1(KOS). The first two lines represent a restriction map of the *Hind*III-*Sal*I fragment of pHS. The location of the wild type *vhs* gene within pHS is shown by the filled box in the lind labeled pHS1. The thick lines represent the coding regions of the *vhs* protein with the unfilled portion of the line marking the position of the six nucleotide insertion. The thin lines represent the 5'- and 3'-untranslated portions of the mRNA.


HindIII
BstEII
HincII
BstEII
HincII
SalI
BalI
BalI
NruI
BstEII
HincII
SmaI
SmaI

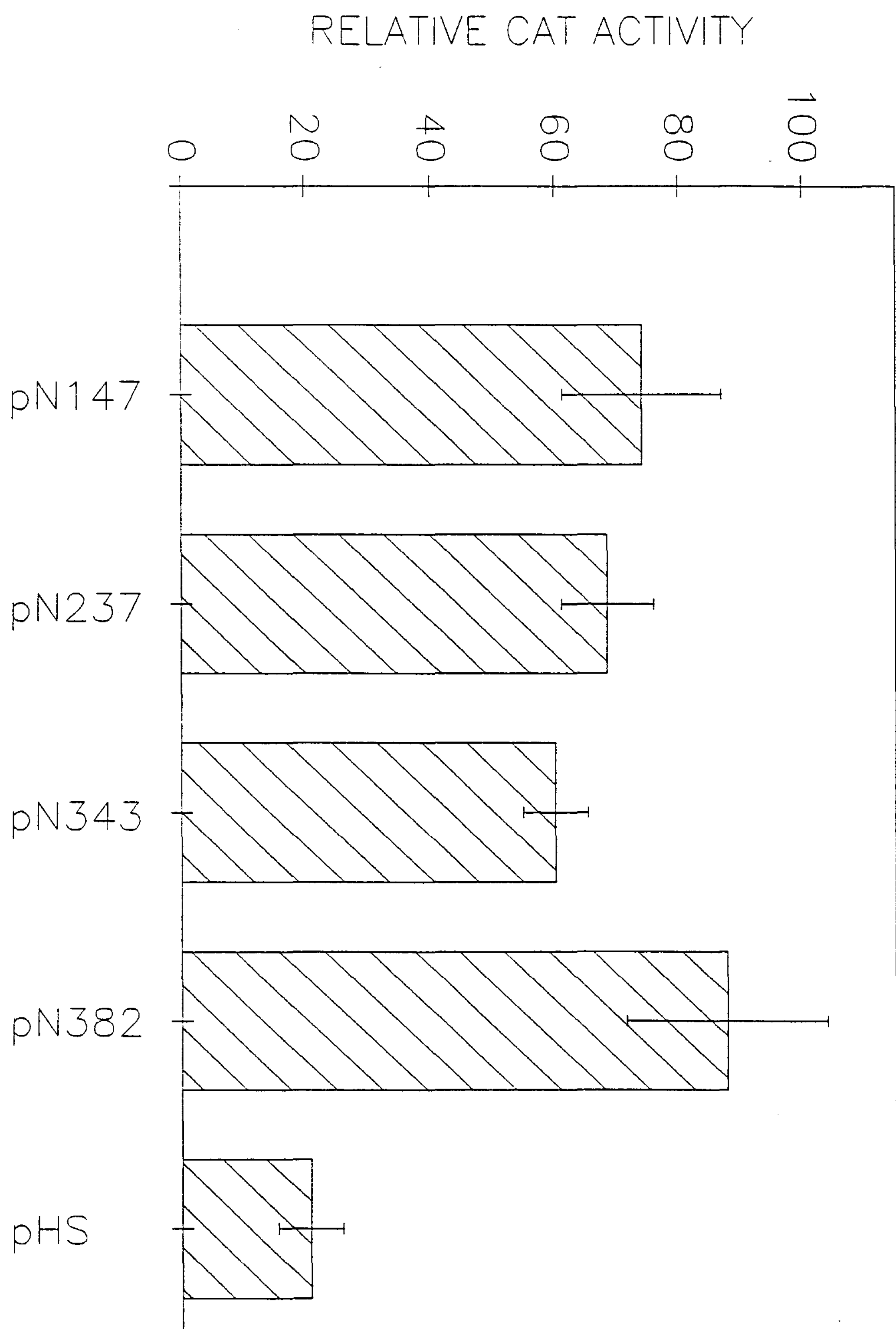

pHS
489 aa
pI382
491 aa
pI343
491 aa
pI237
491 aa
pI147
491 aa

been designated pI382, pI343, pI237, and pI147, corresponding with the locations of the nonsense mutations.

Affect of nonsense and insertion mutations on reporter gene activity.

Each of the four vhs nonsense mutations was analyzed in our cotransfection assay for its ability to CAT inhibit reporter gene activity. Cells were first transfected with pN382 which encodes a truncated vhs protein incorporating only the first 382 amino acids of the 489 amino acid of the wild-type vhs polypeptide. As is shown in Figure 14, the CAT activity measured from this transfection resulted in little or no reduction of CAT activity compared to cells cotransfected with pSV3CAT and the control plasmid Bluescript KS. These results demonstrate that truncating the vhs protein by 107 amino acids nearly abolished the inhibitory activity of the vhs polypeptide. Similarly transfections which plasmids pN343, pN237 and pN147 which contain nonsense mutations encoding the first 343, 237, and 147 amino acids of the wild type vhs polypeptide respectively, resulted in a three to four fold reduction in CAT activity compared to transfections with the wild-type vhs gene. The observation that all four nonsense mutations significantly reduced the ability of the vhs gene to inhibit CAT activity implies that the inhibitory activity is dependent on the UL41 polypeptide and that the 107 carboxyl amino acids are essential for its vhs function.

Figure 14 Effect of vhs nonsense mutants on CAT gene expression. Vero cells were transfected with mixtures containing 5 μg pSV3CAT, 10 μg pSG1, 10 μg pKX2P4, 65 μg of salmon sperm DNA, and 10 μg of one of the following plasmids: pN147, pN237, pN343, pN382, pHS, or Bluescript KS (not shown). Cultures were harvested 48 h. after transfection and assayed for CAT activity as described in the text. Relative CAT activity is defined as the ratio of the percent acetylation by the indicated plasmid to the percent acetylation by the control plasmid, Bluescript KS. The values for CAT activity represent the means of multiple experiments and the error bars represent the standard errors of the mean.



RELATIVE CAT ACTIVITY
0
20
40
60
80
100
pN147
pN237
pN343
pN382
pHS

The four insertion mutations were also cotransfected with the CAT gene and HSV transcriptional activators. Somewhat unexpectedly, all four insertion mutants significantly reduced CAT activity. Transfection of cells with pI147, which encodes a full-length vhs polypeptide with a two amino acid insertion 147 residues from the carboxyl terminus, resulted in production of almost as much CAT activity as did control transfections using Bluescript KS. The remaining three insertion mutations which were carried in plasmids pI382, pI343, and pI237, also exhibited a significant reduction in vhs activity compared to the wild-type host shutoff activity found in HSV-1. These results are illustrated in Figure 15.

Affect of nonsense mutations in vivo.

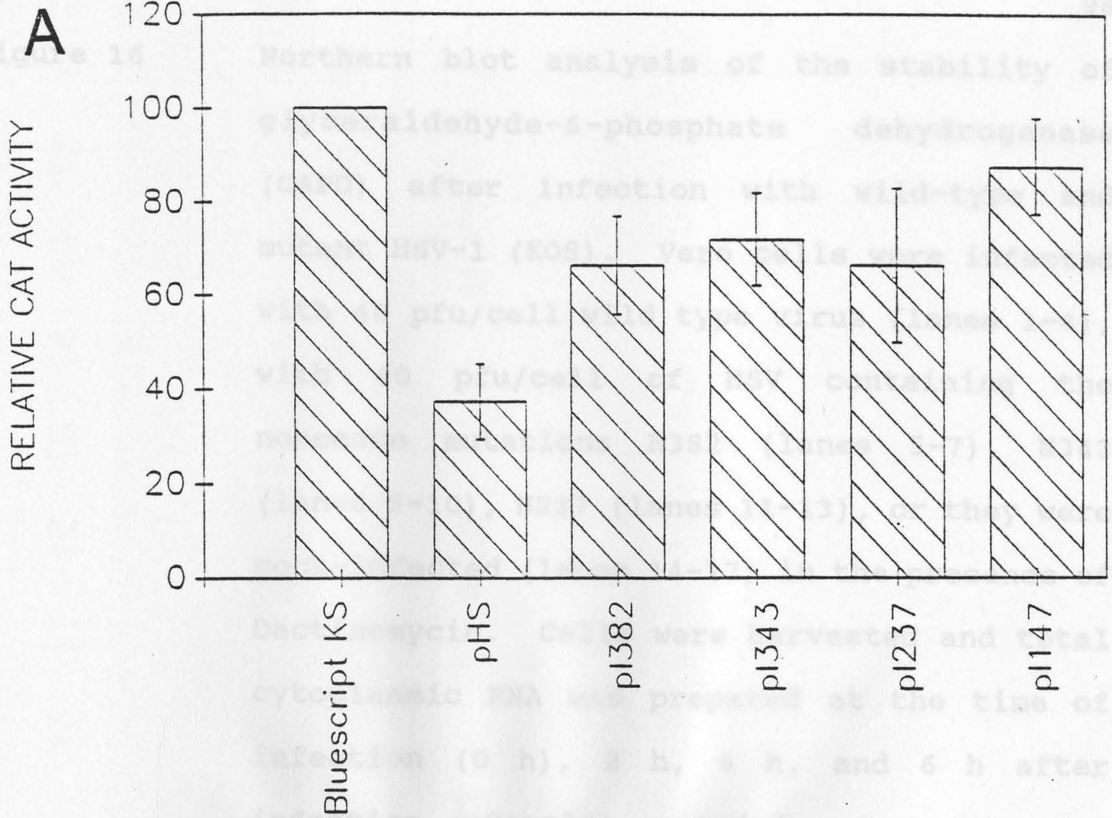
Three of the nonsense mutations, (N382, N343, and N237) were transferred to the HSV-1 genome via marker rescue experiments. The virus vhsB, kindly provided by J. Smiley, McMaster University, Hamilton, Ontario, Canada, contains a lacZ gene inserted into a unique BamHI site in the vhs gene. Upon infection of Vero cells and in the presence with X-gal, vhsB forms blue plaques. pN382, pN343, or pN237 was cotransfected with vhsB and plaques from the recombinant virus were selected by the lacking blue color after staining with X-gal. Vero cells were infected at a multiciplicity of infection of 40

plaque forming units per cell with either wild type virus, one of the three nonsense mutant viruses, or mock infected with whole cell lysate. Infections were carried out in the presence of Dactinomycin to inhibit transcription from the time of infection. The cells were harvested at 0, 2, 4, and 6 h post infection and by total cytoplasmic mRNA was extracted, size fractionated in agarose electrophesis, and blotted onto nitrocellulose. The mRNA was then probed with the cellular glyceraldehyde-3-phosphate dehydrogenase (GAPD) gene. The results of this experiment are shown in Figures 16 and 17. As early as two hours post infection, the wild type infected cells showed a decrease in GAPD message. By six hours post infection, nearly all the host GAPD mRNA had been degraded. In contrast, the GAPD message in mock-infected cells was found to be stable throughout the experiment. When the cells were infected with any of the three viruses containing the vhs nonsense mutations, the GAPD mRNA also remained stable.

Quantitation of mRNA from cells transfected with vhs gene.

The results from the preceding set of experiments suggested that our in vitro assay for vhs function provides a representation of how a specific vhs gene or vhs mutant gene functions in vivo. Virion host shutoff function ,however, is at the level of mRNA stability. A more direct analysis of our in vitro assay would be to measure the mRNA levels of the target gene when it is cotransfected with the vhs gene. This

Figure 15 Effect of <u>vhs</u> insertion mutants on CAT gene expression. Vero cells were transfected with mixtures containing 5 μg pSV3CAT, 10 μg pSG1, 10 μg pKX2P4, 65 μg of salmon sperm DNA, and 10 μg of one of the following plasmids: pI147, pI237, pI343, pI382, pHS, or Bluescript KS. Cultures were harvested 48 h after transfection and assayed for CAT activity as described in the text. **A.** Relative CAT activity is defined as the ratio of the percent acetylation by the indicated plasmid to the percent acetylation by the control plasmid, Bluescript KS. The values for CAT activity represent the means of multiple experiments and the error bars represent the standard errors of the mean. **B.** Autoradiogram of a representative chromatogram.


A
RELATIVE CAT ACTIVITY
120
100
80
60
40
20
0
Bluescript KS
pHS
pI382
pI343
pI237
pI147

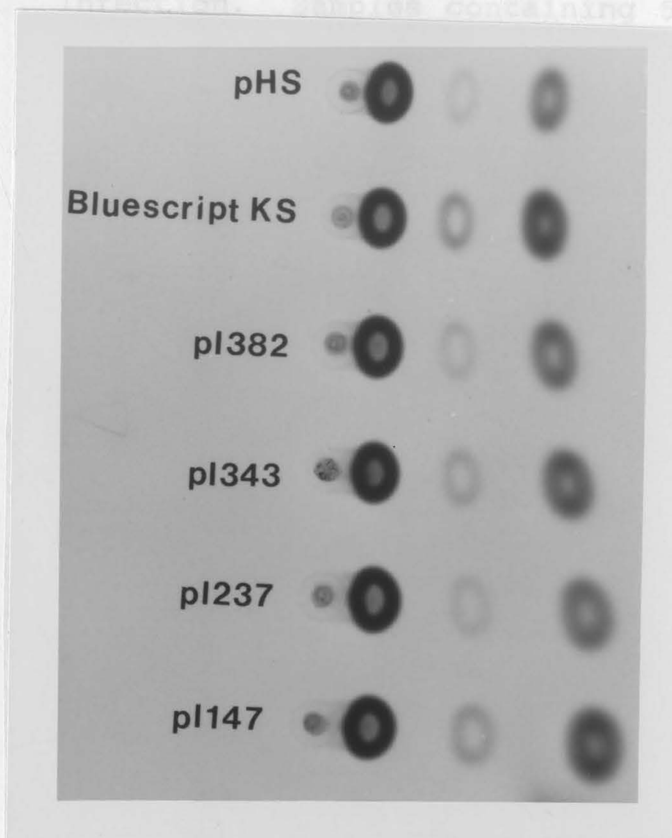
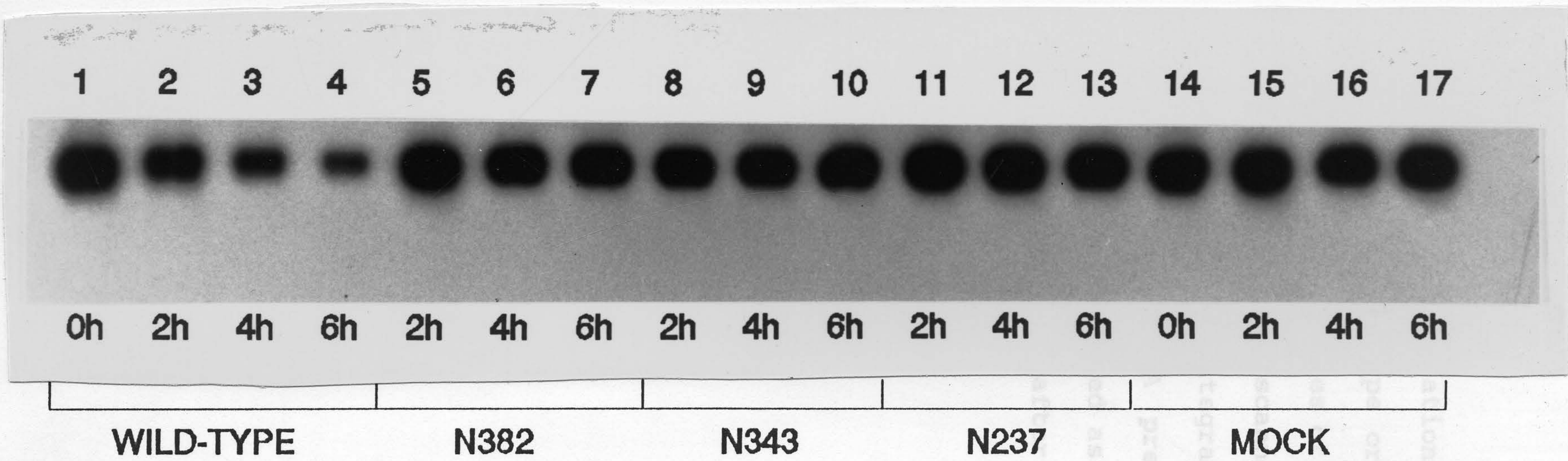
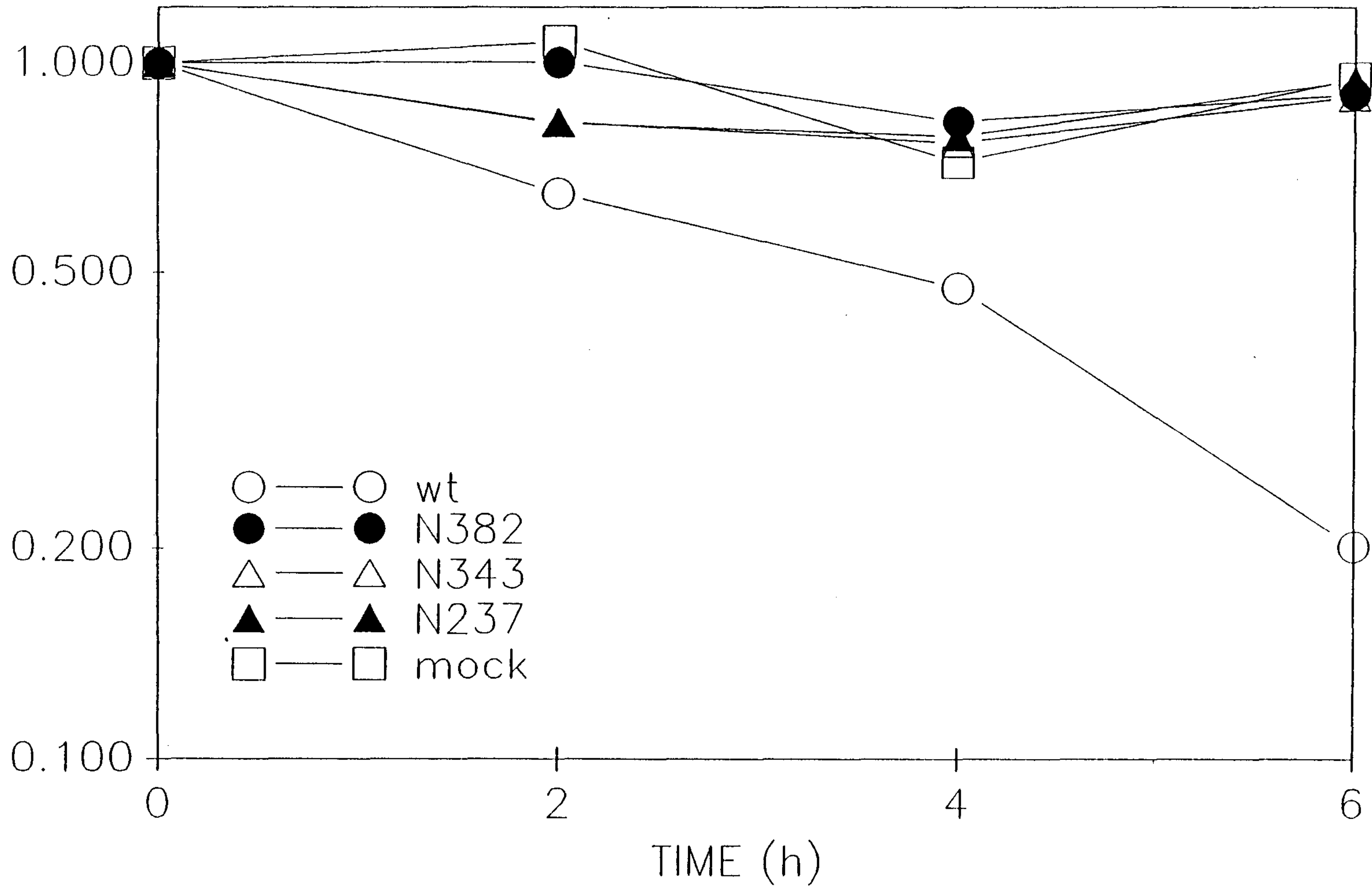

B
pHS
Bluescript KS
pI382
pI343
pI237
pI147

Figure 16 Northern blot analysis of the stability of glyceraldehyde-6-phosphate dehydrogenase (GAPD) after infection with wild-type and mutant HSV-1 (KOS). Vero cells were infected with 40 pfu/cell wild type virus (lanes 1-4), with 40 pfu/cell of HSV containing the nonsense mutations N382 (lanes 5-7), N343 (lanes 8-10), N237 (lanes 11-13), or they were mock-infected (lanes 14-17) in the presence of Dactinomycin. Cells were harvested and total cytoplasmic RNA was prepared at the time of infection (0 h), 2 h, 4 h, and 6 h after infection. Samples containing 5 μg RNA were analyzed by electrophoresis and Northern blot hybridization with a ^{32}P-labeled GAPD probe.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
0h 2h 4h 6h 2h 4h 6h 2h 4h 6h 2h 4h 6h 0h 2h 4h 6h
WILD-TYPE
N382
N343
N237
MOCK

Figure 17 Quantitation of GAPD mRNA after infection with wild-type or mutant HSV-1(KOS). Appropriate exposures of the autoradiogram shown in Figure 16 was scanned, and the areas under the peaks were integrated. For each virus, the amount of mRNA present at 0, 2, 4, and 6 h is expressed as a fraction of the amount present at 0 h after infection.



FRACTION OF mRNA REMAINING
1.000
0.500
0.200
0.100
0
2
4
6
TIME (h)
wt
N382
N343
N237
mock

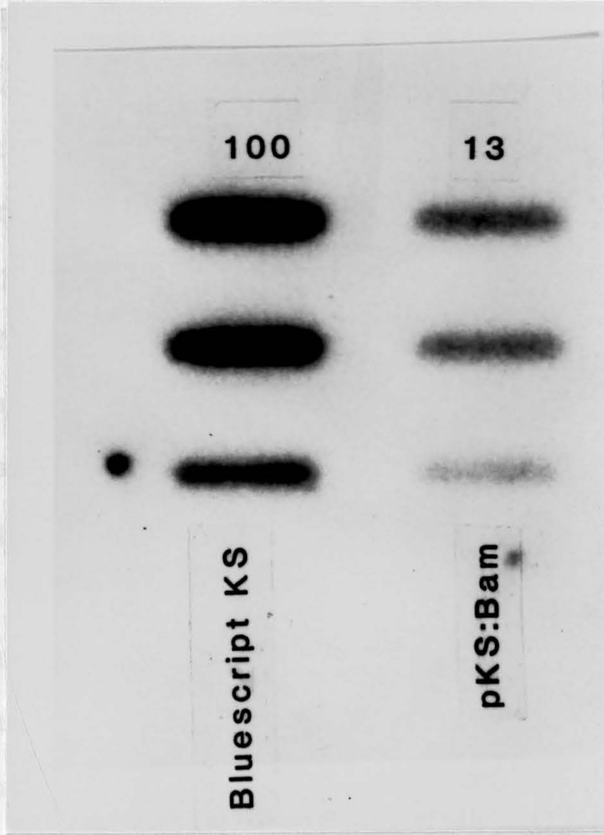
experiment was carried out by transfecting pSV3CAT and pKS:Bam in the same manner as the previous transfections. Forty-five hours after transfection, cells were harvested and total cytoplasmic RNA was extracted. The RNA was quantified by UV spectroscopy and equal amounts were loaded into the wells of a slot blot apparatus. The slot blot was then probed with a ^{32}P labeled CAT gene. As can be seen in Figure 18, the message hybridizing to the pSV3CAT probe is significantly reduced in the pKS:Bam transfected cells as compared to the bluescript KS control transfection. To confirm that the total amount of mRNA loaded onto each lane of the gel was equal, the blots were stripped and re-probed for 28S ribosomal RNA. These bands were determined to be of equal intensities. These data suggest that in cotransfections which included the vhs gene, the quantity of mRNA is indeed reduced.

Construction of HSV-1 x HSV-2 recombinants.

The results shown in Figure 10 demonstrated that the vhs genes from HSV-1 strain KOS and HSV-2 strain 333 could be distinguished by their levels of host shutoff activity. We sought to localize the region of the vhs gene responsible for the difference in efficiency of the two host shutoff genes. To this end, four HSV-1 X HSV-2 recombinant vhs genes were constructed and tested for their degrees of shutoff activity.

The first two recombinants constructed made use of a unique SacI restriction site found two thirds of the way down the coding region of the vhs genes of both strains. In the

Figure 18 Effect of the vhs gene on the level of CAT mRNA. Vero cells were transfected with mixtures containing 5 μg pSV3CAT, 10 μg pSG1, 10 μg pKX2P4, 65 μg of salmon sperm DNA, and 10 μg of either pKS:Bam or Bluescript KS as indicated in the figure. Cultures were harvested 48 h after transfection and total cytoplasmic RNAs were prepared. Serial two-fold dilutions of RNA were loaded onto a Zeta-probe membrane using a slot blot apparatus. The filter was probed with ^{32}P-labeled CAT gene and the levels of CAT mRNA were determined by densitometric scanning of the resulting autoradiogram. The membrane was then stripped and reprobed with pX1r11 to detect the levels of 28S rRNA. The amount of CAT mRNA in each sample was normalized to the amount of 28S rRNA in order to control for variations in the amount of RNA loaded onto different slots of the blot. The relative amount of CAT mRNA is expressed as a percent of the amount found in control cultures transfected with Bluescript KS.


100
13
Bluescript KS
pKS:Bam

first recombinant, pKS:Sac1-2, each gene was cut with SacI and religated so that the 5'-two thirds of the gene was from HSV-1 and the 3'-one third of the gene was from HSV-2. An important reason for choosing the SacI site for construction of these recombinants was that the reading frame of both vhs genes would be conserved after ligation. The second recombinant gene, pKS:Sac2-1 was constructed by ligating the 5'-two thirds of the type 2 vhs gene to the 3'-one-third of the type 1 vhs gene. A diagram of these recombinants is shown in Figure 19.

The next two vhs gene recombinants were constructed using a PvuI site found one third of the way down the coding region of the vhs genes in both HSV-1 and HSV-2. In Figure 19, pKS:Pvu1-2 is diagrammed showing that the first third of the vhs gene is from HSV-1 and the remainder of the gene is from HSV-2. Also shown is pKS:Pvu2-1 in which the first third of the vhs gene is from HSV-2 and the remainder of the gene is from HSV-1.

HSV-1 X HSV-2 recombinant vhs genes.

Each of the HSV-1 X HSV-2 recombinant genes was assayed for vhs function. Of the four recombinants, only pKS:Sac1-2 exhibited shutoff activity comparable to the type 2 vhs gene carried in pKS:Bam. Both pKS:Bam and pKS:Sac2-1 transfected cells had a 20-fold reduction in CAT activity compared to the control cells. Cells transfected with pHS (containing the HSV-1 vhs gene) had a 3-fold decrease in CAT activity compared

to the control transfection. The three remaining HSV-1 X HSV-2 recombinants displayed a level of host shutoff activity intermediate to HSV-1 and HSV-2 activities. Cotransfection of these plasmids (pKS:Sac2-1, pKS:Pvu1-2, and pKS:Pvu2-1) with pSV3CAT reduced the level of CAT activity by approximately five fold. These results are shown in Figure 20.

Figure 19 Construction of HSV-1 x HSV-2 vhs gene recombinants. The top lines represent HSV-1 (solid line) and HSV-2 (dashed line) vhs genes. Each of these genes is contained in the vector, Bluescript KS (arrows). The arrows underneath the repective vhs genes depict the right to left direction of the vhs gene as it exists in the prototype orientation of HSV-1 and HSV-2. The final four lines depict the recombinant vhs geneswhich were constructed as described in the text.

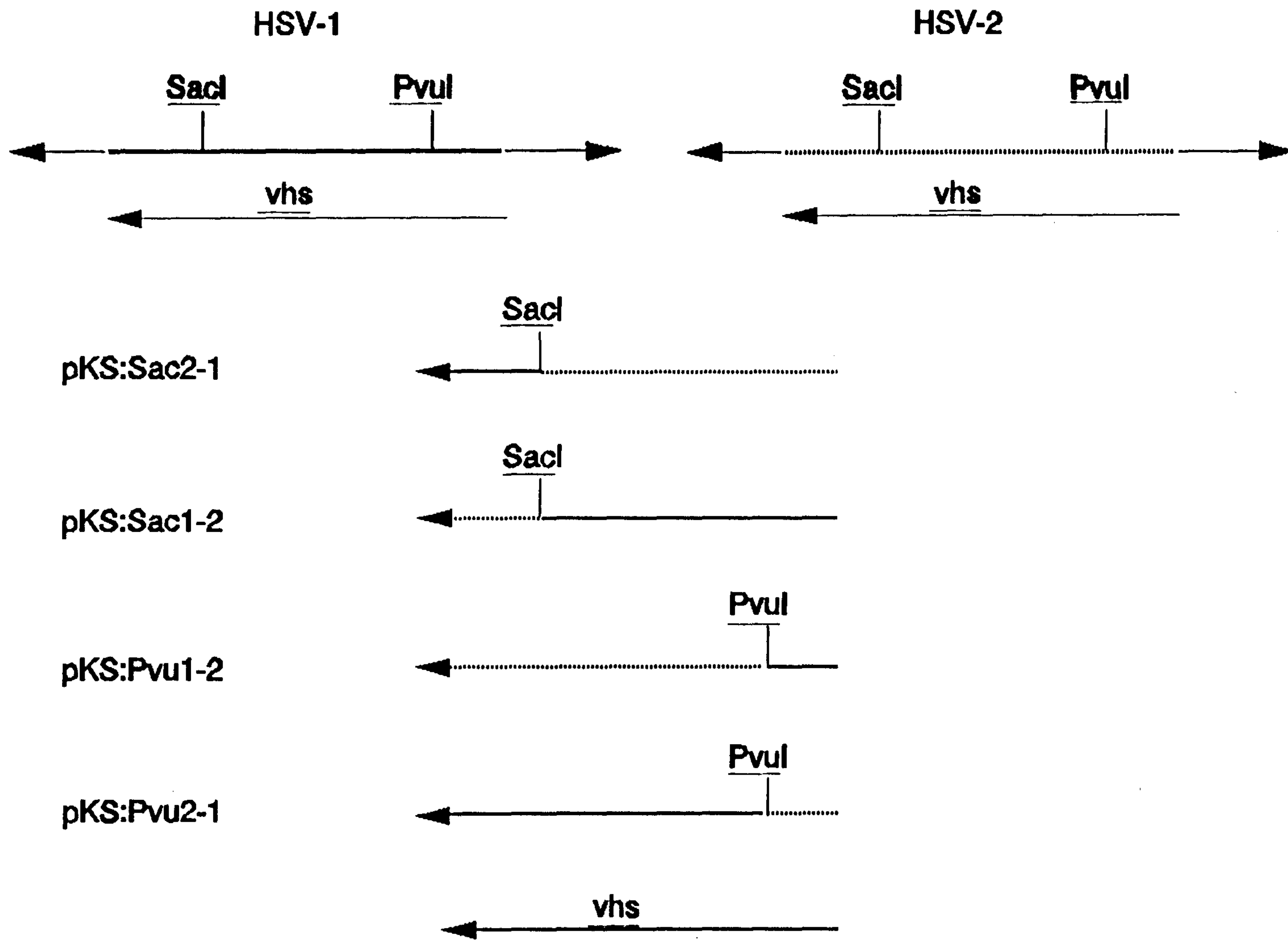

HSV-1
HSV-2
SacI
PvuI
SacI
PvuI
vhs
vhs
SacI
pKS:Sac2-1
SacI
pKS:Sac1-2
PvuI
pKS:Pvu1-2
PvuI
pKS:Pvu2-1
vhs

Figure 20 Effect of HSV-1 x HSV-2 recombinant vhs genes on CAT gene expression. Vero cells were transfected with mixtures containing 5 μg pSV3CAT, 10 μg pSG1, 10 μg pKX2P4, 65 μg of salmon sperm DNA, and 10 μg of one of the following plasmids: Bluescript KS, pHS (HSV-1 vhs gene), pKS:Bam (HSV-2 vhs gene), pKS:Sac2-1, pKS:Sac1-2, pKS:Pvu1-2, or pKS:Pvu2-1. Cultures were harvested 48 h after transfection and assayed for CAT activity as described in the text. **A.** Relative CAT activity is defined as the ratio of percent acetylation of the indicated plasmid to the percent acetylation of the control plasmid, Bluescript KS. The values for CAT activity represent the means of multiple experiments and the error bars represent the standard errors of the mean. **B.** Autoradiogram of a representative chromatogram.

HSV−1 x HSV−2 RECOMBINANTS

RELATIVE CAT ACTIVITY

120
100
80
60
40
20
0

KS
pHSI
pKS:Bam
pKS:Sac2−1
pKS:Sac1−2
pKS:pvu1−2
pKS:Pvu2−1

BluescriptKS
pHS
pKS:Bam
pKS:Sac2-1
pKS:Sac1-2
pKS:Pvu1-2
pKS:Pvu2-1

DISCUSSION

The first objective of this project was to develop an *in vitro* assay for the host shutoff function of HSV. Once developed, this procedure was not only useful for measuring *vhs* activity, but it also allowed us to limit the number of HSV polypeptides present during the analyses. Thus, it was possible to ascertain exactly which viral genes are required for the HSV host shutoff activity. The simplicity of this assay also permitted a rapid screening of a number of *in vitro* generated mutants of the HSV-1 *vhs* gene. This enabled us to obtain preliminary information regarding the host shutoff activity of particular mutants before attempting to reintroduce the mutant genes back into the virus and test for *vhs* function *in vivo*. Moreover, the *vhs* activities of several *in vitro* generated HSV-1 X HSV-2 recombinant genes were examined in an effort to localize the region of the *vhs* gene responsible for the difference in efficiencies between HSV-1 and HSV-2 host shutoff activities.

Use of CAT gene for analysis of mRNA degradation.

The chloramphenicol acetyl transferase (CAT) gene is frequently used in experiments designed to measure eucaryotic promoter function. The most direct analysis of promoter function is, of course, a direct quantitation of the message

under control of the promoter being analyzed. Unfortunately, quantitation of mRNA may be tedious and difficult unless the promoter is particularly strong. In such cases, the promoter of interest is often joined to the easily assayed CAT reporter gene and the recombinant gene is transfected into tissue culture cells. After an incubation period required for the expression of the transfected gene(s), the cells are assayed for CAT activity. Gorman and coworkers have shown that the CAT enzymatic activity measured in these types of experiments does indeed correspond with promoter strength and consequently steady state levels of CAT mRNA (Gorman et al. 1982). In view of the correlation between CAT activity and actual mRNA levels, we sought to use the CAT assay as an indicator of vhs function on mRNA stability.

As mentioned previously, the vhs1 mutation was mapped to a 265 base pair restriction fragment spanning map coordinates 0.604 to 0.606 of the HSV-1 genome (Kwong et al. 1988). Sequencing data placed this mutation within the UL41 open reading frame of HSV-1 (McGeoch et al. 1988). Mapping the HSV-2 virion-associated host shutoff function had been less precise. Experiments using random HSV-1 x HSV-2 recombinants mapped the host shutoff function to a region spanning map coordinates 0.52 to 0.60 of the HSV-2 genome (Morse et al. 1978; Fenwick et al. 1979). The colinearity of HSV-1 and HSV-2 genomes had allowed us to predict that the HSV-2 vhs gene is positioned within the BglII N fragment of HSV-2.

Characterization of the transcripts mapped within this fragment suggested that the homologue for the _vhs_ gene of HSV-1 is indeed located within the _Bgl_II N fragment and encodes a 1.9 kb mRNA transcript spanning map units 0.599-0.590 (Jenkins and Howett 1988). This putative _vhs_ transcript was also translated _in vitro_ to form a 61 kD protein (Galloway _et al_. 1982). These data supported our decision to use the HSV-2 _Bgl_II N fragment as a source of the _vhs_ gene in our initial experiments.

At the onset of this project, two opposing hypotheses regarding the function of the _vhs_ gene product had been proposed. Fenwick and Owen (1988) had proposed that the UL41 (_vhs_) gene product protects mRNAs which are rendered susceptible to degradation by another virion component or by the virions themselves. Conversely, Oroskar and Read (1987,1989) had provided evidence that the _vhs_ protein induces the degradation of both host and viral mRNA's. We proposed to use the CAT gene to supply a mRNA target for the _vhs_ gene product. Our assay involved the cotransfection of the CAT reporter gene, the _vhs_ effector gene, and the HSV transcriptional activators ICP4 and ICP0 which are required for efficient transcription of genes belonging to the beta-gamma class of HSVs such as the _vhs_ gene. If the transient expression of the transfected _vhs_ gene in tissue culture cells affected the stability of the other transfected gene

transcripts, a reduction in CAT protein and thus CAT enzymatic activity would be observed.

Transfection of the HSV-2 BglII N fragment with CAT gene.

The first attempt at assaying vhs activity using transfected HSV DNA used pKC7BglN to provide the vhs gene. As was shown in Figure 3, CAT activity was reduced five-fold when pKC7BglN was cotransfected with pSV3CAT into Vero cells. This suggested that sequences which are believed to contain the vhs gene of HSV-2 in the 7.3 kb BglII N fragment were responsible for a decrease in CAT activity. These initial results also indicated that upon cotransfection with the vhs gene, a decrease in the expression of the CAT reporter gene could be observed. This corroborates earlier findings which reported that 1) the vhs gene product induces the degradation of mRNA (Scheck and Bachenheimer 1985; Strom and Frenkel 1987; Oroskar and Read 1987,1989) and 2) the mRNA targets are nonspecific in that both viral and infected cell messages were destabilized (Oroskar and Read 1987;1989). The data presented here also extended the range of target mRNA, showing that messages of transfected genes could also be targets of the vhs protein.

Notwithstanding the decrease in CAT activity upon cotransfection with pKC7BglN, sequences within the pKC7 vector sequences could have influenced the levels of CAT activity. To eliminate this possibility, the same BglII N fragment was inserted into Bluescript KS and the plasmid was designated pKS:BglIIN. Transfecting only Bluescript KS had no affect on

CAT activity indicating that the vector sequences do not alter the results. The plasmid pKS:Bgl N, however, reduced CAT activity five fold. The Bluescript vector was used for all further cloning procedures so that the transfection of non-HSV DNA could be eliminated as a factor in subsequent experiments.

Having established that the CAT enzymatic activity was reduced as a result of sequences in the BglII N fragment, I set out to more precisely map the sequence responsible for this effect. A 4.6 kb Bgl-Sal fragment was subcloned into Bluescript KS. After transfecting the plasmid pKS:Bgl-Sal in the assay, a reduction in CAT activity was again observed demonstrating that vhs function was unimpaired. Next, a smaller BamHI fragment, approximately 3.4 kb long, was subcloned to form pKS:Bam. Again, this plasmid conferred vhs activity to the tissue culture cells. The only intact open reading frame which has been mapped to this fragment is the 1.9 kb transcript which had been predicted to be the vhs transcript. These results have been substantiated in an experiment by Fenwick and Everett (1990) who excised a 3.6 kb SstI fragment of HSV-2 strain G and inserted it into the TK gene of HSV-1 strain 17. The recombinant virus which carried vhs genes from both HSV-1 and HSV-2 had a strong shutoff activity corresponding to the vhs function of HSV-2 and not the parent HSV-1 gene (Fenwick and Everett 1990). Examination of my data together with that of Fenwick and Everett has allowed me to narrow down the sequences responsible for the

host shutoff function to the 3177 bp between BamHI and SstI sites shown in Figure 6.

To further examine the sequences associated with vhs function, efforts were focused on determining more precisely which sequences are necessary for host shutoff function. DeLuca and Schaffer (1985) had previously described a method in which one gene in a cotransfection experiment was inactivated while all others were expressed. This was accomplished by cutting within the coding region of the HSV-1 ICP4 gene before cotransfecting it with the other intact genes. These experiments were employed to correlate the expression of the ICP4 polypeptide with its activities. I used this technique to further correlate the inhibition of CAT activity with expression of the vhs protein and also to examine sequences to the 3' side of the translated portion of the gene.

To be certain that cutting pKS:Bam does not in itself destroy CAT vhs activity, pKS:Bam was first cut with either BamHI or KpnI. The vhs gene does not contain restriction sites of either of these enzymes and as expected, vhs activity was not altered when the cut plamids were used in the transfection. The plasmid pKS:Bam was then cut at restriction sites found within the coding region of the vhs gene. If the decline in CAT activity in the preceding experiments was actually due to the expression of the vhs gene, cutting the gene with restriction enzymes should eliminate this effect.

As illustrated in figure 6, there are unique PstI, PvuI, and XhoI restriction sites located near the center of the coding region of the vhs gene. As is shown in Figure 7, all vhs activity was indeed eliminated by cutting the gene at any of these central sites. These results further correlate the inhibition of expression of the CAT reporter gene with vhs activity.

The next sequences examined using the same type of experiment were in the 3' noncoding region of the HSV-2 vhs gene. The plasmid pKS:BAM was cut with either HindII or BstEII for which there are restriction sites 230 bp and 620 bp downstream from the poly(A) signal of the vhs gene respectively. The digested plasmids were cotransfected with the reporter gene and transcriptional activators and their effect on CAT activity was measured. Transfecting the BstEII cleaved plasmid showed no loss of vhs activity when compared to the control transfection of intact pKS:Bam. However, when pKS:Bam was cut with HindII before transfecting the plasmid, vhs activity was to be reduced two fold. It has been demonstrated that removal of sequences downstream from the poly(A) addition sites can prevent the correct formation of mature mRNA (McDevitt et al. 1984). In fact, the primary transcript may extend 500-2000 bp past the poly(A) site (Birnstiel,M.L. et al.1985). Our results suggest that sequences between the HindII and BstEII sites may be important for the proper expression of the vhs gene.

The foregoing data demonstrated that the *vhs* gene cotransfected with a CAT gene causes an inhibition of CAT-gene expression. However, the effect of the cotransfected *vhs* gene on the target message was not directly measured. For this reason, I decided to examine the CAT mRNA from one of the cotransfection experiments. After cotransfecting Vero cells with the *vhs* and CAT genes, total cytoplasmic RNA was harvested and blotted onto Nytran membranes using a slot blot apparatus. The blot was probed with a ^{32}P-labeled CAT gene DNA. The autoradiogram shown in Figure 18 indicates that CAT mRNA is indeed reduced due to the cotransfection of the *vhs* gene.

Despite the correlation between the transfected *vhs* gene and decline of CAT mRNA shown in the preceding experiment, *in vivo* studies of the *vhs* gene have more precisely delineated the action of the *vhs* gene (Oroskar and Read 1985). These studies have demonstrated that the decrease in protein synthesis caused by the *vhs* polypeptide is due to a decrease in the half-lives of target mRNAs. A direct analysis of CAT mRNA stability in this transient-expression assay was hampered by the low levels of CAT mRNA in cells cotransfected with pSV3CAT and the *vhs* gene. Consequently, I sought to correlate the *in vitro* *vhs* activity observed in the cotransfection assay with the destabilization of mRNAs observed *in vivo*.

A comparison of the shutoff activities of several mutant *vhs* genes was carried out both *in vivo* and *in vitro*. The

first _vhs_ gene examined was the ΔSma deletion mutant which encoded a shortened protein lacking 196 amino acids from the center of the _vhs_ polypeptide. _In vivo_ studies of the ΔSma mutation inserted into the HSV-1 genome showed that the destabilization of host GAPD mRNA evident in wild type infected cells was completely abolished in the virus containing the ΔSma mutation. Analogous results were obtained when the same mutant gene was transfected with the CAT gene.

A similar outcome was observed when the _vhs_ activities of three _in vitro_-generated nonsense mutants were analyzed both _in vitro_ and _in vivo_. In each type of experiment, the nonsense mutants were whown to have lost all _vhs_ activity. The parallels found in the _in vivo_ and _in vitro_ experiments support my position that the _in vitro_ CAT assay does indeed reflect _vhs_ activity.

The _in vitro_-generated nonsense mutants described above were also used to provide information on the structural domains of the _vhs_ protein that are important for its activity. The length of the wild type protein of HSV-1 strain KOS is 489 amino acids whereas the nonsense mutant genes were predicted to encode truncated _vhs_ proteins 382 ,343, 237, and 147 amino acids in length. When any of the nonsense mutants was cotransfected with the CAT gene, the CAT activity measured indicated that _vhs_ function had been completely eliminated. From these results it may be inferred that the 107 carboxyl terminal amino acids play an important role in

the function of the vhs polypeptide. Everett and Fenwick (1990) also presented evidence that the carboxyl end of the HSV polypeptides is functionally important. Their work described HSV-2 strain HG52 which is deficient in host shutoff activity. DNA sequence analysis of the vhs gene from this virus showed that the first 342 residues of the protein were identical to the vhs gene of HSV-2 G which possesses very high shutoff activity. However a change in reading frames caused the last residues of the protein to deviate from the HSV-2 G sequence. These results indicate that the carboxyl third of the vhs protein of HSV-2 strain G is functionally important for the polypeptide.

Plasmids containing each of the four insertion mutants, pI147, pI236, pI382, and pI343 were also cotransfected with pSV3CAT and assayed for their effect on CAT-gene expression. Of the four mutations, I147 (located 147 amino acids downstream from the amino terminus) caused the greatest reduction in vhs function. This mutant had only 10% of the activity measured for the wild-type protein. The three remaining mutants displayed vhs activities which were about 30% of the normal wild type activity. Whether these mutations are located at functionally important regions of the protein or that the protein is particularly sensitive to any perturbations of its native structure cannot be determined by this small number of mutants. To answer this question, a large number of in frame insertion mutants could be

constructed over the entire sequence of the vhs gene. This has been accomplished successfully in an analysis of the HSV-1 immediate early gene ICP0 (Everett 1987). Using 43 insertion mutants, Everett (1987) was able to show specific regions within the gene that affected gene function to varying degrees. A similar experiment with the vhs gene may be useful in better locating functionally important regions of the gene products.

In addition to examining the structure and function of the vhs gene, I was interested to learn which viral genes are absolutely required for HSV host shutoff activity. It had been shown previously that host shutoff activity does not exist without a functional vhs gene. This was demonstrated when cells infected with HSV mutant, vhsΔSMA, showed no decline in levels of host cell message.

My in vitro assay is unique in that it does not use whole viruses for analysis of vhs function. In fact, it conveniently allowed to "pick and choose" which viral genes were to be expressed during analyses. The viral genes transfected in the majority of our experiments were the vhs, ICP0, and ICP4 genes. The transcriptional activators were used to obtain maximum expression of the vhs gene. However, in experiments in which the vhs gene was the only viral gene present, host shutoff activity was still observed. These results indicate that no viral genes in addition to the vhs gene are required for host shutoff activity.

Differences between HSV-1 and HSV-2 vhs protein activity.

The initial attempts at mapping the HSV-2 vhs gene had used early observations that HSV-2 strains possessed greater shutoff activities than HSV-1 strains (Powell and Courtney 1975; Morse et al. 1977). Whether the difference was due to a larger quantity of the vhs protein packaged into HSV-2 virions or that the packaging allowed the polypeptide to be more accessible had not been investigated. Our data supports the theory that the vhs polypeptides of HSV-2 strains are more efficient than those of HSV-1 strains. By transfecting HSV-2 or HSV-1 vhs genes in our assay, the effects of packaging within the virion are eliminated.

By constructing HSV-1 x HSV-2 recombinants I had hoped to find a region of the gene required for the difference in efficiencies of shutoff activity. Of the four recombinants tested, only pKS:Sac 2-1 has vhs activity comparable to the wild-type HSV-2 vhs gene. The pKS:Sac 2-1 construct contains the carboxy terminal third (123 amino acids) of the HSV-1 vhs fused to the amino two-thirds (369 amino acids)of the HSV-2 vhs gene. The only inference that can be made from this experiment is that the amino two thirds of the vhs protein encodes the region of the vhs protein responsible for the greater efficiency of the HSV-2 polypeptide. A comparison of the two vhs genes which have been sequenced (HSV-1 strain 17 and HSV-2 strain 333) does not suggest any obvious differences between the two vhs genes that might be responsible for their

different efficiencies. However, two other recombinants can be constructed which may delimit the region further.

A HSV-1 x HSV-2 recombinant may be constructed using the same restriction enzyme sites as the four recombinants already constructed. These restriction sites were chosen previously because upon religation of the fragments, the reading frames from each gene would remain intact. A construct using the Pvu1 and Sac1 sites could be made which consists of HSV-1 sequences on the amino and carboxyl ends with the center third of the vhs protein encoded by the HSV-2 vhs gene.

The second recombinant could be constructed using ScaI sites found in the center of the vhs genes from both types of virus. By cutting each or the genes with ScaI and religating the 5'-half o the HSV-1 vhs gene with the 3'-half of the HSV-2 vhs gene, the reading frame would again remain intact and a recombinant vhs protein could be formed. Each of the above recombinants would be tested for vhs activity and perhaps limit the region of the gene responsible for the high vhs activity of the vhs gene further.

Some intriguing observations that have not yet been addressed involve the dominance relationships between various wild type and mutant vhs genes. Mixed infections of wild type HSV-1 (KOS) and the mutant vhs1 (which shows no shutoff activity) were carried out in Vero cells. When equal amounts of each virus were used in the coinfection experiment, the mutant phenotype was found to be dominant. Only when the

ratio of wild-type to mutant viruses was increased to 4:1 did the wild type phenotype appear to be dominant (G.S. Read, unpublished data). These observations may be explained easily if the vhs function is being carried out by a multimer of vhs polypeptides. For instance, if the functional vhs protein is a dimer and one or both subunits are composed of the vhs1 mutant protein, shutoff function might be abrogated. When the ratio of the wild type to mutant is raised, more of the dimers will be likely to be composed of two wild type polypeptides, thus giving rise to a wild-type phenotype. Although this model of the functional vhs polypeptide can explain how these ratios of coinfections can give rise to observed results, it does not answer the question of why the mutant phenotype is dominant. The most likely explanation is that mixed multimers are non-functional. Fenwick and Everett have recently performed a similar experiment coinfecting BHK cells with HSV-1 (17^+) and HSV-1 (17G42). Again, the virus with the weak-shutoff function (17^+) inhibited the strong shutoff of strain 17G42. The mechanism of vhs activity must be determined before this question can be resolved.

These data are consistent with several models for the mechanism for vhs function. The vhs protein may itself be a ribonuclease, or it may induce a cellular ribonuclease. Krikorian and Read (1990) developed an in vitro mRNA degradation system which can be used to address this question. In this system, the rate of degradation of cellular or viral

mRNAs is measured using in vitro translation extracts. The extracts are prepared from HSV-infected or mock-infected HeLa cells and the rates of in vitro degradation of specific mRNAs are measured. In an experiment to localize the vhs function, the in vitro translation extract was first fractionated on a sucrose gradient. This allowed separation of a polysome pellet and an S13 post-polysomal supernatant fraction. It was discovered that upon resuspension of the polysomes in the appropriate buffer, no in vitro degradation occurred. Degradation of host mRNAs, however, could be recovered when the post polysomal supernatant fraction was added to the mixture. These results indicate that one or more factors in the post polysomal supernatant fractionvare required for host shutoff function. These factors may include the vhs protein itself and additional cellular factors.

To address the question of whether the vhs protein is a ribonuclease, purified vhs proteins could be added to naked host cell mRNA. If host mRNA degradation is observed, the vhs protein can be concluded to be a ribonuclease. If the message remains stable, the question remains: Does the vhs protein induce a cellular ribonuclease or is it a ribonuclease which requires additional cellular factors?

It has been demonstrated in this dissertation that the only viral gene required for HSV host shutoff function is the vhs gene. On the other hand, the number of cellular factors required for HSV induced mRNA degradation is unknown. The

elucidation of cellular factors involved in the HSV host shutoff function will be multifarious. The task could be achieved using a monoclonal antibody specific for the *vhs* protein. If the *vhs* protein forms a complex with cellular proteins, a monoclonal antibody may be used to isolate the protein aggregate. Once isolated, the cellular and *vhs* proteins would be eluted from the monoclonal antibody and size fractionated on a SDS-polyacrylamide gel. After separating the proteins, they could be transferred to nitrocellulose and isolated for protein micro-sequencing (Matsudaira 1989). With the sequencing data, small peptides could be synthesized and antibodies against the individual proteins raised using the peptides. In addition, the protein sequence data could be used to create DNA probes for the cellular genes making it possible to isolate the corresponding cellular genes.

Although the use of a monoclonal antibody to the *vhs* protein could quickly isolate a protein complex, it would not be useful if the *vhs* protein interacts with the cellular proteins transiently without forming a stable complex. In this case, standard cellular fractionation procedures would be necessary to isolate the relevant proteins.

Scientists are becoming increasingly aware that a major component in the control of gene expression is the regulation of mRNA stability. Initially, the rate of transcription was believed to be the major control point of protein expression.

Now it is widely accepted that the rate of protein synthesis is governed by both transcription controls and by the rate of mRNA degradation.

The _vhs_ protein of HSV has been shown to nonspecifically induce the destabilization of both viral and cellular mRNAs. The continuing investigation of the _vhs_ gene should increase the understanding of, not only HSV regulation, but also the regulation of cellular gene expression. With this in mind, it is also possible to gain new insights into the mechanisms by which genes become unregulated and consequently cancerous.

Using the assay described in this dissertation, I have shown that the _vhs_ gene is the only viral gene required for HSV host shutoff activity. We have also begun to dissect portions of the _vhs_ gene important for its activity. For instance, the carboxyl end of the _vhs_ gene appears to be functionally important. The construction of additional mutations in this region should provide further information.

The _in vitro_ assay for _vhs_ function will facilitate the analysis of the _vhs_ gene by allowing further rapid screening of _in vitro_ generated mutations. A combination of this kind of analysis along with the determination of any cellular components in _vhs_ function will lead to a greater fundamental understanding of cellular gene regulation.

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VITA

Annette Schmidt Pak was born January 3, 1962, in Chicago, Illinois. Ms. Pak received her Bachelor of Arts degree with a major in biology from Washington University, St. Louis, Missouri in May, 1984. From 1984 to 1985 she worked as a research technician at Washington University in the laboratory of Dr. Simon Silver. In August 1985 she entered the graduate program of the Department of Microbiology and Immunology at Loyola University Medical Center. Ms. Pak is the co- author of the following publications and abstracts:

Schmidt, A., L Haberstroh, S. Silver and T.K. Misra. 1985. Mercuric Ion resistance operons of plasmid R100 and transposon Tn 501. ASM annual meeting.

Misra, T.K., N.L. Brown, L. Haberstroh, **A. Schmidt**, D. Godette and S. Silver. 1985. Mercuric reductase structural genes from plasmid R100 and transposon Tn 501: functional domains of the enzyme. Gene **34**:253-262.

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APPROVAL SHEET

The dissertation submitted by Annette Schmidt Pak has been read and approved by the following committee:

Dr. G.S. Read, Director
Assistant Professor
Division of Cell Biology and Biophysics
University of Missouri, Kansas City

Dr. Kenneth Thompson
Associate Professor
Departments of Microbiology and Pathology
Loyola University Chicago

Dr. Leonard Erickson
Professor
Departments of Medicine and Pharmacology
Loyola University Chicago

Dr. Robert Miller
Professor and Chairman
Department of Microbiology
Oklahoma State University

Dr. John Nawrocki
Assistant Professor
Department of Medicine
Loyola University Chicago

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

12/10/91
Date

Sullivan Read
Signature