1989

Control of Viral mRNA Stability by the Virion Host Shutoff Function of Herpes Simplex Virus

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by

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A Dissertation Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

May

1989

ACKNOWLEDGEMENTS

The author would like to thank her director, Dr. G. Sullivan Read, for his constant encouragement in the achievememt of the goals set for this study. His esteemed guidance has been invaluable in the shaping of this project. His confidence in the success of this project has played a key role in its completion.

The author would also like to thank her committee, Drs. S.K. Farrand, A. Frankfater, R.E. Malone, and R.V. Miller, for their advice and constructive criticism in the analysis of the data.

Finally, the author wishes to dedicate this dissertation first, to her parents, Krishna and Dharambir Gulati, whose progressive foresightedness was instrumental in enabling her to pursue her career; and secondly, to her beloved husband, Anil, whose unwavering support under seemingly insurmountable problems was a significant factor in the completion of this project.

VITA

The author Asha Anil Oroskar, is the daughter of Krishna D. Gulati and Dharambir Gulati. She was born in New Delhi, India.

Her secondary education was obtained at the Fatima High School, Bombay, India, where she graduated in May 1971. Ms. Gulati received her Bachelor of Science degree in Microbiology, with Honors, from the University of Bombay, India, in the year 1975. In 1974, she was ranked first in the University of Bombay, amongst all candidates who tested for Chemistry as the minor subject in the Bachelor of Science degree.

Ms. Gulati received her Master of Science degree in Biophysics from the University of Bombay, in the year 1978. Her masters thesis was written on the topic "Studies on cell migration in vitro. Quantitative evaluation of murine splenocyte migration". The research for the masters degree was carried out at the Cancer Research Institute, Bombay, India. During the period of her study, Ms. Gulati was awarded the Cancer Research Institute Scholarship from 1975 to 1978. In 1981, Ms. Gulati married Dr. Anil R. Oroskar.

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Ms. Oroskar has co-authored the following publications.

1. Gothoskar, B.P., A.D. Gulati*, and V.K. Jain. 1983. Studies on murine splenocyte migration in vitro: effects of energy modifiers. Indian J. Exp. Biol. 21:225-228.

   *maiden name

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

3. Oroskar, A.A., and G.S. Read. 1989. Control of mRNA stability by the virion host shutoff function of herpes simplex virus. J. Virology. To be published in the upcoming May issue.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Act-D.....Actinomycin-D

ATP.....Adenosine triphosphate

bp.....Base pair

cm.....centimeter

CTP.....Cytosine triphosphate

DNA.....Deoxyriboncleic acid

DNase I.....Deoxyribonuclease I

DNA pol.....DNA polymerase

g.....gram

gB.....glycoprotein B

gC.....glycoprotein C

gH.....glycoprotein H

GTP.....Guanosine triphosphate

HSV-1.....Herpes simplex virus 1

HSV-2.....Herpes simplex virus 2

h.....hour

ICP.....Infected cell polypeptide

IR_L.....Internal repeat of the L component

IR_S.....Internal repeat of the S component

kb.....kilobases

M.....Molar

MEM.....Minimal essential medium

μCi.....microCurie

μg.....microgram

μl.....microliter

ml.....milliliter

mM.....millimolar

min.....minute

MOI.....multiplicity of infection

mRNA.....messenger ribonucleic acid

ng.....nanogram

pfu.....plaque forming units

poly(A).....polyadenylic acid residues

RNA.....Ribonucleic acid

RNAse.....Ribonuclease

SDS.....sodium dodecyl sulfate

TK.....Thymidine kinase

TR_L.....Terminal repeat of the L component

TR_S.....Terminal repeat of the S component

ts.....temperature sensitive

TTP.....Thymidine triphosphate

vhs.....virion host shutoff

wt.....wild type

INTRODUCTION

Infection of permissive cells with herpes simplex virus (HSV) types 1 and 2 results in the sequential expression of at least four classes of viral genes whose synthesis is coordinately regulated (Honess and Roizman, 1974, Roizman and Spear, 1981, and Roizman and Batterson, 1986). For most viral gene products, the rate of protein synthesis is proportional to the level of correponding mRNA in the cytoplasm (Marsden et al., 1983, and Smith and Sandri-Goldin, 1988). Thus factors that affect cytoplasmic mRNA accumulation, such as the transcription rate, mRNA processing and transport, as well as cytoplasmic mRNA stability, will necessarily influence HSV viral gene expression (Raghow, 1987). To date, most of the literature on herpes simplex virus has concentrated on unraveling transcriptional regulation of HSV gene expression. In the case of alpha and beta gene products however, it has been shown that posttranscriptional control may play an important role in maintaining the cascade of viral gene expression (Roizman and Spear, 1981). Studies on the posttranscriptional controls in HSV gene expression became much more amenable after the isolation of vhs mutants of the herpes simplex virus type 1 (Read and Frenkel, 1983). Prelimnary characterization of the virion associated host shutoff or vhs mutants, which were defective in the virion function responsible

for shutoff of host polypeptide synthesis, suggested that the vhs components may be involved in the posttranscriptional regulation of cellular as well as viral alpha, beta, and gamma messages (Read and Frenkel, 1983). Further studies on the mode of vhs inhibition of host polypeptide synthesis showed that vhs-mediated degradation of cellular messages was one of the important aspects of HSV-associated suppression of cellular polypeptide synthesis (Schek and Bachenheimer, 1985). The purpose of this study therefore, was to determine the role of the vhs gene product in regulating mRNA stabilities in HSV-infected cells and to assess the importance of the control of mRNA stability in the overall scheme of HSV viral gene expression.

The mechanism of host shutoff with other viruses, varies from virus to virus. As is the case for other virus-host interactions, infection with herpes simplex viruses leads to a massive suppression of host protein synthesis (Roizman and Spear, 1981, Fenwick, 1984, and Roizman and Batterson, 1986). However, the process of HSV induced host shutoff differs from that found in other viral systems, in that the inhibition of host protein synthesis is initially caused by a virion component which induces the disaggregation of host polyribosomes (Sydiskis and Roizman, 1967) and causes the degradation of host mRNAs (Schek and Bachenheimer, 1985). Until the late 1970's, studies on the mechanism of HSV virion shutoff had not been fully explored beyond the obvious effects seen on the inhibition of cellular protein synthesis. In an effort to characterize this function, Read and Frenkel (1983) isolated several vhs mutants which were defective in this virion associated shutoff of host protein synthesis. Only those mutants that

were unable to shutoff cellular protein synthesis when infected in the presence of Actinomycin-D, were considered to be true vhs mutants. Read and Frenkel further noted that not only were these vhs mutants defective in the virion-associated host shutoff of host polypeptide synthesis but they were also defective in the posttranscriptional regulation of viral alpha messages. The discovery that the vhs components modulated alpha mRNA translation was made quite accidently. The authors were actually studying the role of alpha polypeptides in vhs mediated inhibition of host protein synthesis. Cells were infected with either the wild type or the vhs 1 mutant virus in the presence of cycloheximide. Five hours after infection, the cycloheximide was washed off and the infection continued in the presence of Actinomycin-D. About 3 hours after the reversal of cycloheximide, alpha protein synthesis was significantly inhibited only in the wild type but not vhs 1 infected cells. Furthermore, the presence of alpha polypeptides did not help the inhibition of host infection in vhs 1 infected cells. On the other hand, the alpha protein synthesis in vhs 1 infected cells could be inhibited by wild type virion components. At the time when cycloheximide was removed and Actinomycin-D added, if vhs 1 infected cells were superinfected with the wild-type HSV-1 virions, then alpha polypeptide synthesis was found to be inhibited. Thus, wild-type virion components were capable of modulating alpha polypeptide synthesis in the absence of viral gene expresssion. These studies suggested that the vhs mutants could be the perfect tools for dissecting the posttranscriptional regulation of viral mRNA in HSV infected cells.

Several investigators had focussed their efforts on studying vhs mediated posttranscriptional regulation of cellular messages in HSV infected cells. These studies indicated that the wild-type vhs function affected cellular mRNAs in a manner that led to their functional destabilization as determined in an in vitro translation system (Fenwick and McMenamin, 1984) and their physical degradation as seen in Northern blots (Schek and Bachenheimer, 1985. Strom and Frenkel (1987) subsequently showed that, in contrast to the wild-type virus, the vhs 1 mutants were defective in degrading host mRNAs in the absence of viral gene expression.

Recently, Kwong and coworkers (1988) succeeded in mapping the vhs gene. On the basis of the published sequence of the HSV genome of McGeoch et al. (1988), the vhs gene is predicted to code for a 56 kilodalton protein. The vhs 1 mutation has also been sequenced. Sequence analysis of the vhs encoding region revealed that a single nucleotide difference between the wild-type KOS virus strain and the vhs 1 mutant virus changed a threonine to an isoleucine amino acid residue (Kwong, 1988). The vhs gene was mapped through marker rescue, a procedure where specific fragments from the wild-type virus were transfected along with the vhs 1 viral DNA and the resultant progeny virus was screened for wild type vhs gene function. Kwong et al. (1988) discovered that the resultant wild-type progeny virus recombinants obtained through marker rescue were not only proficient in the degradation of cellular mRNAs but also in the postranscriptional regulation of viral mRNAs belonging to all kinetic classes (Kwong et al, 1988).

It became apparent then that the vhs gene product played a very significant role in determining the cytoplasmic expression of viral genes in HSV infected cells. Furthermore, it seemed very likely that similar to its posttranscriptional modulation of cellular mRNA stability, the vhs mediated posttranscriptional control of viral mRNAs would involve the modulation of viral mRNA stability. To date, however, few studies have directly addressed the role of the vhs gene product in the regulation of viral mRNA half lives in HSV infected cells. We were the first to demonstrate that the wild type vhs gene product affected both the functional and physical stabilities of alpha mRNAs (Oroskar and Read, 1987). Kwong and Frenkel (1987) subsequently showed that the wild-type vhs gene product could destabilize preexisting alpha mRNAs in the absence of viral gene expression. In cells infected with the vhs 1 mutant in the presence of cycloheximide, the alpha mRNAs were physically degraded when superinfected with the wild-type but not the vhs 1 mutant virus in the presence of Actinomycin-D. Furthermore, several studies suggested that the vhs gene product may also be involved in regulating the cytoplasmic expression of beta, beta/gamma, and gamma genes:

1. SDS-polyacrylamide gel electropherograms (SDS-PAGE) of cellular extracts from cells infected with each of the six vhs mutants indicated that in comparison with wild-type infections, the cascade of viral protein synthesis for beta, beta/gamma, and gamma polypeptides was also affected in the mutant infected cells (Read and Frenkel, 1983). Thus alpha, beta, beta/gamma, and gamma polypeptides seemed to overaccumulate, and be synthesized for much longer periods in vhs mutant infected

cells.

2. Strom and Frenkel (1987) had also performed some very prelimnary studies on the effects of the vhs gene product on the accumulation profile of some viral mRNAs in HSV infected cells. They found the US10 beta/gamma mRNA, and the US11 gamma mRNA to be overaccumulated in vhs 1 infections in comparison with their levels from wild-type infections.

3. Finally, Kwong and Frenkel (1987) had studied the ability of viral mRNAs to direct polypeptide synthesis in the presence of Actinomycin-D, which inhibits de novo transcription. Cells were infected with either the wild-type virus or the vhs 1 mutant for varying periods up to 16 hr and then pulse labeled with ^{35}[S]-methionine in the presence of Actinomycin-D. SDS-PAGE of extracts from wild type and vhs 1 infected cells indicated that six hours after the addition of Actinomycin-D, beta, beta/gamma, and gamma proteins were synthesized only in vhs 1 infected but not wild-type infected cells. These studies are consistent with their suggestion that the vhs 1 mutant gene product was defective in regulating the stability of beta, beta/gamma, and gammma mRNAs. However, these experiments were designed to test the rates of beta, beta/gamma, and gamma polypeptide synthesis but not the stability of beta, beta/gamma, and gamma mRNAs.

We therefore set out to systematically address the role of vhs gene product in controlling viral mRNA stability in HSV infected cells. The dissertation is designed to specifically focus upon the following questions:

1. whether the vhs gene product affects the functional and physical

stabilities of all or only some viral mRNAs.

2. whether the vhs gene product affects steady state levels of viral mRNAs belonging to all four kinetic classes.

3. whether the vhs mediated control of viral mRNA stability exhibits any target selectivity.

Our studies indicate that the wild-type vhs gene product affects the half lives of viral mRNAs belonging to all four kinetic classes. The vhs-mediated decay of viral mRNAs thus involves little, if any target mRNA selectivity. Results on the accumulation profiles of ten different viral mRNAs indicate that the vhs 1 mutation caused a dramatic overaccumulation in the steady state levels of alpha and beta mRNAs but not a very significant accumualtion in the cytoplasmic steady state levels of beta/gamma and gamma mRNAs.

General definition

Members of the family Herpesviridae are viruses of eukaryotes. The HSV viral genome typically consists of a single linear, double stranded DNA molecule that is packaged into icosahedral nucleocapsids with 162 capsomers, assembled in the nucleus. The viral envelope consists of a virus modified glycoprotein and lipid layer derived by budding through the inner nuclear membrane (Roizman and Batterson, 1986).

Classification

The herpesviruses comprise a group of structurally similar viruses. This family includes six important human viruses: Herpes simplex

virus type 1 (HSV-1), Herpes simplex virus type 2 (HSV-2), Varicella zoster virus (VZV), Cytomegalovirus (CMV), Epstein Barr virus (EBV), and the recently identified Human herpesvirus-6 (HHV-6). The members of the family Herpesviridae (Gr. herpein, to creep) have been classified into three subfamilies on the basis of differences in host range and the tissues involved in acute and latent infections in vivo, and by differences in host range, growth rate, and cytopathology of infections in cell cultures in vitro (Matthews, 1982, and Roizman, 1982). Alphaherpesvirinae members are characterized by their wide host range, short reproductive cycle (less than 18 hrs) and capacity to establish latent infections in sensory ganglia. The alpha herpesviruses are exemplified by herpes simplex viruses associated with common oral (HSV-1), and genital (HSV-2) infections of man. The subfamily also includes varicella zoster virus, the causative agent of human chicken pox and shingles. In contrast,members of the subfamily Betaherpesvirinae have a relatively restricted host range and long replication cycle in cell cultures. These viruses are typically species specific in vivo and in vitro, and have been isolated from a wide range of vertebrates. Betaherpesviruses include the cytomegaloviruses, or "salivary gland" viruses which are capable of producing disseminated infectious diseases involving a wide range of tissues in neonates or immunocompromised adults of their natural hosts. Gammaherpesvirinae members infect only the relatives of their natural hosts. In the laboratory, these viruses replicate mostly in lymphoblastoid cells with some using epitheliod and fibroblastic cells to multiply. The gammaherpesviruses include Epstein-Barr virus (EBV), the agent of human infectious mononucleosis.

This agent is also associated with African Burkitt's lymphoma and with nasopharyngeal carcinoma. This subfamily also includes the human herpes virus 6. The wide host range and short reproductive cycle have made Alphaherpesviruses most amenable for studies on the herpes viruses. Almost all the information cited here therefore, has been gleaned from the simplex viruses such as HSV- 1, and HSV-2.

HSV virions

Morphology of subunits

HSV particles have a diameter of about 100 nm. The herpesvirion consists of four structural elements.

1) a DNA containing toroidal core about 75 nm in diameter (Furlong et al, 1972 and Heine et al, 1974)

2) an icosahedral capsid 100 nm in diameter. The capsid is composed of 162 elongated hexagonal prisms called capsomers, which are 9.5 X 12.5 nm with a central hole about 4 nm in diameter (Wildy and Watson, 1963).

3) an electron dense granular zone made up of globular protein (Schwartz and Roizman, 1969 and Morgan et al, 1968), constitutes the area between the capsid and the envelope. This zone was given the term tegument by Roizman and Furlong (1974). The thickness of the tegument varies upon the location of the virion within the infected cell; the tegument is thicker when the virus accumulates in the cytoplasmic vacuoles than in the perinuclear space (Fong et al, 1973). Infact, the highly variable diameter of herpesviruses derives in part from differing thickness of the tegument.

4) an outer covering called the envelope, is derived from patches of

altered cellular membrane (Asher et al, 1969, and Morgan et al, 1959). The HSV envelope contains many short periodic projections or spikes. Wildy and Watson (1963) estimated the size of the size of these spikes to be about 8 nm in length.

In short, the purified enveloped virions are made up of 25-30 virus specified proteins which form 70% of the virion by weight. The virion envelope is acquired by the process of budding through the inner nuclear membrane of the cell. It consists mainly of phospholipids and glycoproteins and forms 22% of the virion by weight. Detectable amounts of spermine are found in the nucleocapsid. The phospholipid nature of the envelope makes herpesviruses highly susceptible to inactivation by lipid solvents.

Conformation and sequence arrangement of HSV DNA

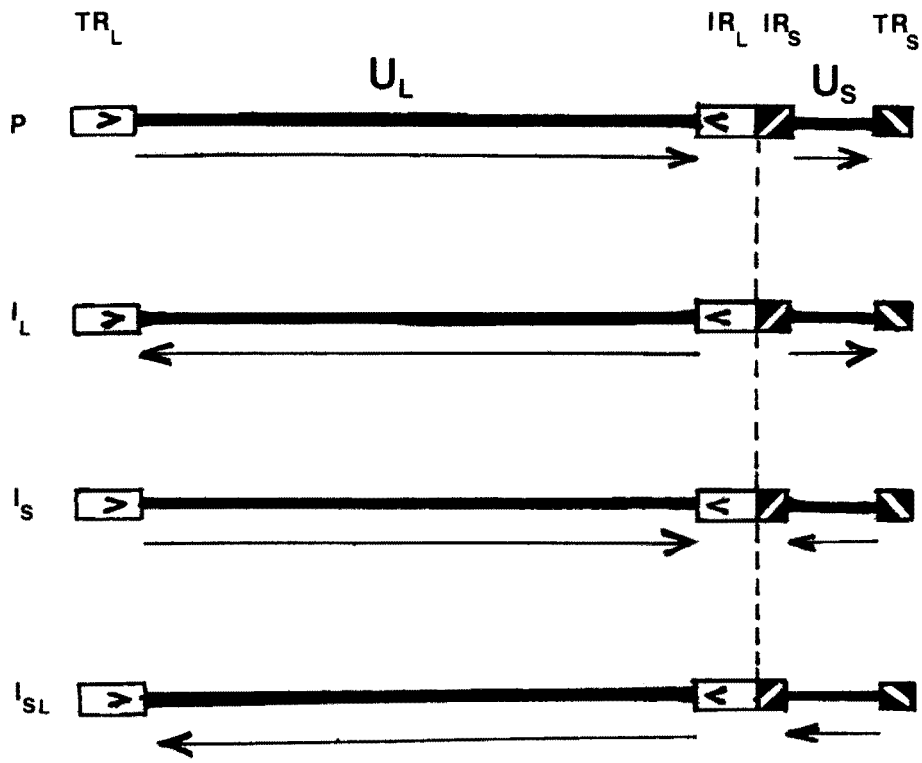
The genomes of HSV-1 and HSV-2 are linear double stranded DNA molecules, containing approximately 152 kilo basepairs, with a base composition of 67 and 69 moles% G+C respectively (Buchman and Roizman, 1978a and 1978b ; Kieff et al, 1971). The genome of HSV-1 consists of two covalently linked DNA segments: a long segment consisting of 82% of the genome and a short segment made up of the remaining 18% (Sheldrick and Berthelot, 1975). Both the long(L) and the short(S) segments consist of unique sequences U_L and U_S bounded by inverted repeats; ab (terminal repeat TR_L) and b'a' (Inverted repeat IR_L) flank U_L and contribute 6% of the genome while, a'c' (Inverted repeat IR_S) and ca (Terminal repeat TR_S) together make up 4.3% of the genome and flank the U_S (see Figure 1).

The HSV-1 DNA structure has been deduced from two different kinds

of analyses. First, intact single strands upon self annealing form two different loops held together by short double stranded regions of DNA (Wadsworth et al, 1975). Second, analysis of HSV-1 genomic fragments obtained through restriction digests (Sheldrock and Berthelot, 1975) provided further proof of the DNA sequence arrangement in HSV-1 DNA. Sheldrock and Berthelot (1975) proposed that, because the terminal regions are inverted internally, recombination between the ends of the molecule and their inverted repeats might readily occur, leading to inversion of L and S components. Evidence for the occurrence of such inversions was obtained through analysis of restriction fragments obtained with an enzyme that cleaves the DNA outside the inverted repeats. Cleavage of the HSV-1 DNA with this enzyme Hsu I, yeilds four distinct terminal fragments, each present in concentrations of 0.5 M relative to the molarity of intact viral DNA and, four distinct junction fragments, each present in concentrations of 0.25 M relative to the molarity of intact DNA. All other fragments located between the terminal and junction fragments were present in concentrations of 1.0 M relative to the concentration of intact DNA. This data is consistent with the existence of four equimolar populations of HSV-1 DNA which differ solely in the orientation of L and S components. These four populations are designated as prototype (P), inversion of S component (I_S), inversion of L component (I_L), and inversion of both S and L components (I_{SL}) (Roizman et al 1974, and Hayward et al, 1975).

Recent work of Weber and colleagues (1988) indicated that the HSV-1 viral DNA replication machinery is entirely sufficient for mediating inversion events in the HSV-1 genome. Furthermore, their evidence

Figure 1 Structure of the herpes simplex virus type 1 genome. The mature genome is composed of a long (L) and a short (S) segment divided by a junction. Each segment is composed of unique sequence components (U_L and U_S) bounded by inverted repeats. The terminal and internal repeats TR_L and IR_L of the L segment consist of ab and b'a' whereas the terminal and internal copies TR_S and IR_S of the S segment consist of ca and c'a'. Due to the ability of unique sequences and their associated repeats to invert with respect to each other, populations of mature genomes are normally composed of an equimolar mixture of the four sequence isomers: P or prototype, I_S with an inversion of the S segment, and I_L with an inversion of the L segment and I_{SL} with inversion of both the S and L segments.


TR_L
IR_L
IR_S
TR_S
U_L
U_S
P
I_L
I_S
I_SL

indicates a lack of sequence specificity involved in the process that gives rise to these inversions. These results were fortuitously obtained during the process of analyzing HSV-1 genes that were non-essential for replication in cell culture. Cloned HSV-1 restriction fragments containing the Tn5 insertions in three S component genes were recombined into the viral genome without impairment of virus reproduction in cell culture (Weber et al, 1987). However, copies of Tn5 inserted into the HSV-1 genome were able to undergo sequence inversion via high frequency recombination between the duplicated IS50 elements of the transposon (Weber et al, 1988). In addition, cloned HSV-1 fragments containing Tn5 insertions were added to the mixture of plasmids required for transient DNA replication and analyzed in transient expression assays to study cis and trans acting functions required for Tn5 inversion. The results of this assay revealed that the seven HSV-1 DNA replication enzymes alone mediated Tn5 inversion; no additional viral gene products were required for detectable recombination to occur (Weber et al, 1988). The herculean task of sequencing the entire HSV-1 genome has only recently been completed (McGeoch et al, 1988). The whole genome of HSV-1 has 72 recognizable genes, which encode 70 distinct proteins since two of the genes are diploid.

Different strains of virus vary in the lengths of their a sequence (Locker and Frenkel, 1979, Tognon et al,1983,and Wagner and Summers, 1978). Specifically, the HSV-1 (F) a sequence consists of a 20 base pair direct repeat (DR1), a 65 base pair unique sequence (U_b), a 12 base pair sequence (DR_2) reiterated 19-23 times, a 37 base pair sequence (DR_4) repeated 2-3 times, and a 58 base pair unique sequence

(U_C) and another copy of DR1 (Mocarski and Roizman, 1982). In the case of HSV-1, the terminal a sequence of the S component is represented only once. On the other hand, several copies of a sequences may be present in the terminal L component and at the LS junction (Locker and Frenkel, 1979, and Wagner and Summers, 1978). Most interestingly, the terminal a sequence of the L component has a 3' protruding nucleotide followed by the distal 18 base pairs of DR1. The terminal DR1 of the unique a sequence of the S component also has a 3' protruding nucleotide followed by only one base pair of the DR1. The terminal DR1 of the L segment and the terminal DR1 of the S segment together form a complete DR1 (Mocarski and Roizman, 1982). This sequence has profound implications in the biogenesis of packaged viral DNA. Thus, HSV-1 DNA is generated by cleavage of concatemeric or circular DNAs at the DR1 shared by adjacent a sequences (Mocarski and Roizman, 1982).

Anywhere from 15-35 species of structural polypeptides have been reported in purified virions of herpesviruses (Cassai et al., 1975; Dolynuik et al., 1976; Spear and Roizman, 1972; and Strnad and Aurelian, 1976). Denaturing gel electrophoresis of purified virions from HSV-1 and HSV-2 show at least four glycoproteins present on the surface (Cassai et al, 1975; Spear, 1976; and Spear, 1984) Glycoprotein B, glycoprotein C, glycoprotein D, and glycoprotein E all appear to be sulfated (Hope and Marsden, 1983). HSV-2 has an additional glycoprotein gG (Roizman et al, 1984). Glycoprotein gH has recently been identified in the HSV-1 (Gompels and Minson, 1986).

Glycoprotein B is apparently required for fusion of envelope and cell membrane during viral entry into cells (Little et al, 1981, and

Cai et al, 1988). The evidence that gB is required in cell plasma membranes for virus induced-cell fusion and in virions for viral penetration, raises the possibility that this protein may play a direct role in mediating membrane fusion. Glycoprotein E is thought to be an Fc receptor (Johnson and Spear, 1982). The herpes simplex viruses are known to induce the expression of Fc-binding activity on the surfaces of infected cells (Westmoreland and Watkins, 1974). The virus induced Fc receptors have an affinity for normal IgG and appear to be operationally equivalent to the Fc receptors expressed on certain cells of the immune system. It has been speculated that the binding of normal immunoglobulin or antiviral immunoglobulin to the Fc receptors can somehow interfere with cytotoxic immune reactions, thus sparing the infected cells and perhaps favoring the establishment of latency (Westmoreland and Watkins, 1974). Glycoprotein C, or gC, is characteristic of the true gamma class of messages. Glycoprotein C is known to have C3b binding activity (Friedman et al, 1984), although the function of glycoprotein C is not known. Mutants that lack the ability to express glycoprotein C are viable. Infact, both glycoprotein E and glycoprotein C are not required for infection in cell culture (Neidhardt et al., 1987, and Ruyechan et al., 1979), but are both present in clinical isolates (Friedman et al, 1986, and Pereira et al, 1982) suggesting that they may be important in viral infections in vivo.

Mutants defective in glycoprotein C (Ruyechan et al, 1979) and those that are temperature sensitive for gB accumulation (Manservigi et al, 1977) also show a syncytial phenotype. However,several other loci in the HSV-1 genome have been identified by genetic studies to be

involved in the syncytial phenotype. Infact four different syn mutations have been mapped in the HSV-1 and the MP strain viruses (Cai et al., 1988). At least in the case of the mutant ts B5, temperature sensitive for gB, the syncytial and fast-entry phenotypes, mapped in the gB region, are separate from each other and from the ts lesion (DeLuca et al., 1982). Thus expression of specific viral gene products one of which has been identified as gB, is required for HSV-induced cell-fusion. The expression of a second set of viral gene products somehow prevents cell fusion from occurring, even though the products required for cell fusion may be present. Bzik and coworkers (1982) found that within 6 h after infection, the wild type infected cells inhibited the fusion activity of the syn 20 mutant infected cells. They further noted that fusion inhibition was an active process that required the synthesis of RNA, protein, and glycoproteins. Thus, the functions and interactions of several viral products determine whether a virus will express the syn+ or syn$^-$ phenotype.

Glycoprotein D, another beta/gamma protein has been identified as a major target for neutralizing antibodies (Para et al, 1985), and anti-gD antibodies have been shown to inhibit virus induced cell fusion (Noble et al, 1983). Another glycoprotein mapped in HSV-1, called glycoprotein H, or gH, also belongs to the beta/gamma class of messages (Gompels and Minson, 1986). It is suspected that glycoprotein H may play a role in virus egress since an anti-gH antiboy can inhibit plaque formation when added in an overlay after virus adsorption (Buckmaster et al, 1984). Gompels and Minson (1986) found that some of the properties of gH were similar to those determined for gD through

anti-gD antibody. Thus, monoclonal anti-gH antibody efficiently neutralized virus infectivity, blocked cell fusion by syncytial virus strains, and prevented the passage of virus from infected to uninfected cells (Gompels and Minson, 1986). Since both anti-gH and anti-gD antibodies gave similar results, the data suggest that glycoprotein D and glycoprotein H may be functionally related.

At least six polypeptides are known to be present in the capsid (Gibson and Roizman, 1972). The rest of the virion polypeptides are most likely present in the tegument or core and others proteins may function to anchor the virion structures to each other.

Virions contain several important regulatory proteins. Vmw65, or alpha trans inducing factor, also called alpha TIF (McKnight et al, 1987), is a virion protein that specifically stimulates alpha gene transcription, and presumably accelerates the infectious cycle (Post et al, 1981, and Campbell et al, 1984). Polypeptide Vmw65 is located in the tegument layer between the nucleocapsid and the envelope of the virion (Roizman and Furlong, 1974). The virion host shutoff or the vhs protein, (Read and Frenkel, 1983, and Kwong et al, 1988), whose identity and location in the virion are still unknown, is responsible for destabilizing preexisting host mRNAs (Fenwick and McMenamin, 1984, Schek and Bachenheimer, 1985, Kwong and Frenkel, 1987, and Strom and Frenkel, 1987), and, for the initial shutoff of host protein synthesis (Fenwick, 1984, Roizman and Batterson, 1986, and Roizman and Spear, 1981). Furthermore, an ATPase (Epstein and Holt, 1963) and a protein kinase (Lemaster and Roizman, 1980) also copurify with the capsid-tegument structures.

Replication of Herpesviruses

Herpesviruses initiate infection through sequential steps of attachment to cell surface receptors, followed by fusion of viral envelope with the plasma membrane, transport of encapsidated DNA core to the nuclear pore and ejection of viral DNA into the nucleus. The exact nature of both the cell membrane receptors and the antigens on the viral envelope with which they interact, have not been well delineated (Spear,1984). However, it is known that glycoprotein B plays an important role in fusion of viral envelope with the cell membrane. The role of glycoprotein B in viral entry has been studied by using gB temperature sensitive mutants (Little et al, 1981). At nonpermissive temperatures, these mutants fail to synthesize mature glycoprotein B or to produce infectious virions. Virions formed at the nonpermissive temperature are apparently able to adsorb to, but not enter the cells. Treatment of ts B5 adsorbed cells with polyethylene glycol (PEG), a chemical fusogen, causes the fusion of the envelope with the plasma membrane and increases the number of plaques produced (Little et al, 1981). Data obtained from the temperature sensitive mutants are complicated due to the presence of inactive glycoprotein B. The conclusion has however, been confirmed using glycoprotein B-null mutants by Cai and coworkers (1988). Through transient expression assays, Cai and coworkers (1988) also found that mutations located within the cytoplasmic domain markedly increased fusion activity of glycoprotein B, whereas others, located within the extracytoplasmic domain, decreased the fusion activity.

Finally, a viral function helps in the ejection of viral DNA through the nuclear pore into the nucleus. HSV-1(HFEM)tsB7 mutants are defective in this function and tend to accumulate full capsid containing DNA at the nuclear pore at nonpermissive temperatures (Batterson et al,1983). The parental viral DNA is transcribed in the nucleus by host RNA polymerase II in conjunction with viral regulatory proteins (Wagner, 1984). Replication of viral DNA requires several virus specified proteins (Challberg, 1986, and, Wu et al, 1988) and is thought to occur by a rolling circle mechanism (Roizman and Spear, 1981). Interestingly, progeny DNA is assembled into capsids within the nucleus. Finally, progeny viral particles acquire their envelopes by budding through the inner nuclear membrane.

Several virion components are thus responsible for the success of herpesvirus infection. While some guide the entry of viral DNA into the nucleus, others are involved in the trans activation of immediate early genes (Batterson and Roizman, 1983, and Campbell et al, 1984). One of the most conspicuous features of HSV viral infections on the host cell however, is the virion dependent shutoff of host protein, RNA and DNA synthesis (Fenwick, 1984, Fenwick and Walker, 1978, and Read and Frenkel, 1983).

Viral gene expression

Herpes simplex viruses utilize the cellular RNA polymerase II to transcribe some 70 odd genes within the nucleus. The HSV-1 viral genome is expressed as four groups of genes whose products are coordinately regulated and sequentially ordered in a cascade fashion. This cascade pattern of HSV gene expression is generally mirrored in other

herpesviruses (Wagner,1984).

Alpha or immediate-early genes are the first to be expressed. The operational definition of alpha genes is those viral genes that can be transcribed prior to the onset of viral protein synthesis. The transcription of alpha genes is positively regulated by the virion stimulatory protein , Vmw65 (Campbell et al, 1984), or alpha trans inducing factor, TIF (McKnight et al, 1987). The mode of Vmw65 mediated alpha regulation is only recently being deciphered. Studies by O'Hare and Goding (1988) and, Preston and colleagues (1988), showed that activation of alpha gene transcription involves the specific interaction of a complex containing the Vmw65 with the TAATGARAT DNA sequence located far upstream within the alpha gene noncoding regions. They further showed that this activation was not the result of direct interaction between Vmw65 protein and the target DNA sequence. Instead, a protein-protein complex of Vmw65 and nuclear factors from either infected or uninfected cells was required for its association with the TAATGARAT DNA sequences.

According to standard terminology, infected cell polypeptides, ICP4, -0, -22, -27, and -47 comprise the alpha gene products. Their synthesis can be detected as early as 1 hr post infection (p.i.), reaches a maximum 2-4 hr post infection and decreases thereafter (Honess and Roizman, 1974). Alpha proteins are regulatory proteins; at least one of them, ICP4, is continuously required for the synthesis of all subsequent classes of RNA (Watson and Clements, 1980). This was demonstrated with the use of the temperature sensitive K (ts K) mutant defective in the alpha ICP4 coding gene (Stow et al , 1978) which

accumulates only alpha mRNAs at the nonpermissive temperature (NPT) (Watson and Clements, 1978). Cells infected at the permissive temperature with the ts K mutant were shifted to the nonpermissive temperature, 3 to 7 hours after infection. After 30 min of temperature equilibration, cellular RNA was labeled for 3 hours with ^{32}P-orthophosphate. Analysis of RNA extracted from cells labeled after the temperature shift showed that the RNA pattern corresponded to that of cells making only immediate early or alpha mRNAs. The lanes on the gels were completely devoid of viral RNA representing the beta, beta/gamma, and gamma classes. The studies thus showed that transfer of ts K-infected cells from permissive to nonpermissive temperature resulted in the inactivation of a virus-specified activity, ie. the alpha ICP4 protein which was continuously required for beta, and gamma viral mRNA synthesis.

Beta or early polypeptides reach peak rates of synthesis at 5-7 hr post infection; the synthesis of beta proteins subsequently declines (Honess and Roizman,1974). The beta polypeptides are subclassified into beta 1 and beta 2 subgroups. Ribonucleotide reductase (ICP6) and the major DNA binding protein (ICP8) are members of the beta 1 group which is detected very soon after alpha protein synthesis. The beta 2 group includes the viral thymidine kinase and DNA polymerase. Beta/-gamma or the early late mRNAs appear along with the beta mRNAs but continue to be synthesized for prolonged period of time. In addition, the beta/gamma mRNAs differ from the true gamma mRNAs in that they do not require DNA synthesis as a prerequisite for their expression. The major capsid protein, ICP5, and glycoprotein B, and glycoprotein H are

some of the well characterized beta/gamma subgroup of genes. The true gamma or late proteins are those that are known to have a very stringent requirement for viral DNA synthesis before these genes can be expressed in virus infected cells (Honess and Roizman, 1974; Honess and Watson, 1977; and Costa et al, 1981). To date, Glycoprotein C, is one of the best studied true gamma proteins of the herpes simplex virus type 1 virus.

Properties of HSV-1 transcripts

HSV-1 mRNA is similar to host mRNA in many respects. It is capped at the 5' terminus, polyadenylated at the 3' end, and internally methylated (Bachenheimer and Roizman, 1972; Bartkoski and Roizman, 1976; Bartkoski and Roizman, 1978; and Silverstein et al, 1976). In contrast to many eukaryotic messages, most HSV-1 messages are not spliced.

Functional organization of the herpes simplex virus genome

On the basis of data gleaned from four different protocols it has been possible to map most of the HSV-1 genes. These include the study of HSV-1 X HSV-2 recombinants (Morse et al, 1977 and Morse et al, 1978), marker rescue (Knipe et al, 1978 and Parris et al, 1980), and marker transfer studies (Post et al, 1981 and Ruyechan et al,1979), and in vitro translation of hybrid selected mRNAs (Conley et al, 1981 and Lang et al, 1983). Studies on the mapping of HSV-1 genes have led to the conclusion that the viral genes in the herpes simplex virus are not clustered according to their temporal regulation.

Of the five alpha genes, two have diploid regulatory and coding

sequences located entirely within IR_L/TR_L (ICP-0) and IR_S/TR_S (ICP-4), one is located within U_L (ICP-27) (Clements et al, 1979, and Mackem and Roizman, 1980), and transcripts for the other two are initiated at identical control sequences within TR_S/IR_S but are spliced onto non-identical coding sequences at opposite ends of U_S (ICP-22, and ICP-47). Thus there are alpha genes in unique and repeated sequences of the "short" and "long" genome segments. The promoter regions of these alpha genes contain the classical RNA polymerase II consensus sequences. In addition, alpha genes are equipped with class-specific regulatory elements which are functionally separable from the promoter sequences. These accesory cis-regulatory elements contain multiple direct and inverted copies of (G+C)-rich sequences and components that function in cis as "enhancers" for the expression of heterologous genes (Mackem and Roizman, 1982a, Mackem and Roizman 1982b, and Lang et al, 1984). The U_L and U_S components each contain beta and gamma as well as alpha genes and each region contains genes for envelope glycoproteins (Lee et al., 1982, and Spear, 1984), capsid protein genes and DNA binding protein genes (Weller et al., 1983). Thus, there is no segregation of genes between long and short components on the basis of their regulatory class or function.

Regulation of viral gene expression

The expression of herpes simplex virus genes resembles a cascade (Roizman and Spear, 1981, and Roizman and Batterson, 1986). According to the model originally proposed by Honess and Roizman (1974), alpha gene products turn on the expression of beta genes which together with the alpha gene products turn on the transcription of gamma genes. Both

beta and/or gamma gene products turn off alpha gene expression and a gamma gene product (Vmw65, see Campbell et al, 1984) activates alpha gene transcription in the next round of replication. For most gene products the rate of protein synthesis is proportional to the level of corrresponding mRNA in the cytoplasm (Marsden et al, 1983, and Smith and Sandri-Goldin, 1988). Data from Yager and Coen (1988) however indicates that, at least in the case of the DNA polymerase transcript, translational controls may supercede this basic relationship and modulate HSV gene expression at the cytoplasmic level.

At the transcriptional level, only a few regulatory proteins have been identified in HSV infected cells. The alpha ICP4 protein appears to exert both positive and negative effects on HSV gene expression. At the nonpermissive temperature, ts mutants defective in alpha ICP4 overproduce alpha mRNAs and proteins but fail to induce the expression of beta, beta/gamma, or gamma mRNAs (Dixon and Schaffer, 1980, Holland et al, 1979, Knipe et al, 1978, and Watson and Clements, 1980). Beyond the central role of the ICP4, gene activation studies through transfection have shown that the alpha ICP0 gene product has a role in activation of beta genes (O'Hare and Hayward, 1985a, and O'Hare and Hayward, 1985b). The role of ICP0 in viral infections in culture has been studied through the use of ICP0 deletion mutants by Sacks and Schaffer (1987). The ICP0 deletion mutants are generally grown on ICP0 transformed cells. However, when the ICP0 deletion mutants were grown in Vero cells, their burst sizes were 10 to 100-fold lower compared to those from wild type infections. Significant viral DNA synthesis and only slight reductions in the quantities of late proteins compared to

those in wild-type infected cells were seen with the ICP0 mutant virus infections. Taken together these results indicate that although the ICP0 gene product is not essential for infection in culture, it does play a significant role in viral growth.

In addition to the generalized transactivating role of the ICP4 protein, the alpha ICP27 protein has been implicated in the HSV-1 gene-specific transactivation in infected cells. In studies using the ICP27 temperature sensitive mutants in transient expression assays, Rice and Knipe (1988) showed that the expression of glycoprotein B showed a marked dependence on functional ICP27 protein. Finally, a defective beta ICP8 protein results in the increased accumulation of mRNAs from genes of the alpha, beta, beta/gamma, and gamma classes (Godowski and Knipe, 1983). Through nuclear 'run-on' assays in isolated nuclei from HSV-1 infected cells, Godowski and Knipe (1986) showed that defects in ICP8 actually lead to increased level of transcription of alpha ICP4, beta ICP8, beta/gamma ICP5, and gamma gC. Thus ICP8 may play an important role in maintaining the highly ordered cascade of viral gene expression. Finally, the transcription of alpha genes is positively regulated by a trans acting component of the HSV virion, Vmw65 (Mackem and Roizman, 1982b, Post et al, 1981, and Campbell et al, 1984), or the alpha trans inducing factor (McKnight et al, 1987). That transcription plays a major role in HSV gene expression is underscored by experiments on the regulation of chimeric genes. When the structural sequences for the beta thymidine kinase (TK) were fused to promoters of an alpha or gamma gene, the regulation of the thymidine kinase, or TK gene reflected the temporal class of the promoter (Post et al., 1981, and Silver

and Roizman, 1985).

Superimposed on transcriptional control are the instances of translational control in which different mRNAs are translated with different efficiencies (Yager and Coen, 1988). Yager and Coen (1988) have recently demonstrated that even when thymidine kinase, DNA-binding protein (ICP8), and DNA pol mRNAs were present in comparable amounts in infected cells, the proportion of large polysomes involved in translating the DNA polymerase transcripts did not correspond to the levels of DNA polymerase mRNA. Nonetheless, for many viral genes the rate of polypeptide synthesis is proportional to the amount of functional mRNA in the cytoplasm (Inglis and Newton, 1981, and Marsden et al, 1983, and Smith and Sandri-Goldin, 1988). Infact, Smith and Sandri-Goldin (1988) have recently established this for glycoprotein B, glycoprotein C, and glycoprotein D, and the DNA-binding protein, ICP8, by showing that a close relationship exists between the levels of steady state mRNA, polysome associated RNA, and the rate of protein synthesis.

Finally, the herpes simplex viruses are known for their posttranscriptional control of both cellular and viral gene expression in infected cells. The posttranscriptional control of cellular mRNAs is effected through the virion host shutoff, or vhs component, which is responsible for the inbition of host protein synthesis (Fenwick and Walker, 1978, and Read and Frenklel, 1983) and the concomitant disaggregation of polyribosomes (Sydiskis and Roizman, 1967) and degradation of cellular messages (Fenwick and McMenamin, 1984, Schek and Bachenheimer, 1985, and Strom and Frenkel, 1987). In addition, viral alpha polypeptide synthesis is regulated at the posttranscriptional

level by beta/gamma gene products. Infected cells that were enucleated after the onset of beta/gamma polypeptides synthesis were still capable of down regulating alpha protein synthesis (Fenwick and Roizman, 1977, and Honess and Roizman, 1974). The isolation of virion host shutoff or, vhs mutants by Read and Frenkel (1983), provided proof of the phenomenon of inhibition of host protein synthesis by virion components which occurs in the absence of viral gene expression. Surprisingly, the vhs component was also found to be involved in the posttranscriptional regulation of alpha polypeptide synthesis. Read and Frenkel discovered that in contrast to the wild type virus, the vhs 1 mutant was also defective in the down regulation of alpha polypeptide synthesis. Thus structural virion components form another category of trans regulatory molecules that influence viral gene expression.

Metabolism of host macromolecules in HSV infected cells

Effects on protein synthesis

The infection of permissive cells with HSV-1 or HSV-2 leads to a rapid decline in host protein synthesis with a concommitant rise in the translation of viral polypeptides (Honess and Roizman, 1973, and Sydiskis and Roizman, 1966). The herpes simplex virus type 2 however, causes a more rapid and complete shutoff than that caused by HSV-1 (Fenwick et al, 1979, Hill et al, 1983, Pereira et al, 1977, and Powell and Courtney, 1975). Evidence to date indicates that cellular protein synthesis is inhibited through a multitude of steps. Virion associated shutoff of host protein synthesis has been extensively characterized by several investigators. Through various protocols,

Fenwick and Walker (1978) and Nishioka and Silverstein (1978), demonstrated that the initial inhibition of cellular protein synthesis indeed did not require viral gene expression and was mediated by virion components. Thus, virion host shutoff was evident in HSV infected enucleated cytoplasts. In addition, HSV infections performed in the absence of de novo protein synthesis (in presence of cycloheximide) or absence of transcription (in presence of Actinomycin-D) and those with UV-inactivated viruses still showed inhibition of cellular protein synthesis and disaggregation of host polyribosomes (Bastow et al, 1986, Kwong and Frenkel, 1987, Fenwick and Walker, 1978, Read and Frenkel, 1983, and Schek and Bachenheimer, 1985). However, viruses that had been inactivated by heating or neutralized with antibody were incapable of causing such an inhibition. Indeed, it was only after the isolation of the virion host shutoff, or vhs mutants (Read and Frenkel, 1983) that the complexity of HSV mediated host shutoff began to be revealed. The vhs mutants were defective in the virion structural component that is responsible for the early decline of host polypeptide synthesis in HSV-1 infected cells. However, when viral gene expression was permitted, the vhs mutants were able to inhibit host protein synthesis albeit not as completely as their wild-type counterparts. This later shutoff was termed secondary shutoff by Read and Frenkel (1983). Secondary shutoff becomes evident with the onset of HSV beta/gamma polypeptide synthesis.

In an effort to elucidate the mechanism involved in suppression of host polypeptide synthesis, Nishioka and Silverstein (1978) studied HSV-1 infection in murine erythroid cells transformed by Friend

leukemia virus. Under conditions of induction by dimethyl sulfoxide, the degradation of globin mRNA required viral protein synthesis; UV-inactivated virus could only disaggregate polysomes. On the other hand, data from Smiley and coworkers (1987) suggests that following infection with HSV-1, the Friend erythroleukemia cells may be similar to other permissive cells in the regulation of mRNA stability. The authors showed that when a rabbit β-globin gene was inserted into the viral genome, the accumulation kinetics of the resulting beta- globin mRNA were similar to those of the beta viral messages.

However, degradation of cellular mRNA was not dependent on viral gene expression, at least not in case of HSV-1 strain F or HSV-2 strain G infections in Vero or HeLa cells (Fenwick and McMenamin,1984, Strom and Frenkel, 1987, and Schek and Bachenheimer, 1985). It is likely that such differing results may be the result of different techniques. Nishioka and Silverstein (1978) had used liquid hybridization to determine the integrity of globin mRNA in HSV-1 infected cells. Since the probe detected a small portion of globin mRNA, it is quite possible that they may not have detected a few breaks. On the other hand, Northern blotting (Schek and Bachenheimer, 1985, and Strom and Frenkel, 1987), and in vitro translation (Fenwick and McMenamin, 1984) was used by other investigators to study the effects of vhs on mRNA stability. These techniques are sensitive enough to detect even the most minor breach in the integrity of mRNAs.

Effects on DNA and RNA synthesis

A striking feature of all HSV infections is the rapid shutoff of most of the host macromolecular synthesis (Batterson and Roizman, 1986,

and Fenwick, 1984). Experiments with UV-inactivated HSV-1 and HSV-2 have showed that host DNA synthesis can be suppressed in the absence of detectable viral protein synthesis (Newton, 1969, and Fenwick and Walker, 1978). It is conceivable that the massive suppression of cellular protein synthesis may indirectly affect synthesis of DNA in HSV infected cells. Massive inhibition of cellular protein synthesis may be a signal that the cell was in distress; this may activate mechanisms that lead to the inhibition of cellular DNA synthesis. It is also possible that short lived proteins may be required for DNA synthesis; in the absence of these proteins, cellular DNA synthesis may be affected. Infection with HSV-1 also leads to the inhibition of synthesis of small (4S) and large (45S) cellular RNA and also the processing of large molecules to form ribosomal RNA (rRNA) (Wagner and Roizman,1969). However, preexisting ribosomal RNAs in the cytoplasm are not degraded by HSV-1.

Characterization of vhs 1, the HSV-1 host shutoff mutant

Read and Frenkel (1983) isolated vhs mutants on the basis of their inability to inhibit host protein synthesis in the absence of viral gene expression. Viral polypeptide profiles of infections with all six independently derived vhs mutants showed that every one of them overproduced the alpha polypeptides. In addition, the expression of beta and gamma gene products was delayed. Under conditions where only alpha mRNAs are transcribed and then allowed to be translated in the absence of beta/gamma gene expression, Read and Frenkel (1983) found that the vhs 1 mutant was defective in the posttranscriptional regula-

tion of alpha mRNAs. Cells were infected in the presence of cycloheximide with either the wild-type or the vhs 1 mutant virus. After six hours of infection, cycloheximide was removed, Actinomycin-D added, and cells were monitored for the synthesis of alpha polypeptides in the presence of Actinomycin-D. Under these conditions, Read and Frenkel found that synthesis of alpha polypeptides was regulated only in cells infected with the wild-type virus but not the vhs 1 mutant. Three hours after the removal of cycloheximide and addition of Actinomycin-D, alpha polypeptides had ceased to incorporate label in wild-type infected cells; however, alpha polypeptides continued to incorporate ^{14}[C]-labeled amino acids for atleast six hours after the removal of cycloheximide in vhs 1 infected cells. This implied that structural virion components were responsible for the posttranscriptional control of alpha mRNA expression that was previously postulated to require the expression of beta/gamma viral genes (Fenwick and Roizman, 1977). Taken together, these reports suggest that those virion components that come in with the infecting virus (vhs component), as well as those vhs copies that are newly synthesized during the course of the infection, can each regulate the synthesis of alpha polypeptides in HSV-1 infected cells. Subsequent characterization undertaken as part of this dissertation revealed that the stability of alpha mRNAs was increased in vhs 1 mutant infected cells, whether assayed by in vitro translation or Northern blotting (Oroskar and Read, 1987).

Effect of the vhs 1 mutation on host and alpha mRNA stability was also studied by Strom and Frenkel (1987), and Kwong and Frenkel

(1987). In comparison with wild-type infected cells, in presence of Actinomycin-D, vhs 1 infected cells failed to show any evidence of degradation of host mRNAs such as beta actin, alpha tubulin and heat shock protein 70. Under conditions of cycloheximide reversal , alpha mRNAs for Infected Cell Polypeptide 4, or ICP4, ICP22/47 also showed increased stability as studied by Northern blots (Kwong and Frenkel, 1987). Furthermore, steady state levels of the beta/gamma mRNAs for glycoprotein E and US10 were found to be significantly increased between 5-14 hr post infection in vhs 1 infected cells. Synthesis of the gamma protein ICP15 was also delayed in vhs 1 infected cells (Strom and Frenkel, 1987).

Kwong, Kruper and Frenkel (1988) recently mapped the vhs 1 mutation, that affects the virion shutoff of host protein synthesis,to a 265-base pair NruI-XmaIII fragment spanning map coordinates 0.604-0.606 of the HSV-1 genome. Based on the sequence of the viral genome, this mapped the vhs 1 mutation to an open reading frame capable of encoding a protein with a predicted molecular mass of 56 kilodaltons (McGeoch et al., 1988). The vhs gene was mapped through marker rescue, a procedure where specific fragments from the wild type virus were transfected along with the vhs 1 viral DNA and the resultant progeny virus was screened for wild-type vhs gene function. Kwong and her colleagues discovered that the resultant wild-type progeny virus recombinants obtained through marker rescue were not only proficient in the post-transcriptional regulation of host mRNAs but also of viral mRNAs belonging to all kinetic classes (Kwong et al., 1988). The posttranscriptional regulation of alpha mRNAs was studied by determining alpha

polypeptide synthesis in cells that were first infected for 7 hr in the presence of cycloheximide. At that time cycloheximide was removed, Actinomycin-D added and the incorporation of ^{35}[S]-methionine was determined at zero and 4 hr after the addition of Actinomycin-D. The posttranscriptional regulation of beta and gamma mRNAs was actually measured 11 hr after infection by studying the incorporation of ^{35}[S]-methionine at early (0 to 4 hr) and late (4 to 8 hr) times after the addition of Actinomycin-D. Through such studies, Kwong and colleagues (1988) found that the rates of synthesis of alpha, beta, and gamma proteins was downregulated only in those cells infected with wild-type recombinants obtained through marker rescue, but not in vhs 1 infected cells. In the case of beta, and gamma proteins, however it cannot be ruled out that late in HSV infections, the cellular translation apparatus is altered so as to allow preferential translation of beta, and gamma mRNAs, regardless of the posttranscriptional control by the vhs component. Nevertheless, the composite data does imply that control of mRNA stability, both host and viral, must play an important role in the overall scheme of HSV gene regulation.

Modulation of mRNA stability in eukaryotic cell systems

The cytoplasmic steady state level of an mRNA is determined by the rate of its biosynthesis and degradation. The decay rates of eukaryotic messages vary from mRNA to mRNA. Infact, it is becoming increasingly clear that several mRNAs in mammalian cells can quickly respond to altered physiological conditions through modulation of their stabilities (Marzluff and Pandey, 1988, Brock and Shapiro, 1983, Paek

and Axel, 1987, and Guyette et al, 1979). Thus decay rates of eukaryotic mRNAs may indeed play a very important role in their cytoplasmic accumulation profiles.

Examples of the regulation of mRNA stability have been documented in a variety of systems. Early during infection, mRNAs of the adenovirus early viral transcription units E1A and E1B have half lives of 6-10 minutes. Soon after adenovirus DNA replication begins their half lives show a tenfold increase (Wilson and Darnell, 1980). Babich and Nevins (1981) discovered that a 72 kd DNA binding protein was required for regulating the E1A and E1B mRNA half lives early in infection.

Responsiveness to hormones through increase in cytoplasmic stability is a hallmark of many important mRNAs. Estrogen increases the half life of vitellogenin mRNA from 16 hr to 3 weeks (Brock and Shapiro, 1983). Prolactin exposure leads to 100-fold increase in the levels of casein mRNA. This increase was acheived through a 2-4 fold increase in transcription rate and a 17-25 fold increase in the stability of casein mRNA (Guyette et al, 1979). Other examples include the case of human growth hormone mRNA, whose stability is enhanced by the glucocorticoid hormones (Paek and Axel, 1987).

Cell cycle regulation of histone gene expression is an important example of posttranscriptional control (Schummperli, 1986, and Marzluff and Pandey, 1988). The concentration of histone mRNAs varies 30-50 fold during the cell cycle (Heintz et al, 1983, and DeLisle et al, 1983), and the changes in mRNA concentrations occur very rapidly in parallel with changes in DNA synthesis. Such a significant change is acheived through a 3-5 fold increase in histone gene transcription as

cells progress from G0 or G1 to S phase and a 4-6 fold increase in histone mRNA half lives in the S phase (Heintz et al, 1983, and Sittman et al, 1983).

Posttranscriptional events such as those affecting mRNA turnover are increasingly recognized as important in the expression of transiently expressed genes. A destabilizing sequence present in the 3' untranslated region of the transferrin receptor mRNA allows iron dependent regulation to take place at a posttranscriptional level in the cytoplasm (Mullner and Kuhn, 1988). The regulation of human transferrin receptor was studied through measurement of transient expression assays that determined the transcription rate and cytoplasmic mRNA stability in mouse L cells. The studies involving human transferrin receptor cDNA deletion mutants revealed that two distinct domains in the 3' untranslated region were sufficient to confer iron-dependent regulation by modulating mRNA stability of the human transferrin receptor. A highly conserved AU sequence from the 3' untranslated region also mediates selective mRNA degradation of granulocyte monocyte-colony stimulating factor, GM-CSF (Shaw and Kamen, 1986), and the c-fos mRNA (Fort et al, 1987, and Miller et al, 1984). Previous studies have shown that this region contains a 67-nucleotide AU rich segment that acts to suppress the transforming activity of the c-fos gene (Meijlink et al, 1985). The authors however failed to determine the effects of deleting this 67 base pair sequence on c-fos mRNA stability. Shaw and Kamen (1986) postulated that the AU sequences probably constitute the recognition signals for an mRNA processing pathway. This pathway may specifically be designed for the mRNAs encoding

several lymphokines such as alpha, beta, and gamma Interferons, Interleukins -1, -2, and -3, and proto-oncogenes like the c-sis, c-myc, and c-myb.

Destabilizing 5' terminal sequences may also be important in regulating the steady state levels of c-myc mRNA (Piechazyk et al, 1985). Due to a 12;15 reciprocal chromosome translocation involving the c-myc gene in the J558L and MPC-11 plasmacytomas, the c-myc gene is transcribed as a truncated transcript which lacks most of the normal c-myc leader sequence. The normal c-myc promoter is lost due to the loss of the first exon during the process of chromosomal translocation. However a cryptic promoter within the first intron of the normal gene gives rise to the truncated c-myc transcript. However, both the normal c-myc and the truncated c-myc transcripts code for the same protein since the translational start codon is located within the second exon of the normal c-myc gene. Despite these differences in the transcripts, the authors found that transcription rate of the truncated c-myc mRNAs was the same as that of the normal c-myc in ABPC20 pre-B lymphoma; however, the half life of the truncated c-myc mRNAs was considerably increased (Piechazyk et al, 1985).

The stability of β tubulin mRNA has been comprehensively dissected through very elegant experiments by Cleveland's group. The synthesis of tubulin is shut off by colchicine which disassembles microtubules but its synthesis is enhanced by drugs like taxol which stabilize microtubules (BenZe'ev et al, 1979). Thus the rate of beta tubulin synthesis is tied to the concentration of unpolymerized β tubulin in the cytoplasm. Further analyses brought out that altered rates of

tubulin synthesis were achieved through autoregulation of tubulin mRNA stability (Cleveland and Havercroft, 1983, and Pittenger and Cleveland, 1985). It was further shown that exon 1 sequences of β tubulin mRNA confer autoregulation by modulating mRNA stability (Gay et al, 1987). A recent publication from Cleveland and his colleagues has taken us a step further in characterizing the mechanism of β tubulin mRNA autoregulation. The instability of tubulin mRNA depends on the recognition of the first four amino acids of β tubulin, Met-Arg-Glu-Ile, as they emerge from the ribosome, and not on the sequence or the structure of the mRNA as such (Yen et al, 1988). Alterations in the tubulin mRNA sequence permitted normal regulation as long as the N-terminal amino acid sequences remained the same. In addition, tubulin mRNA regulation also depended on whether the correct N-terminal peptide sequence was located at the amino terminus rather than being buried deep within the transcript. The model suggests that excess unpolymerized beta tubulin components could interact with this N terminal nascent peptide and cause the activation of ribonucleases associated with the translational apparatus.

Posttranscriptional regulation may well be a prevalent form of control of gene expression for many housekeeping genes. Carniero and Schibler (1984) determined that the transcription rates of genes specifying moderately abundant mRNAs were not significantly different from those specifying the rare mRNAs. Thus differential mRNA stabilities were responsible in modulating the steady state levels of these mRNAs.

Host shutoff by other viruses

Viral infection of eukaryotic cells frequently results in a dramatic inhibition of cellular protein synthesis. In many cases, this inhibition is often accompanied by extensive viral protein synthesis without the concommitant functional inactivation or degradation of cellular messages. Thus virus-induced host shutoff can be achieved through translational control as well as posttranscriptional control of message stability.

Translational competition between viral and cellular messages is utilized by many RNA viruses, a prime example of which is the Vesicular Stomatitis Virus (Lodish and Porter, 1980). After viral infection, the total amount of translatable mRNA increases threefold. Through sheer magnitude, the vesicular stomatis viral mRNA is translated at the cost of host messages, although both viral and cellular mRNAs initiate translation with similar efficiencies.

Following poliovirus infection, the inhibition of protein synthesis in HeLa cells correlates with the proteolysis of a 220 kd polypeptide (p220) associated with eukaryotic initiation factor 3 and a cap-binding protein complex (Etchison et al, 1982). Eukaryotic messages are generally capped at the 5' end; these capped messages are highly dependent on the intact cap-binding protein for their translation. In poliovirus infected cells where p220, the high molecular weight component of the eukaryotic initiation factor was inactivated, Bonneau and Soneneberg (1987) found that the translation of cellular mRNAs was reduced only to 30% of control levels. Thus other changes may be responsible for the complete inhibition of protein synthesis in

poliovirus infected cells.

Translation of poliovirus RNA occurrs differently from that of the classical eukaryotic and viral mRNAs. Poliovirus RNAs are not capped at the 5' ends; they are therefore independent of the cap-binding proteins for their translation. Pelletier and Sonenberg (1988) recently showed that translation initiation on poliovirus RNA in poliovirus infected cells occurred by internal binding of ribosomes to the 5' noncoding regions. In addition, they also found that the internal binding to the 5' noncoding region of poliovirus RNA can occur in HeLa cell extracts *in vitro*, in the absence of poliovirus proteins (Pelletier and Sonenberg, 1989).

Alterations in the intracellular ionic concentration favorable to the translation of virus rather than host mRNAs seems to be yet another mode of inhibiton of cellular translation. Apparently, hypertonic salt concentrations lead to a block in cellular translational initiation, without affecting mengovirus translation (Nuss et al, 1975). This belief is based on the fact that the highly efficient mengovirus RNA can complex with the initiation factor, eIF-2, under hypertonic salt conditions which destabilize even the most efficiently translated cellular messages (Rosen et al, 1982).

Finally, inhibition of host protein synthesis in influenza virus infected cells is accompanied by degradation of cellular mRNAs (Inglis, 1982).

It is obvious that eukaryotic viruses utilize every possible point of attack to achieve the goal of selective viral translation at the expense of cellular translation. Whether this goal is achieved

through translational control or posttranscriptional control of cellular mRNAs will most likely depend on several things. Most importantly, these will include intrinsically superior viral capabilities, as is the case with mengovirus RNA; and also, whether the particular mode of cellular mRNA degradation has important implications in the regulation of viral gene expression, as in the case of the herpes simplex virus.

Postulated mode of mRNA decay in prokaryotic and eukaryotic system

The half life of prokaryotic mRNAs is very short indeeed. On an average, bacterial mRNAs decay with a half life of 2-3 minutes. In comparison with the studies on eukaryotic mRNA decay, a great deal is known about the factors that determine prokaryotic mRNA stability (Brawerman, 1987).

No obvious relationship exists between the size of the message and its decay rate. Bellasco and colleagues (1986) have concluded these results from the β-lactamase constructs which, although differing in length by 50%, had similar decay rates. Also, in comparison to the nonpolyribosomal mRNA fraction , the polyribosome bound mRNA was found to be no more protected from mRNA decay. Stanssens and his colleagues (1986) found that although the modified lacZ transcripts differed in their ability to initiate translation, they were both equally susceptible to mRNA degaradation. The studies involved comparison of the stability of lacZ transcripts that were so constructed as to have a set of different ribosome binding sites at their 5' ends. The results showed that the transcripts differed in their ability to initiate

translation. In addition, the investigators found that although the number of ribosomes covering the coding region of lacZ transcripts varied with its ability to initiate translation, there were no differences in the lacZ decay rates.

However, structures at the 3' ends were found to influence the rate of mRNA decay. Stem-loop forming terminator sequences of Bacillus thuringensis mRNA coding for the crystal protein is known to positively regulate its expression. Transfer of this sequence to other genes also conferred stability to the resultant transcripts (Wong and Chang, 1986).

In short, very limited information is available on the cellular machinery and the probable mode of mRNA decay in prokaryotes. Donovan and Kushner's data (1986) suggest that bacterial mRNA is normally degraded by two 3' exonucleases, RNAase II and polynucleotide phosphorylase. Absence of these exonucleases led to accumulation of several mRNAs. Endonucleases recognizing specific sequence could trigger the rate limiting step which would be followed by 3' exonucleolytic attack. RNAase III recognizes the sib site next to the int gene of the bacteriophage lambda. Destruction of the sib site by RNAase III leaves the transcript with an exposed 3'terminus and leads to its rapid decay (Guarneros et al., 1982, and, Schmeissner et al., 1984). Thus, terminal structures at the 3' end are important barriers to the physical decay of mRNA since, removal of this terminus results in the loss of the integrity of the mRNA.

The study of mRNA decay mechanisms in eukaryotic systems is still in its infancy. One of the significant problems is that degradation

intermediates are rarely seen. The situation is probably similar to the prokaryotic systems where the first endonucleolytic attack is rapidly followed by exonucleolytic attack leading to rapid mRNA decay. Under normal circumstances special structures at the 3' terminus protect mRNA chains against exonucleolytic attacks. Darnell's group first proposed that poly(A) tails may have a role in protecting mRNA against degradation (Sheiness et al., 1975). To date, most of the published work has supported this idea. Jeffrey Ross and his colleagues have identified a 3' to 5' exonuclease that degrades histone H4 mRNA (Ross et al., 1987) in a cell-free mRNA decay system (Ross and Kobs, 1986). The enzyme is loosely associated with polysomes, does not require ATP or GTP and it functions efficiently at low monovalent ion concentrations. In characterizing its substrate specificity, Peltz and colleagues (1987) found this exonuclease degraded a host of different RNAs that lacked the poly(A) tail. Polyadenylated histone mRNA was degraded more slowly than unmodified histone mRNA. Jeffrey Ross and colleagues have further shown that histone H4 mRNA degradation also occurs in the 3' to 5' direction in vivo (Ross et al, 1986). These conclusions were based on S1 nuclease protection assays; S1 nuclease does not degrade the mRNA sequences which are hybridized to its complementary DNA. Histone H4 mRNA lacks a poly(A) tail but contains conserved sequences at its 3' end capable of forming stem-loop structures, providing it partial but regulated protection (Marzluff and Pandey, 1988). In fact polyadenylated histone mRNA is more stable than unmodified histone mRNA in oocytes (Woodland and Wilt, 1980) and, in mammalian cells (Bird et al, 1985, and Luscher et al, 1985).

Wilson and Triesman (1988) recently showed a close correlation between removal of poly(A) sequences and degradation of c-fos mRNA. Serum stimulation of susceptible cells leads to a 50-fold increase in the transcription rate of c-fos mRNA (Greenberg and Ziff, 1984). This c-fos mRNA is exceedingly unstable and is rapidly degraded (Greenberg and Ziff, 1984). Studies on the process of c-fos mRNA degradation showed that c-fos mRNA poly(A) tail is rapidly removed in a translation dependent manner, leading to the accumulation of deadenylated c-fos mRNA (Wilson and Triesman, 1988). These conclusions were made through studies on the non-denaturing gel electrophoresis of c-fos mRNA-3' complementary nuclease T1 resistant hybrids. Under conditions of non-denaturing gel electrophoresis, the migration of RNA hybrids is dependent on poly(A) tail lengths; heterogeneity in poly(A) length will cause migration as a smear rather than a sharp band. A precursor product relationship between polyadenylated and deadenylated c-fos mRNA was established by studying poly(A+) c-fos mRNA under non-denaturing gel electrophoresis; and, the integrity of the corresponding c-fos mRNA was studied under denaturing gel electrophoresis. The results showed that c-fos mRNA was stable until the poly(A) tail was substantially removed; after this, the c-fos mRNA was degraded. Finally, transient expression assays revealed that deletion or replacement of the 3' AU-rich sequences dramatically slowed down the poly(A) shortening rate and also stabilized the c-fos mRNA. Thus, the destabilization of both c-fos (Wilson and Triesman, 1988) and c-myc (Brewer and Ross, 1988) mRNAs is apparently dependent on the 3' AU-rich sequences which act to destabilize the mRNA by directing the rapid removal of the mRNA poly(A)

tract.

It is conceivable then, that vhs mediated decay of cellular and viral messages might well involve a process that leads to the initial step which causes the progressive loss of their poly(A) tails. Messages with no intact 3'ends would then be good substrates for the exonuclease that degrades poly(A) lacking eukaryotic messages.

Proposed studies in the dissertation

At the outset of this study, two facts were known about the vhs 1 phenotype. First, posttranscriptional regulation of host and alpha mRNAs was defective in vhs 1 infected cells (Read and Frenkel, 1983). In addition, Fenwick and McMenamin (1984), had shown that HSV-1 and HSV-2 infection led to structural changes in the cellular mRNA that made them untranslatable in vitro. Second, in Vero cells, posttranscriptional control of host mRNAs was accomplished in part,through the degradation of cellular messages, and the vhs 1 was defective in this function (Schek and Bachenheimer, 1985). These findings had raised the possibility that the wild-type vhs gene product was involved in controlling the stability of both host and alpha messages. Our initial studies were thus designed to determine whether the wild-type vhs gene product affected the functional and physical half lives of alpha messages.

Cells were infected with either the wild-type or vhs 1 mutant virus in the presence of cycloheximide. Five hours post infection, cycloheximide was removed and the infection was allowed to continue in presence of Actinomycin-D. Thus, alpha mRNAs made in the presence of

cycloheximide could be translated in the absence of beta or gamma viral gene expression. Analysis of total cytoplasmic mRNA immediately after cycloheximide reversal from wild-type and vhs 1 infections, indicated that alpha mRNAs were both physically intact as well as translatable in vitro. However, five to six hours after reversal, alpha mRNAs from only vhs 1 but not wild-type infections were translatable in vitro and physically intact. The results provided the first clear evidence that the wild-type vhs gene product regulated the physical and functional stabilities of alpha mRNAs and that the vhs 1 mutant was defective in this function (Oroskar and Read, 1987).

Our subsequent plan was formulated to determine the requirements for vhs-mediated decay of alpha mRNAs in infected cells. The question we pursued was whether the contribution of alpha polypeptides was important in the vhs mediated decay of alpha mRNAs. Cells infected in the presence of cycloheximide with the vhs 1 mutant were superinfected at five hours with either the wild type or the vhs 1 virions in the presence of Actinomycin-D. Cycloheximide was either removed or not removed at the time of superinfection. Presence of cycloheximide would inhibit the synthesis of alpha polypeptides. On the other hand absence of the cycloheximide inhibitor would allow the synthesis of alpha polypeptides. In addition, Actinomycin-D would inhibit all subsequent viral and cellular transcription. Finally, through the superinfection protocol, we could effectively test the effect of virion components brought along with the superinfecting virus. Five hours after superinfection, alpha mRNAs were found to be degraded under the influence of wild type vhs components only from the group where cycloheximide was

removed, but not from the group containing cycloheximide. The vhs 1 superinfecting virions were unable to affect the stability of alpha mRNAs both in the absence or the presence of alpha polypeptides. These results raise the possibility that alpha polypeptides play a role in vhs-mediated mRNA decay of alpha mRNAs. However, other explanations are also possible and will be discussed in subsequent chapters.

We next planned extensive experiments which would allow us to evaluate the role of the vhs gene product in regulating the half lives of viral mRNAs belonging to different kinetic classes, and to assess the importance of control of mRNA stability in the overall scheme of gene regulation in HSV-1 infected cells. These experiments were meant to determine whether the vhs modulation of viral mRNA half lives showed any target selectivity. To this end, the accumulation kinetics and half lives of ten different viral mRNAs were determined in cells infected with either the wild-type or vhs 1 mutant virus. The accumulation patterns of viral mRNAs were studied through Northern blots of total cytoplasmic RNA extracted at 2 hr intervals between 2 and 16 h post infection in the absence of drugs. The half lives of viral mRNAs were studied by two different protocols. Viral mRNA stability was determined under de novo inhibition of transcription through the addition of Actinomycin-D, and secondly, through pulse-chase. The first method involved the measurement of viral mRNA decay in the presence of Actinomycin-D starting at 6 h post infection. Total cytoplasmic RNA was extracted at 45 minute intervals upto 3 h after the addition of Actinomycin-D. The levels of viral messages were then determined through Northern blotting and hybridization. The second

method was chosen so as to determine the half lives of viral mRNA from wild-type infected cells in the absence of any drugs. Cells were infected with wild-type virus for 5.5 h and then pulsed for 30 min with ^{3}[H]-uridine. The radioactive label was then removed and the cells incubated for various intervals in medium containing an excess of unlabeled cytosine and uridine. Total cytoplasmic RNAs were prepared at various times and the radioactivity specific for each mRNA was determined through filter hybridization. The results indicate that the vhs gene product significantly affects the stabilities of viral mRNAs belonging to all kinetic classes. The lack of such a control in vhs 1 infected cells significantly affects the steady state levels and kinetics of accumulation of almost all classes of viral mRNA. The half life measurements show that in wild-type infections, the half lives are similar for viral mRNAs belonging to all kinetic classes. On the other hand, the vhs 1 mutation caused a significant increase in the half lives of all viral messages.

Our present findings reveal that the vhs protein induces destabilization of many, if not all, viral mRNAs in HSV-1 infected cells. This suggests that the mechanism of vhs-mediated mRNA decay apparently lacks target mRNA selectivity. Our studies have identified a unique system functioning in eukaryotic mRNA decay. Its easy accessibility to researchers makes it a very attractive system in studies on the factors that govern the life expectancy of eukaryotic messages.

MATERIALS AND METHODS

Cells

Vero and HeLa S3 cells were obtained from the American Type Culture Collection. Both Vero and HeLa cells were maintained in Minimum essential medium (MEM) (GIBCO, Grand Island, NY) containing 10% calf serum (CS) and a mixture of antibiotic and antimycotic solution consisting of Penicillin at 100 units/ml, Streptomycin at 100 μg/ml, and Amphotericin B at 0.25 μg/ml. These cells were passaged in 75 cm^2 polystyrene flasks from Corning, by splitting them at a 1:5 dilution twice a week. Cell stocks were frozen in medium containing 80% MEM, 10% glycerol (Sigma) and 10% calf serum. Unless noted otherwise, all tissue culture plasticware was obtained from Corning.

Virus

The herpes simplex virus type 1 (HSV-1) KOS strain, originally isolated by K.O.Smith, Baylor University, Houston, Texas, was obtained from Bernard Roizman, University of Chicago, Chicago, Illinois. vhs-1, the HSV-1 mutant virus, was originally isolated by Read and Frenkel (1983).

Preparation of virus stocks

Wild-type and mutant viruses were plaque purified and small sto-

cks were prepared and stored frozen as mother stocks. To prepare working stocks of virus for use in experiments, aliquots from the mother stocks were used to infect 75 cm^2 flasks of confluent Vero cells at a multiplicity of infection (MOI) of 0.01 to 0.05 plaque forming units (pfu)/cell. Virus was harvested after complete CPE was evident. The infected cells were brought into suspension by vigorous shaking of the flasks. Infected cells were then centrifuged and resuspended in a very small volume of MEM containing 10% calf serum. HSV-1 was released from cells by disrupting them with three cycles of freeze thawing. The broken cells were centrifuged and both the pellet and supernatant fractions were stored frozen in MEM + 10% calf serum at -90°C until further use.

Bacterial strains and bacteriological media

DH-1, a derivative of Escherichia coli K-12, transformed with pSG28 and pSG87 (Goldin et al, 1981), and, HB101, transformed with pXlr11 (Dawid and Wellauer, 1976), were propagated in Luria Bertani (L Broth) medium. Both of these strains were kept frozen at -90°C in L Broth containing 15% glycerol (Sigma). Whenever needed, ampicillin selection was used at 40 µg/ml of L Broth (Maniatis et al., 1982). The JM107 bacterial strain is a derivative of Escherichia coli strain K. It is proficient in the modification of but deficient in the restriction of E. coli DNA of the "K" type (r_K^-, m_K^+). It has a chromosomal deletion of lac Z and pro A , B genes but carries an F episome with pro A,B,lacI^{q}ZΔ M15 (Yanisch-Perron et al.,1985). JM107 was obtained from Bethesda Research Laboratories (BRL). JM107 strains were propagated on

M9 minimal medium (Maniatis et al,1982).

Whenever needed, Bacto agar was used at a concentration of 1.5% and top agar at 0.8% in L Broth (Maniatis et al., 1982).

Plasmids

pSG28 and pSG87 were the kind gifts of Myron Levine, Univ. of Michigan, Ann Arbor, Michigan. Each of these plasmids contain EcoR1 fragments of HSV-1, KOS strain, cloned into the pBR325 vector (Goldin et al., 1981). pSG28 contained the EcoR1 EK fragment which spanned map units 71.6 through 85.5. The cloned EK fragment contains genomic segments coding for ICP-4, ICP-0 and ICP-27 (Anderson et al., 1980, Clements et al., 1979, Watson et al., 1979, and Watson et al., 1981). pSG87 contained the EcoR1 N fragment and was used to detect the thymidine kinase and the glycoprotein H mRNAs (McKnight et al., 1980). pXlr11 has a 4.6 kb Xenopus laevis rDNA fragment cloned into the EcoR1 site of Colicin E1. The cloned fragment contains most of the 28S rRNA coding region and a small portion of the 18S rRNA region (Dawid and Wellauer, 1976). pXlr11 was kindly provided by Jeff Doering, Department of Biology, Loyola University of Chicago, Chicago, Illinois.

HSV-1 intragenic sequences in M13 phages

Most of the HSV-1 gene probes utilized were recombinant M13 phages containing inserts of HSV-1 intragenic DNA sequences (Weinheimer and McKnight, 1987). These probes were the generous gifts of Drs. Steven Weinheimer and Steven Mcknight (Carnegie Institution of Washington, Department of Embryology, Baltimore, Maryland. The recombinant

phages contained the following HSV-1 DNA inserts:a 1.6 kb Sst-BamH1 fragment for the alpha ICP0 gene (Mackem and Roizman, 1982b) , a 0.6 kb BglII-EcoR1 fragment for the DNA polymerase gene (Gibbs et al,1985), a 1.1 kb BglII-HindIII fragment for the thymidine kinase gene (McKnight, 1980), a 1.6 kb SalI-BamH1 fragment for ICP8, the DNA-binding protein (Holland et al., 1984) , a 0.6 kb BamH1-HindIII fragment for the major capsid protein ICP5, (Frink et al., 1981), a 0.6 kb Pst1-SalI fragment for the glycoprotein B (Holland et al., 1984), a 0.6 kb BamH1-EcoR1 fragment for the glycoproyein H gene (McKnight, 1980), 6 kb EcoR1-BamH1 fragment for the glycoprotein C (Frink et al., 1981). For a negative control for nonspecific hybridization we used a 1.5 kb EcoR1-BglII fragment derived from the chicken thymidine kinase gene (Merrill et al., 1984). All the above probes were provided to us as single stranded (ss) phage DNA containing either the antisense (template) or the sense (nontemplate) DNA strands.

Transformation of JM107 with single stranded M13 recombinant phage DNA

We transformed single stranded phage DNA into the JM107 bacterial strain according to the procedures described in Basic Methods in Molecular biology (Davis et al., 1986). Frozen JM107 cells were first streaked out on M9 minimal agar plates and the plates were incubated overnight at 37°C. To prepare competent JM107 cells, several colonies of JM107 cells from confluent M9 plates were then used to inoculate 50 ml of L Broth in 250 ml flasks and incubated in a shaker incubator at 37°C. When the O.D. at 600 nm (as measured in Spectronic 20, Bausch and Laumb) reached 0.3, 3 ml of these cells were removed and saved at 4

°C. The remaining cells were spun at low speed for 10 min at room temperature in the IEC HN SII table top centrifuge. The LB supernatant was discarded and the cell pellet was then gently resuspended in 3 ml of 50 mM $CaCl_2$. This volume was then brought up to 20 ml with additional 50 mM $CaCl_2$. The cells were treated in 50 mM $CaCl_2$ for 20 minutes on ice. The cells were next pelleted at 2500 RPM for 10 min at 4°C and resuspended in 2.5 ml of ice cold 50 mM $CaCl_2$. These cells designated as competent cells, were held on ice until the addition of transforming DNA.

Transformation of competent JM107 cells was carried out as follows. Aliquots containing 150 μl of competent cells were dispensed into several sterile microfuge tubes. Different concentrations ranging from nanogram to microgram quantities of single stranded M13 phage DNA were added to each of the tubes containing competent cells and incubated for 40 min on ice. During this incubation, melted L Broth top agar was cooled to 50°C and 100 μl of 10% X-Gal in dimethylformamide and 100 μl of 0.1M IPTG was added for every 30 ml of top agar. About 3 ml of this top agar mixture was aliquoted into glass tubes and maintained at 50°C. At the end of 40 min incubation on ice, the transformed cells were heat shocked at 42°C for 2 minutes. To each transformation tube was added 0.1 ml of exponentially growing competent cells which had been saved on ice from the earlier step. The contents of each of the transformation tubes was then transferred to top agar tubes held at 50°C. Transformed cells in the top agar mix were immediately poured onto 90 mm L Broth agar plates. The plates were swirled so as to have an even layer of top agar. After the top agar had hardened, the plates

were incubated overnight at 37°C. White plaques containing the M13 phages with the insert were picked within the next day and tested for presence of the proper insert.

For use in the pulse chase experiment, ICP0 and TK inserts from the recombinant M13 phages were recloned into the appropriate sites of the polylinker of the plasmid Bluescript SK (Stratagene, La Jolla, California).

Rapid small scale isolation of M13 replicative form (RF) phage DNA

M13 is a Escherichia coli male specific bacteriophage which has a single stranded circular DNA packaged in the virus particle. The life cycle of M13 phage however goes through the production of a double stranded replicative viral DNA intermediate called the replicative form (RF). The RFs are not packaged but are used as templates for production of single stranded plus strand DNA which is packaged into mature phage. M13 phage particles are extruded nonlytically into the medium and the RFs are retained into the host cell (M13 Cloning/Dideoxy Sequencing Instruction Manual, BRL).

Preparation of phage stocks from recombinant plaques

Colorless plaques containing M13 DNA with the insert, were picked from transformation plates. Agar plugs containing the plaque and parts of the JM107 bacterial lawn was picked up with a pasteur pipette and used to inoculate 10 ml of L Broth. The L Broth tubes were then incubated at 37°C overnight on a shaker incubator (M13 Cloning Manual, BRL).

Preparation of M13 RF DNA

Ten milliliter of phage stock was centrifuged at approximately 2500 RPM for 10 min at 4°C and the supernatant containing the recombinant phages was stored at 4°C as phage stock. The bacterial cell pellet was then transferred to eppendorf tubes. The replicative form of M13 DNA was extracted by the Alkaline lysis method (Maniatis et al., 1982). This method is generally used for rapid isolation of plasmid DNA from recombinant clones. The cells were lysed at room temperature for 5 min in 100 μl ice cold solution containing 50 mM glucose, 10 mM EDTA pH 7.9, 25 mM Tris.Cl pH 7.9 and 4 mg/ml lysozyme. To each tube of cellular lysate was added, 200 μl of a mixture of 0.2N NaOH and 1% SDS. The cell lysate was incubated on ice for 5 minutes. Finally, 150 μl of an ice cold solution of potassium acetate (from a stock solution made up of 60 ml of 5M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml water) was added to the cellular lysate. The tube was gently vortexed and stored on ice for 5 min. The microfuge tubes were then centrifuged for 5 min at 4°C in an eppendorf microfuge. The clear supernatant containing the double stranded RF DNA was next extracted with an equal volume of phenol-chloroform. Two volumes of ethanol were subsequently added to the aqueous phase. The mixture was vortexed and DNA was allowed to precipitate for 10 min at room temperature. The DNA pellet was then rinsed with 70% ethanol and dried briefly in a vacuum desiccator. Each DNA pellet was dissolved in 30 μl TE (10 mM Tris - 1 mM EDTA pH 7.9) and stored at -20°C until further use.

Large scale isolation of plasmid DNA

Plasmid DNA was prepared on a large scale according to procedures described in the Cold Spring Harbor Cloning manual (Maniatis et al, 1982). Essentially, the bacteria harboring the plasmids were grown stepwise in increasing volumes of L Broth and the plasmids were finally amplified in the L Broth. When appropriate, every step contained ampicillin selection at 40 μg/ml of L Broth. Specifically, a 10 ml overnight culture was obtained by inoculating scrapings from a frozen stock of the desired bacterial strain into L Broth. About 0.5 ml of this culture was used to inoculate a 250 ml flask containing 50 ml L Broth and incubated on a shaker incubator at 37°C until the O.D. 600 nm was about 0.6. Twentyfive milliliter of this late log culture was then inoculated into a 2 liter flask containing 500 ml L Broth prewarmed to 37°C. The bacterial cultures were allowed to multiply in a shaker incubator at 37°C until the O.D. at 600 nm reached 0.4. The plasmids were then amplified overnight at 37°C, in the presence of 170 μg/ml of chloramphenicol.

Harvesting and lysis of bacteria

Bacterial cells were harvested in the Sorvall RC-5 centrifuge at low speed for 10 min at 4 °C. The cells were then washed in ice cold STE (0.1 M NaCl, 10 mM Tris.Cl pH 7.9 and 1 mM EDTA pH 7.9) in 50 ml culture tubes (Corning) in the IEC-HNII table top centrifuge. Washed cells were then lysed by the Alkali lysis procedure of Birnboim and Doly (1979), as published in Maniatis et al (1982). Cells from a liter of culture were lysed in 10 ml of a solution containing 50 mM glucose, 25 mM Tris.Cl pH 8.0, 10 mM EDTA pH 7.9 and 5 mg/ml lysozyme. The

cells were transferred to Beckman 60Ti centrifuge tubes and allowed to lyse for 5 min at room temperature. To this cell lysate, 20 ml of solution II containing 0.2 N NaOH and 1% SDS was added and allowed to stand on ice for 10 minutes. Finally, 15 ml of an ice cold solution of 3 M potassium and 5 M acetate was added to the above tube. The contents of the tube were thoroughly mixed by inverting the tube and then incubated on ice for 10 minutes. Cell debris along with most of the chromosomal DNA was separated out by centrifugation in the Beckman 60Ti fixed angle rotor at 30,000 RPM for 30 min at 4°C in a Beckman ultracentrifuge. The resulting supernatant was transferred to 30ml Corex tubes and plasmid DNA was precipitated by addition of 0.6 volumes of isopropanol. The isopropanol was completely mixed with the supernatant and the tubes were allowed to stand for 15 to 20 min at room temperature. The DNA was pelleted by spinning at 10,000 RPM in an SS34 rotor for 30 min at room temperature in a Sorvall RC-5 centrifuge. The DNA pellet was briefly dried in a vacuum desiccator. Plasmid DNA was dissolved in TE and then purified by centrifugation to equilibrium in cesium chloride-ethidium bromide (CsCl-EtBr) gradients.

Cesium chloride-ethidium bromide gradients

Plasmid DNA extracted from one liter of bacterial culture was generally centrifuged in CsCl-EtBr gradients in the Beckman VTi50 rotor in the Beckman ultracentrifuge. One gram of CsCl (U.S.Biochemicals) was added for every ml of DNA solution and 0.8 ml of 10 mg/ml EtBr solution was added for every 10 ml of the DNA-CsCl solution. The refractive index of the final DNA solution was adjusted to about 1.3860. The DNA was centrifuged at 41,000 RPM for 24-36 hours at 20°C.

The lower plasmid DNA band was aspirated with a needle and syringe inserted into the side of the tube. Finally, the Ethidium bromide was removed by extraction with 2-butanol (Eastman Kodak) until the aqueous phase was devoid of the pink color.

Concentration of DNA

DNA was first dialysed against several changes of TE at 4°C. Plasmid DNA was precipitated from the aqueous phase by addition of 0.3 M sodium acetate pH 5.2 and 2.5 volumes of ethanol at -20°C overnight.

Large scale isolation of recombinant M13 phage RF DNA

M13 phage RF DNA was prepared on a large scale according to the procedures described in Basic Methods in Molecular Biology (Davis et al., 1986). The initial steps involved preparation of the phage stock and late log culture of JM107 cells. One hundred ml of LB in 500 ml flasks was inoculated with several JM107 colonies propagated on M9 minimal agar plates. The bacteria were incubated in a shaker incubator at 37°C until the O.D. at 600 nm reached 0.8-1.0. One hundred ml of L Broth in 500 ml flask was then seeded with one ml of this late log JM107 cells. These cells were then inoculated with 3 ml of the desired recombinant phage stock that was previously stored at 4°C. These flasks were incubated in a shaker incubator overnight at 37°C to give a phage stock inoculum. The rest of the late log JM107 cells were stored at 4°C overnight. On the next day, 900 ml of prewarmed L Broth in 2 liter flask was inoculated with the late log JM107 cells stored at 4°C. The 2 liter flask was then transferred to to a 37°C shaker incubator until the O.D. at 600 nm reached about 0.5. These cells were

then inoculated with 100 ml of the overnight grown phage stock. Phage infection was accomplished by shaking at 37°C for 15 min. At this juncture, 0.5 ml of 30 mg/ml chloramphenicol solution in ethanol was added to the 2 liter flask. Flasks were incubated at 37°C on the shaker for about 2 hours to allow accumulation of RF DNA. Recombinant M13 phage RF DNA was then extracted according to the Alkali lysis procedure of Birnboim and Doly (1979).

SDS-polyacrylamide gel electrophoresis

^{35}[S]-methionine labeled proteins were analysed by electrophoresis through sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE). Infected Vero cultures were generally maintained in MEM + 2% calf serum up to the time of labeling. Before labeling, the monolayer was washed with warm phosphate buffered saline (PBS) and labeled with 30 microcuries/ml ^{35}S- methionine (New England Nuclear) in MEM lacking methionine. At the end of labeling period, cells were washed twice with ice cold PBS, scraped with a rubber policeman and harvested into a microfuge tube in cold PBS. Cells were first pelleted and then lysed with about 100 to 200 μl of lysis buffer containing 50 mM Tris pH 7.0, 2% SDS, 5% 2-mercaptoethanol and 3% sucrose (Read and Frenkel, 1983). The cell extract was generally kept frozen at -90°C until electrophoresed. Prior to electrophoresis, the cellular extract was sonicated for 5 minutes and then placed into boiling water for 3 minutes. Equal aliquots were then analysed on SDS polyacrylamide gels. SDS polyacrylamide gel electrophoresis was performed according to the procedures of Morse et al, with the following exceptions. The separation gel was

made with 9.25% acrylamide (Sigma) crosslinked with N,N-diallyl-tartardiamide (DATD) (Biorad), and the stacker gel contained 3% acrylamide cross linked with N,N'-methylenebisacrylamide (BIS) (Biorad). The ratio of crosslinker to acrylamide was 1:37.5(wt/wt) in both the stacker and separation gels. Following an overnight run in a vertical gel apparatus (International Biotechnologies Inc., IBI, Model VCV) the gel was dried under vacuum on a Whatman #2 qualitative filter paper in the Hoeffer Scientific (Model SE 1150) gel drier. Finally, an X-ray sensitive film (Dupont, Cronex) was sandwiched between intensifying screens (Dupont, Cronex Hi Plus) and exposed on the dry gel for autoradiography. In situations where proteins from in vitro translation products were analysed, the gel was treated slightly differently at the end of electrophoresis. First, the gel was fixed with constant shaking, for an hour, in a solution containing methanol:water:glacial acetic acid at 5:5:1 and later treated with a liquid flour, Autoflour (National Diagnostics) for 2 hours. As before, the gel was dried and subjected to autoradiography.

Cytoplasmic RNA extraction

Monolayers of 1 to 2 X 10^7 Vero or HeLa cells were maintained in MEM with 1% calf serum until just prior to RNA extraction. At appropriate time points, cytoplasmic RNA was extracted according to the procedures of Berk and Sharp (1977). At the outset, the monolayer of cells was cooled on a bed of ice. Cells were then washed with 2 to 3 rinses of cold PBS and scraped into tubes. Cells were pelleted by centrifugation at low speed in the cold room. The cell pellet was

resuspended in 2.2 ml of resuspension buffer consisting of 10 mM Tris-.Cl pH 7.9, 0.15 M NaCl and 1.5 mM $MgCl_2$. One hundred and fifteen microliters of NP-40 (Calbiochem) concentrate was added to the resuspended cells. The NP-40 concentrate was the resuspension buffer containing 13% NP-40. Cells were allowed to lyse by incubation for 8-10 minutes at 4°C. During this interval the cells were brought into suspension by manually flicking the tube 3 to 4 times. Nuclei were then pelleted by a quick spin at 2000 RPM at 4°C. The nuclear pellet was subsequently rinsed with 1.2 ml wash buffer consisting of the resuspension buffer with 0.65% NP-40. The combined supernatants were then centrifuged at 10,000 RPM for 10 minutes at 4°C in the Sorvall RC-5 cenrifuge. To the cytoplasmic extract, an equal volume of urea buffer was added and this mixture was extracted with an equal volume of (50-:50) phenol-chloroform by centrifugation at room temperature. Urea buffer was made up of 7 M Urea (IBI), 10 mM Tris.Cl pH 7.9, 0.35 M NaCl, 10 mM EDTA and 1% SDS. The organic phase from the first phenol-chloroform extraction was extracted once again with an equal volume of urea buffer and later extracted with chloroform. The combined aqueous phases were extracted for a second time with a mixture of phenol-chloroform. This aqueous phase was finally extracted with two rounds of chloroform. RNA was precipitated from the final aqueous phase by addition of 2.5 volumes of ethanol and incubation at -20°C overnight. This RNA pellet was precipitated one more time from Tris-EDTA (TE, 10 mM Tris.Cl pH 7.9 and 1 mM EDTA pH 7.9) by the addition of 0.3 M Sodium acetate pH 5.2 and 2.5 volumes of ethanol followed with incubation at -20°C overnight. The final RNA pellet was dissolved in a small volume

of TE and stored frozen at -90°C.

In vitro translation of total cytoplasmic RNA

In vitro translation was carried out in micrococcal nuclease treated rabbit reticulocyte lysates according to protocols provided by the manufacturer, Promega Biotec, Madison, Wisconsin. All components of the in vitro translation reaction were purchased from Promega Biotec. Translation reactions were performed in a total of volume of 25 microliters. Each reaction tube contained either 750 or 375 μg/ml of total cytoplasmic RNA, 2.5 units of RNAsin, 0.5 X 10^{-9} M of each amino acid except methionine, 50 μCi of ^{35}[S]-methionine and 17.5 μl of rabbit reticulocyte lysate. The in vitro translation was allowed to continue for two hours at 30°C. At the end of incubation, the reaction was stopped by transferring the tubes to -90°C freezer for 5 minutes. The untranslated RNA was subsequently inactivated and further translation inhibited by addition of 5 μl of a stock solution consisting of 600 μg/ml cycloheximide and 600 μg/ml RNase A. Finally, 30 microliters 2 X lysis buffer containing 100 mM Tris.Cl pH 7.0, 4% SDS, 10% 2-mercaptoethanol and 6% sucrose were added to the transaltion products. Samples were always placed for 3 minutes in boiling water prior to loading on the SDS-PAGE gels.

Northern blots of total cytoplasmic RNA

RNA electrophoresis was carried out after denaturation with glyoxal (Read and Summers, 1982). Each reaction mix of 28 microliter contained 5 to 10 micrograms of total cytoplasmic RNA, 1 M freshly deioni-

zed glyoxal, 50% dimethylsulfoxide in 10 mM $NaPO_4$ buffer, pH 6.8 and 0.36 mM EDTA pH 7.9. The RNA was denatured with glyoxal by incubation at 50°C for 60 minutes. At the end of the incubation period, 2 microliters of tracking dye consisting of a 1% bromophenol blue and 1% xylene cyanol mixture was added to each RNA reaction tube. The RNA was subsequently electrophoresed in 1.2% agarose. Both the gel and the buffer were made up of 10 mM $NaPO_4$ pH 6.8 and 1mM EDTA pH 7.9. Electrophoresis was carried out in a BRL horizontal gel apparatus (Model H4) at 115 volts/cm for a period of 4 to 5 hours. Throughout the period of electrophoresis, the buffer was constantly recirculated using a series pump (Manostat) and the buffer was also changed every hour. Generally, the gel was stored at 4°C, and electroblotted the next day onto Gene Screen Plus.

Electroblotting RNA onto Gene Screen Plus

The integrity of electrophoresed RNA was always checked before it was electroblotted on the nylon membrane. This was accomplished by determining the intactness of 28S and 18S rRNA bands from any one of the RNA samples; it was electrophoresed in a separate lane specifically for this purpose. This gel lane was cut out and soaked in 50 mM NaOH for 30 minutes to reverse the glyoxal denaturation. Ribosomal RNA was then stained in 0.5 μg/ml EtBr and photographed under ultraviolet light. The 28S and 18S rRNA bands were also used as size markers for each respective gel.

The RNA was electroblotted onto Gene Screen Plus (New England Nuclear, NEN) using the Trans-Blot Cell (Biorad). We followed the

procedure that NEN recommended for electroblotting RNA denatured with glyoxal. Essentially, a sheet of Gene Screen Plus, 4 sheets of Whatman 3MM chromatographic paper, each cut out to the size of the gel, and two Scotch Brite pads were soaked in a solution of 12 mM Tris - 6 mM sodium acetate - 0.3 mM EDTA, pH 7.5 for at least 25 minutes. The gel and the membrane were then sandwiched into the cassete holder as follows. The two wet Whatman 3MM filter papers were laid onto a wet Scotch Brite pad. The Gene Screen Plus membrane with side B facing up was then laid on the filter papers and the gel laid onto the membrane by carefully removing any air bubbles between the gel and the membrane. The remaining two sheets of wet 3MM Whatman paper were finally placed on the gel and the sandwich was completed by placing the second wet Scotch Brite pad on the top of the Whatman papers. The cassete holder containing the sandwich was inserted into the Trans-Blot cell which contained ice-cold 12 mM Tris - 6 mM sodium acetate - 0.3 mM EDTA pH 7.5. Electroblotting was carried out by initially transferring at 10 volts for the first hour and then at 40 volts for an additional three hours. The power source used was the Biorad model 250/2.5 power supply. During electroblotting, cold water was constantly circulated through a cooling coil within the Trans-Blot apparatus. At the end of blotting, the casette was diassembled and the membrane was soaked in 50 mM NaOH for 30 seconds to reverse gloxal. The membrane was next soaked for 30 sec in a solution containing 0.2 M Tris pH 7.5 and 1X SSC (0.15 M sodium chloride - 0.015 M sodium citrate). The membrane was air dried at room temperature prior to hybridization.

Preparation of probes

Isolation of DNA fragments

To prepare probe from recombinant pSG28, pSG87, and pXlr11 plasmids, the fragment of interest was first completely excised from the vector sequences. The DNA fragments were subsequently electrophoresed in low melting point agarose, and the DNA insert was eluted from the low melting point agarose (LMP) (Gafner et al., 1983). Completely cut pSG28 and pSG87 was electrophoresed into 1% LMP agarose (BRL) and the desired HSV-1 " EK" or the "N" fragment, or the 28S rRNA fragment was then cut in a manner such that about 400 μl gel slices were transferred into eppendorf tubes. An equal volume of phenol (equilibrated with TE) was added to each tube; the misxture was then melted at 65°C for 10 to 12 min. To ensure that the agar was completely melted, the eppendorf tubes were vortexed about 2 to 3 times during the course of melting the LMP agarose and the phenol mixture. The aqueous phase was then separated from the phenol phase by spinning the tubes at room temperature for 5 minutes. The phenol phase was next extracted with 50 μl TE pH 7.9 and the combined aqueous phases were extracted once more with phenol. Phenol was removed from the aqueous phase by extracting it twice with chloroform. The DNA was concentrated by addition of 0.3 M sodium acetate pH 5.2 and 2.5 volumes of ethanol and incubation overnight at -20°C. The DNA was pelleted by centrifugation at 4°C for 15 minutes. Finally, the DNA pellet was then dissolved in a small volume of TE and stored at -20°C.

A different protocol was used to prepare intragenic HSV-1 DNA fragments from M13 phage RF DNA and rDNA fragment from Colicin E1 pla-

smid. DNA fragments were completely excised from their respective vectors but were not separated away from the vectors. The completely digested DNA was simply extracted twice with phenol-chloroform and twice with chloroform. The DNA was precipitated at -20°C overnight by addition of 0.3M sodium acetate pH 5.2 and 2.5 volumes of ethanol.

Nick translation

DNA probes were prepared by nick translation. The nick translation kit was obtained from BRL which supplied a protocol necessary for carrying out the nick translation. Each reaction tube consisted of 5 μl of a 0.2 mM solution of deoxyribonucleoside triphosphates containing dATP, dGTP, and dTTP, 0.5 μg to 1 μg of DNA to be nick translated, 156 pmoles or 125 μCi of α-dCT^{32}P, and 5μl of a mixture of DNaseI and DNA polymerase I in a total volume of 50 μl . The labeling reaction was carried out at 15°C for 1.5 hours. Any further reaction was inhibited by addition of 5 μl of the EDTA solution.

Separation of labeled DNA probe from unincorporated radionucleotides

The radioactive probe was separated from unincorporated radionucleotides by exclusion chromatography through Sephadex G-50 beads (Maniatis et al., 1982). Sephadex G-50-80 (Sigma) was prepared through overnight equilibration in a large volume of sterile TE pH 7.9. The swollen sephadex beads were then stored in TE pH 7.9 at 4°C. Approximately 5 to 6ml Sephadex G-50 column was prepared in Dispocolumns bought from Biorad. This column was washed with at least three column volumes of TE pH 7.9. The entire contents of the nick translation reaction were next loaded onto the column followed by 100 μl washings of the reaction tube. A reservoir of about 1 to 2 ml TE pH 7.9 was then applied

to the column and approximately 20 fractions of 0.5 ml each were collected into eppendorf tubes. Using a hand held Geiger counter, exclusion of the probe into the void volume could be followed. The trailing peak consisted of unincorporated radionucleotides. Total counts per minute (CPM) incorporated into the probe fraction were generally determined by Cerenkov radiation. The DNA probe was stored at -70°C until further use.

Hybridization of RNA

Northern blots were hybridized according to the published procedures of Graham et al (1984). Blots were prehybridized for 24 hours at 42°C in a heat sealed bag containing about 25 ml buffer. Prehybridization buffer contained 50% formamide (Mallinkrodt), 10% dextran sulfate (Pharmacia), 10% Denhardt solution, 5 X SSPE (1 X SSPE is 180 mM NaCl, 10 mM $NaPO_4$ pH 6.8, 1 mM EDTA pH 7.9), 1% sodium lauryl sulfate and 250 µg/ml of sheared and denatured salmon sperm DNA. Hybridization was carried out for 17 to 24 hours at 42°C with a minimum of 5 X 10^6 CPM/ml of prehybridization buffer. The probe was first denatured by placing it in boiling water for 10 minutes. The denatured probe was then immediately mixed with the prehybridization buffer. Only after the probe was completely mixed into the buffer, was it spread onto the blot. At the end of hybridization, the blot was extensively washed as follows. The first step included two 45 minute washes with constant shaking at room temperature in a buffer containig 2 X SSPE - 0.4% SDS. This was followed by two 15 minute washes with 0.1 X SSPE at 60°C in a shaking water bath.

Moist blots were generally placed into freezer bags and exposed on X-ray sensitive X-(OMAT)AR films (Kodak) between intensifying screens for varying lengths of time. Autoradiograms were scanned in the Gilford Response spectrophotometer. Peak areas were automatically integrated by the spectrophotometer.

Stripping and reprobing blots

The hybridized probes were stripped from the Northern blots according to recommendations from New England Nuclear, manufacturers of Gene Screen Plus membranes. Blots were placed for 20 to 30 minutes in boiling buffer containing 10 mM Tris pH 7.9, 1 mM EDTA pH 7.9 and 0.1% SDS. Complete stripping of the probe was monitored by autoradiography. Two such boiling treatments were generally found to be sufficient for complete stripping of the hybridized probe.

Studies on the vhs mediated regulation of alpha mRNA stability

Vero cells plated 36 h in advance onto 100 mm Corning dishes were pretreated for 30 minutes with 50 μg/ml cycloheximide in MEM + 1%CS at 34°C. At the end of the pretreatment, cells were infected with 40 pfu/cell of either the wild-type virus or the vhs 1 mutant in MEM + 1% calf serum containing 50 μg/ml cycloheximide. After one hour of virus adsorption the inoculum was removed, and the cells were overlaid with medium containing cycloheximide. At 5 hours after infection, Actinomycin-D was added to every culture at a final concentration of 5 μg/ml of medium. After 30 minutes of exposure to Actinomycin-D, the cultures were first washed with prewarmed medium and then overlaid with MEM + 1% calf serum containing 5 μg of Actinomycin-D/ml. The infection was then

allowed to continue up to the time desired. This protocol is referred to as the cycloheximide reversal protocol.

For the studies on the contribution of alpha polypeptides to vhs mediated alpha mRNA decay, exactly the same procedure was followed except the primary infection was carried out only with the vhs 1 mutant at 30 pfu/cell. After five hours of infection in the presence of cycloheximide, the cycloheximide was removed, cultures were washed and divided into two sets. Each set was superinfected with either the mock lysate, or the wild type or vhs 1 mutant virus at 150 pfu/cell either in the presence or absence of cycloheximide in medium containing 5 μg/ml of Actinomycin-D. This protocol is referrred to as the super-infection protocol.

In both experiments, zero hour was considered as the time when cycloheximide was washed off and Actinomycin-D added. Total cytoplasmic RNA was then extracted thereafter at the times indicated. The total RNA was then processed for in vitro translation or Northern blotting.

Measurement of mRNA half-lives by the Actinomycin-D chase protocol

Vero cells plated 36 h in advance onto 100 mm Corning dishes were infected with either the wild-type virus or the vhs 1 mutant at 20 pfu/cell in MEM with 1% calf serum and incubated at 34°C. At the end of one hour of adsorption, the inoculum was removed and infection was continued for an additional 5 hours. At six hours post infection, Actinomycin-D was added to a concentration of 5 μg/ml of the culture medium and the infection was allowed to continue. Total cytoplasmic RNA

was extracted at 45 min intervals for a period of 3 h after the addition of Actinomycin-D. This RNA was analyzed by Northern blotting. In cells exposed to Actinomycin-D, the integrity of specific mRNAs was monitored by hybridization of the blots with specific probes. The amount of RNA in each lane was normalized to the 28S rRNA in the sample; these values were used to quantitate the mRNA for half-life determinations.

Measurement of mRNA half-lives using the pulse chase protocol

To determine the half-lives of alpha ICP0, and beta TK mRNAs, we used the ^{3}[H]-uridine pulse chase protocol of Pilder and coworkers (1986). However, a slight modification of the protocol gave us good results. HeLa cells plated 36 hours ahead of time onto 100 mm Corning dishes were infected with wild-type virus at 20 pfu/cell in MEM containing 1% calf serum and incubated at 34°C. After 5.5 h of infection infected cells were labeled for 30 min with 200 μCi of ^{3}[H]-uridine (NEN)/ml of medium. At the end of the label pulse, label was removed and cells were overlaid with chase medium containing 5 mM each of unlabeled cytidine and uridine and 35 mM glucosamine. Total cytoplasmic RNA was extracted at 30 min intervals in the first hour and at hourly intervals thereafter up to 6 hr after the start of chase. The label specific for ICP0 and TK mRNAs was analyzed by hybridization to specific probes fixed onto nitrocellulose filter disks. The cytoplasmic RNAs were first digested with RQ1 DNAase (RNase free, obtained from Promega Biotec), according to recommendations of the company. The RNA was then extracted with phenol-chloroform and precipitated with ethanol

before use. Plasmid DNA probes linearized with restriction enzyme were fixed in 15 μg aliquots onto 25 mm nitrocellulose filter disks (Schleicher and Schuell) after denaturation with sodium hydroxide (Ausubel et al., 1987). Labeled RNA from approximately 1.2 X 10^7 cells was hybridized to DNA containing filter disks exactly as described by Zhang and coworkers (1987). Briefly, the disks were prehybridized overnight at 68°C in 2 X SSC (1 X SSC is 0.15 M NaCl and 0.15 M sodium citrate) containing 5 X Denhardt's solution. The labeled RNA samples were then added to the prehybridization buffer, and hybridization was continued at 68°C for 48 hours. The filters were then washed twice for 30 min at room temperature with 2 X SET (1 X SET is 0.15 M NaCl, 20 mM Tris pH 7.8, 1 mM EDTA) containing 0.1% SDS, and twice for 30 min at 60°C with 0.1 X SET containing 0.1% SDS. Finally, the filters were digested with RNase A (Sigma) for 30 min at 37°C, and then washed twice more for 30 min each at 60°C with 0.1 X SET containing 0.1% SDS. The filters were finally air dried and counted using toluene based flour in a liquid scintillation counter.

Statistical analyses

Decay of viral mRNAs after Actinomycin-D addition was analyzed by Northern blotting and autoradiography. The autoradiograms were scanned at 500 nm in a Gilford spectrophotometer. The spectrophotometer integrates the absorbance at 500 nm into peak areas. The ratio of the peak area of a specific mRNA at time was then 't' divided by the peak area of that mRNA at time zero, after the addition of Actinomycin-D. The errors in loading equal amounts of cytoplasmic RNA on gels was

corrected by normalizing the RNA content with the rRNA content within each lane. Thus, fraction of mRNA decay at any time 't' was determined by the following formula:

(mRNA at time 't'/rRNA at time 't') / (mRNA at time 0/rRNA at time 0).

The ratios were then plotted as logarithmic plots based on the first order linear regression analysis on the SigmaPlot Version 3.1 program, written for the IBM PC by Jandel Scientific, Sausalito, California. From the slope and the Y-intercept of the regression line, the half life of a specific viral message was calculated with the following formula: $\log_{10}C = A1Xt + A0$, where, C, is the concentration of mRNA, A1, the slope, and A0, the Y-intercept of the line. If C_2 represents the mRNA concentration at time t_2, and C_1, the initial mRNA concentration at time t_1, then:

$\log_{10}C_2 = A1Xt_2 + A0$, and, $\log_{10}C_1 = A1Xt_1 + A0$.

If we define the half life, $t_{\frac{1}{2}}$, as the time when $C_2 = \frac{1}{2}C_1$, then subtracting, $\log_{10}C_1 - \log_{10}C_2 = A1Xt_1 - A1Xt_2$.

Substituting $C_1 = 2C_2$, we get, $\log_{10}2C_2 - \log_{10}C_2 = A1(t_1-t_2)$, or, $\log_{10} 2\ C_2/C_2 = -A1(t_2-t_1)$.

Thus, $\log_{10} 2 = -A1(t_{\frac{1}{2}})$, where $t_{\frac{1}{2}}$ is defined as the mRNA half life.

The mRNA half life from the SigmaPlot program was therefore calculated as $(-\log_{10} 2) / (A1)$.

Viral mRNA half-lives studied through the pulse chase protocol were calculated using the SigmaPlot 3.1 program written for the IBM PC, and the Expofit program (Elsevier Biosoft, Elsevier Science Publishers, Amsterdam) written for the Apple II E computer. The Expofit program determines the half-life by fitting the mRNA levels to an exponential

decay curve according to the equation $C = C_0\ e^{-kt}$, where, C_0, is the initial concentration, and C, the final concentration, at time 't', and k, the rate constant of the exponential decay plot. When 't' equals the mRNA half-life $t_{\frac{1}{2}}$, the final mRNA concentration $C = \frac{1}{2}C_0$, where C_0 is the initial concentration.

Substituting for C we get, $\frac{1}{2}C_0 = C_0e^{-kt}$.

Taking natural logarithms on both sides, we get, $\ln\frac{1}{2} = -kt\ln e$.

Since $\ln e = 1$, then, $-\ln 2 = -kt$, and, $t = (\ln 2)/k$.

The half-life of mRNA from the Expofit program was thus calculated by dividing ln2 with k,the rate constant of the exponential decay curve. The standard error of the rate constant (S.E.K.) was also determined from the Expofit program.

RESULTS

The virion host shutoff or vhs mutants were isolated on the basis of their inability to suppress host polypeptide synthesis following infection in the presence of Actinomycin-D (Read and Frenkel, 1983). Prelimnary characterization of the vhs mutants indicated that although the wild-type virus exhibited posttranscriptional regulation of alpha mRNA accumulation, the vhs 1 mutant virus was defective in this function. This lack of posttranscriptional control allowed alpha mRNA translation to continue at times when alpha polypeptide synthesis was inhibited in wild-type infected cells. Shortly thereafter, Fenwick and McMenamin (1984), reported that the suppression of host protein synthesis by the HSV-1 and HSV-2 virion functions involved a structural change in the host mRNAs which led to a loss of their in vitro translatabilities. Schek and Bachenheimer (1985) subsequently showed that cellular mRNAs for actin, β-tubulin, and histones H3, and H4 were degraded under conditions that inhibited de novo protein and de novo RNA synthesis in HSV-1 infected cells. This proved that, in the absence of viral gene expression, the vhs function induced degradation of cellular messages. The results of Fenwick and McMenamin (1984), and Schek and Bachenheimer (1985) therefore indicated that virion associated suppression of host protein synthesis was associated with a loss of functio-

nal and physical stability of host mRNAs rather than major alterations in the host translational apparatus.

The experiments described in this disseratation were designed to study whether vhs mediated posttranscriptional control of alpha mRNAs involved control at the level of alpha mRNA stability. Several factors led to this thinking.

1. The posttranscriptional control of both host and alpha mRNAs was apparently effected through the HSV-1 virion components (Read and Frenkel,1983). Further studies had shown that the posttranscriptional control of host mRNAs was achieved through the modulation of both the physical (Schek and Bachenheimer, 1985) and functional stabilities (Fenwick and McMenamin, 1984). It was possible then, that the HSV-1 virion components achieved the posttranscriptional control of alpha mRNAs through a similar mechanism.

2. HSV-1 viral messages are structurally similar to cellular messages. This similarity made it likely that the vhs mediated posttranscriptional modulation of host and alpha mRNAs may involve a similar strategy of the control of mRNA stability.

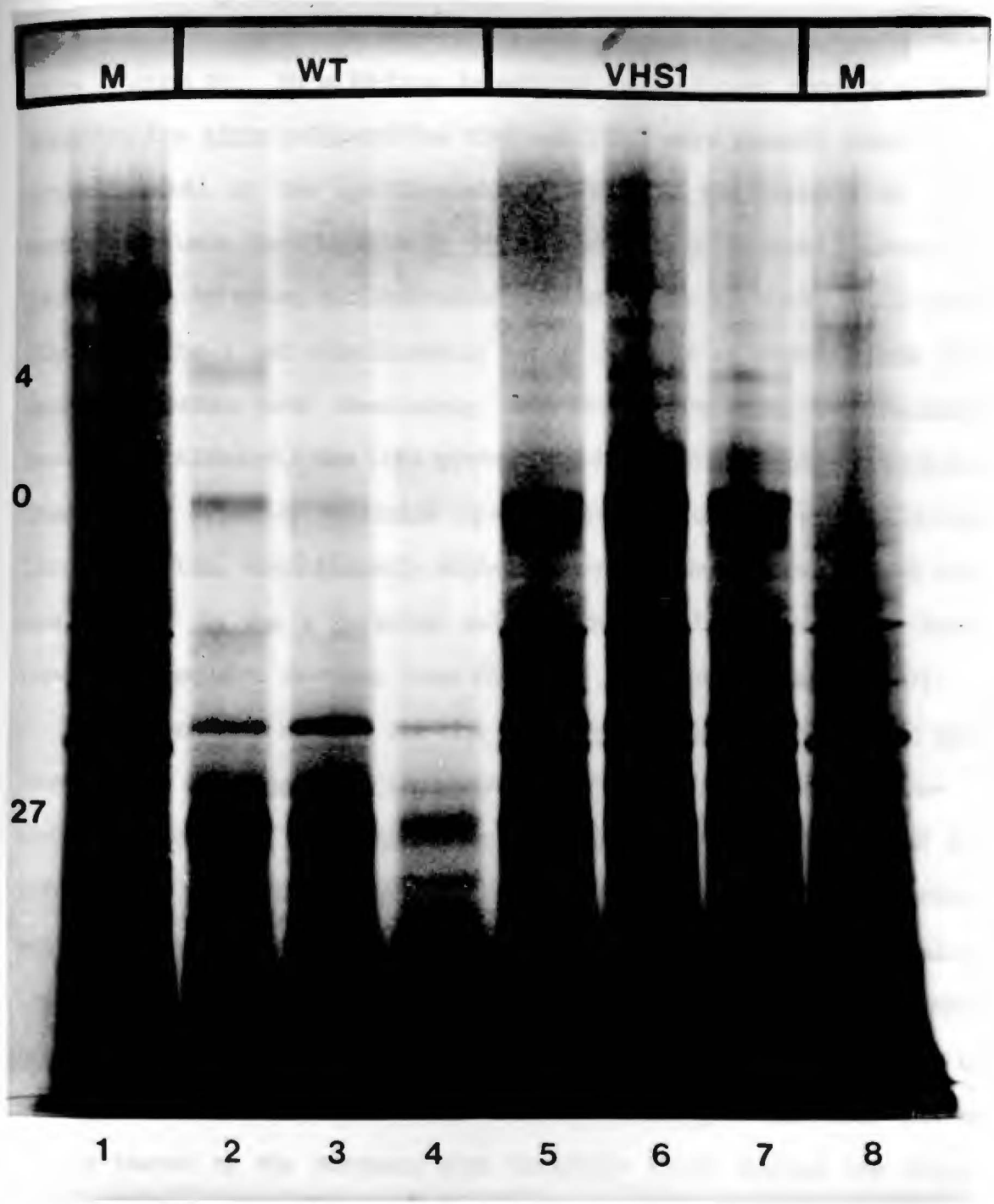
Our initial goal was to study the vhs-dependent destabilization of alpha mRNAs in infected cells. We therefore designed experiments to reproduce the vhs mediated posttranscriptional control of alpha mRNAs, which was first observed by Read and Frenkel (1983). Vero cells were either mock infected or infected with 40 plaque forming units (pfu/cell) of either the wild-type virus or the vhs 1 mutant in the presence of 50 μg of cycloheximide/ml. Under these conditions only alpha mRNAs are transcribed. At 4.5 h post infection, 10 μg of Actino-

mycin-D/ml was added to each culture. The cycloheximide was removed at 5 hr post infection, the cells were washed, and the infection was continued only in the presence of medium containing 10 μg of Actinomycin-D/ml. The rates of alpha polypeptide synthesis were then determined by labeling cells for 1 hr intervals with 30 μCi of ^{35}[S]-methionine/ml starting at 1.5, 3 and 5 h after cycloheximide was removed. Under these conditions the translation of alpha mRNAs was similar to that originally found by Read and Frenkel (1983). Thus, about 3 h after removal of cycloheximide and addition of Actinomycin-D, the rates of synthesis of the polypeptides alpha ICP4, ICP0, and ICP27 were markedly reduced in wild-type infected cells. In contrast, cells infected with vhs 1 showed high levels of alpha polypeptide synthesis as late as 5 h post cycloheximide reversal (Figure 2).

3.1 Effect of the vhs 1 mutation on the stability of alpha mRNAs.

Having confirmed the presence of posttranscriptional control of alpha mRNAs in wild-type infected cells, our first experiment was designed to determine whether the wild-type vhs components affected the functional and/or physical half lives of alpha mRNAs. To examine the difference in posttranscriptional regulation of alpha mRNAs between the wild-type and the vhs 1 virus, a cycloheximide reversal experiment was performed in which total cytoplasmic RNA was extracted from infected cells either immediately after removal of cycloheximide or after an additional 5 h of incubation in the presence of 5 μg of Actinomycin-D/ml. The RNA was then translated in vitro with micrococcal nuclease-treated rabbit reticulocyte lysates. These lysates should be capable

Figure 2 Vhs-mediated posttranscriptional control of the alpha mRNAs. Vero cells were mock infected (M) or infected with 40 pfu/cell of wild-type (WT) or the vhs 1 virus in the presence of 50 μg of cycloheximide/ml. At 4.5 h post infection, Actinomycin-D was added to each culture to a final concentration of 10 μg/ml. The cycloheximide was removed at 5 h post infection, the cells washed, and the infection was continued in the presence of medium containing 10 μg of Actinomycin-D/ml. The relative rates of alpha polypeptide synthesis were determined by pulse labeling the cells with ^{35}[S]-methionine label between 1.5 to 2.5 h (lanes 1, 2, and 5), 3 to 4 h (lanes 3 and 6), or 5 to 6 h (lanes 4, 7, and 8) after the addition of Actinomycin-D. Lysates from equal number of cells were electrophoresed in SDS-polyacrylamide gel and labeled polypepetides were visualized by autoradiography.

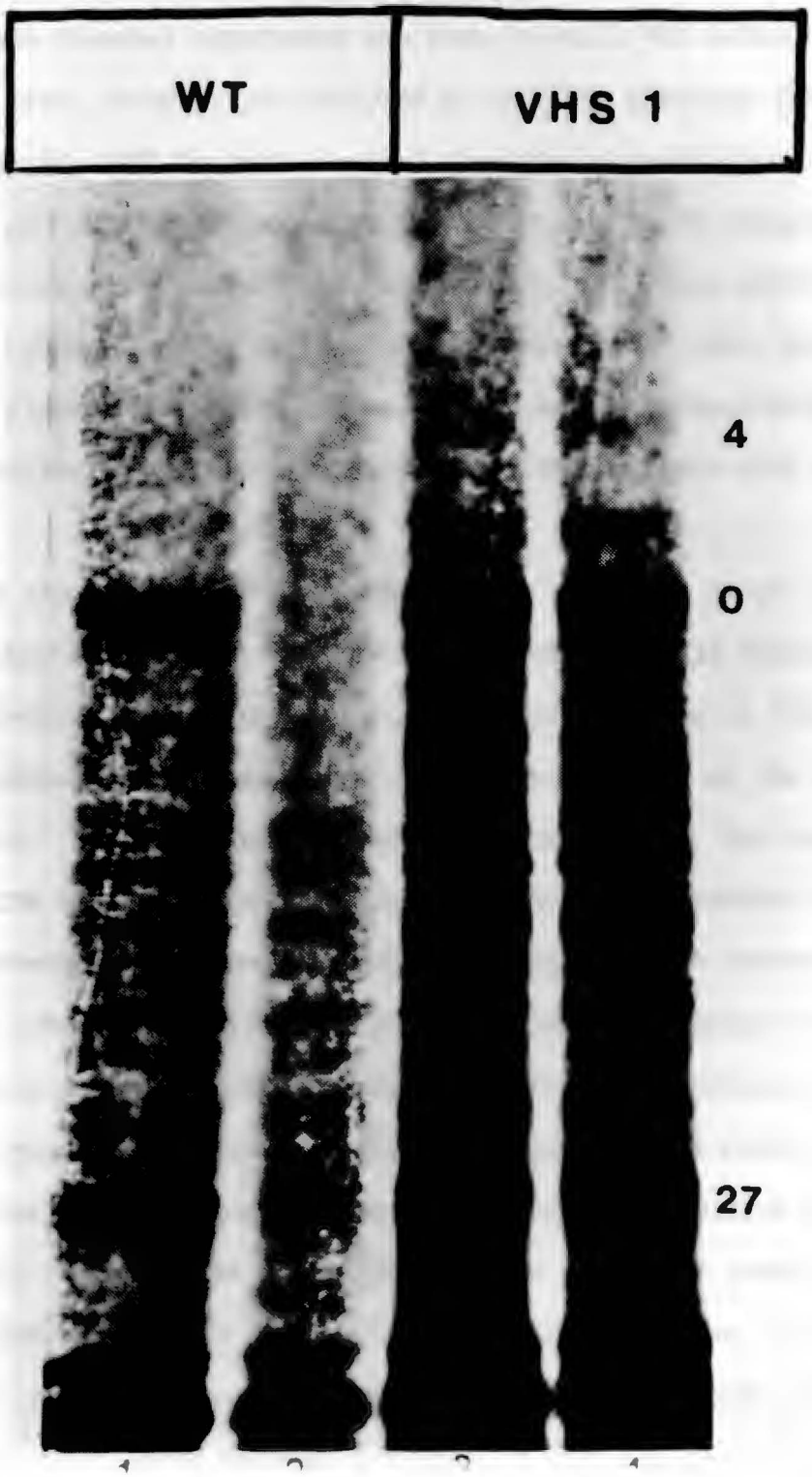

M
WT
VHS1
M
4
0
27
1
2
3
4
5
6
7
8

of translating any potentially translatable alpha mRNAs. In both wild-type and vhs 1 infections cells, the translatabilities of alpha mRNAs in vitro (see Figure 3) paralleled their patterns of translation in vivo (Figure 2). In wild-type infections, significant amounts of mRNA encoding the alpha polypeptides ICP0 and ICP27 were present immediately after removal of the cycloheximide. However, the amounts of these mRNAs that were translatable in vitro, had fallen to barely detectable levels by 5 hr after cycloheximide reversal. In contrast, cells infected with vhs 1 had significantly higher amounts of translatable ICP0 and ICP27 mRNAs both immediately, and five hours after cycloheximide reversal. Although, the ICP4 protein band was only faintly detectable immediately after cyloheximide reversal in RNA samples from wild-type infected cells, significantly higher amounts of translatable ICP4 mRNA was present in vhs 1 infected cells both immediately and five hours post cycloheximide reversal (see Figure 3) (Oroskar and Read, 1987).

The parallel results for the in vitro translatability and in vivo translation of alpha mRNAs indicated that the vhs mediated postranscriptional control of alpha mRNAs involved direct alterations of the mRNAs. In vitro translatability of mRNAs can be altered in various ways. The most direct way of accomplishing it involves the physical degradation of the messenger RNA molecule. Alternatively, the mRNAs might still be physically present, but modified in some way so as to abolish their ability to be translated. These possibilities can be easily tested by the Northern blot technique which enables the determination of alteration in the size of electrophoretically separated mRNA through the use of hybridization probes specific for the mRNA.

Figure 3 Translation of alpha mRNAs in vitro. Vero cells were infected with 40 pfu/cell of wild-type (WT) virus (lanes 1 and 2) or vhs 1 (lanes 3 and 4) in the presence of 50 μg of cycloheximide/ml. At 4.5 h after infection Actinomycin-D was added to each culture at a final concentration of 5 μg/ml. After 5 h the cycloheximide containing medium was replaced with medium containing 5 μg of Actinomycin-D/ml. Total cytoplasmic RNA was prepared either immediately (lanes 1 and 3) or after an additional 5 h of incubation (lanes 2 and 4). Samples of 375 μg/ml were translated in vitro, and analyzed by electrophoresis on 9.25% polyacrylamide gels and autoradiography. The alpha polypeptides ICP4, ICP0, and ICP27 are indicated by their numbers alongside lane 4 (Oroskar and Read, 1987).

61, 1987



To determine whether the decline in levels of translatable alpha mRNAs in wild-type virus infections was due to mRNA degradation, the cycloheximide reversal experiment was done in which RNA extracted at 0, 3, and 6 h after reversal was analyzed by Northern blotting (Figure 4). The HSV-1 EcoR1 "EK" fragment excised from the plasmid pSG28 was nick transalted and used to probe the Northern blots. The EK fragment hybridizes to three alpha mRNAs which encode ICP4, ICP0, and ICP27 (Anderson et al, 1980, Clements et al, 1979, Watson et al, 1979, and Watson et 1981). Figure 5 shows the ratios of peak areas corresponding to the band intensities of all three alpha mRNAs in the Northern blot shown in Figure 4.

There was a clear difference between the half lives of alpha mRNAs in mutant and wild-type virus infections. In cells infected with either wild-type or the vhs 1 virus, significant levels of ICP4, ICP0, and ICP27 mRNAs were present immediately after removal of the cycloheximide block. In cells infected with wild-type virus, the amounts of ICP4 and ICP0 mRNAs fell to approximately 10% of the initial levels by 3 hr postreversal and to undetectable levels by 6 h post reversal. The decline in ICP27 mRNA followed a similar pattern, although the slope was not quite as steep. On the other hand, in cells infected with vhs 1, at 3 h post reversal the amounts of the alpha mRNAs ranged from 65 to 90% of the initial values, and at 6 h, amounts ranged from 42 to 55% respectively (Oroskar and Read, 1987). The data also enabled us to calculate the approximate range of alpha mRNA half lives. A decay of 50% of the original levels of mRNAs coding for alpha ICP4, ICP0, and ICP27 was brought about between 1.5 and 2 h post cycloheximide rever-

Figure 4

Northern blot analysis of alpha mRNAs. Vero cells were mock infected (lanes 1 to 3) or infected with 30 pfu/cell of wild type virus (lanes 4 to 6) or the vhs 1 mutant (lanes 7 to 9), in medium containing 50 μg/ml of cycloheximide. At 4.5 h after infection Actinomycin D was added to each culture at a final concentration of 5 μg/ml. After 5 h, the cycloheximide containing medium was replaced with medium containing 5 μg/ml of Actinomycin-D. Total cytoplasmic RNA was prepared immediately (lanes 1, 4, and 7) after 3 h (lanes 2, 5, and 8) or after 6 h (lanes 3, 6, and 9) of additional incubation. Samples containing 5 μg RNA were analyzed by electrophoresis and Northern blotting using nick-translated EcoR1 fragment "EK" as a hybridization probe. The mRNAs encoding alpha polyprptides ICP4, ICP0, and ICP27 are indicated by their numbers (Oroskar and Read, 1987).

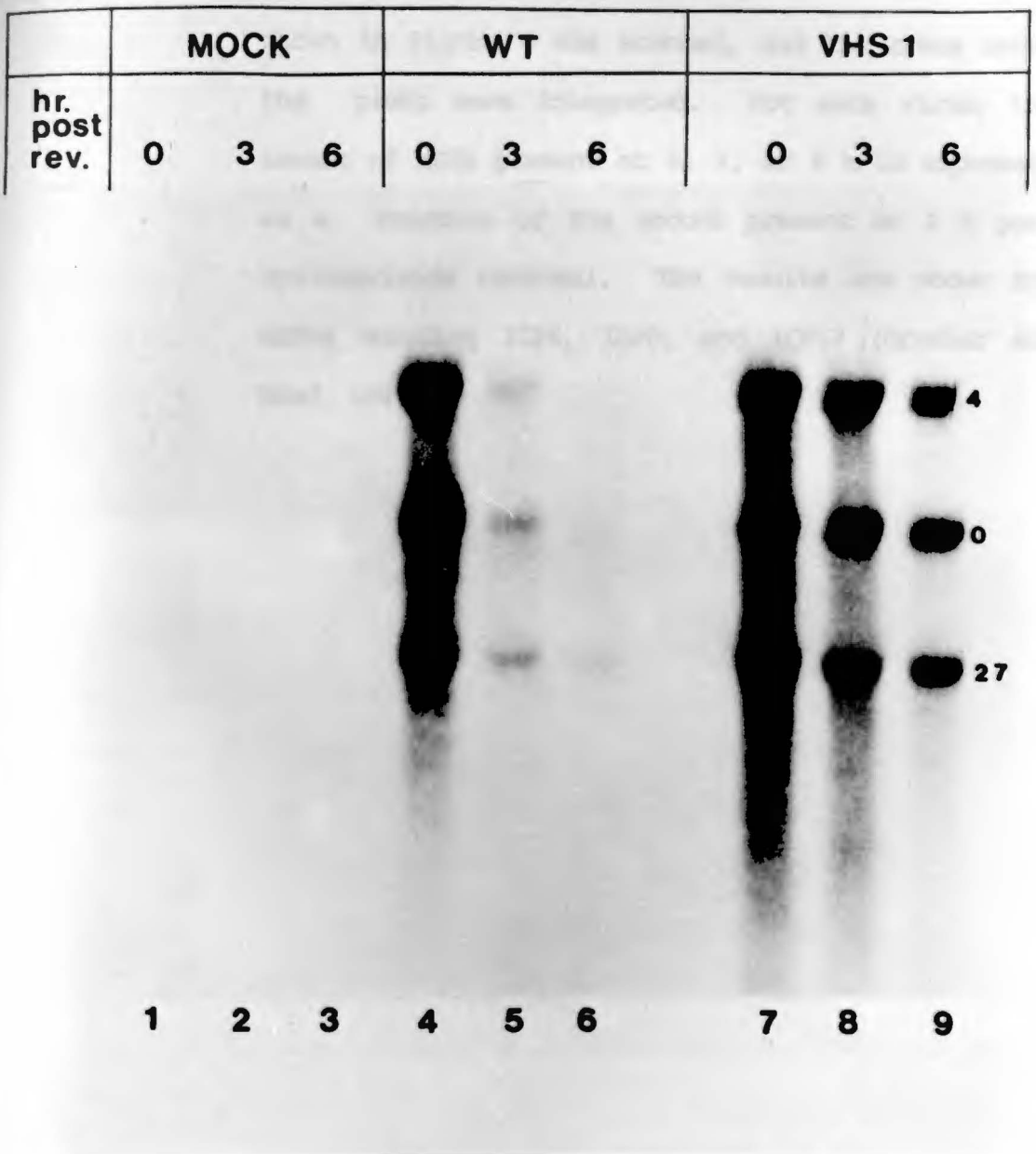
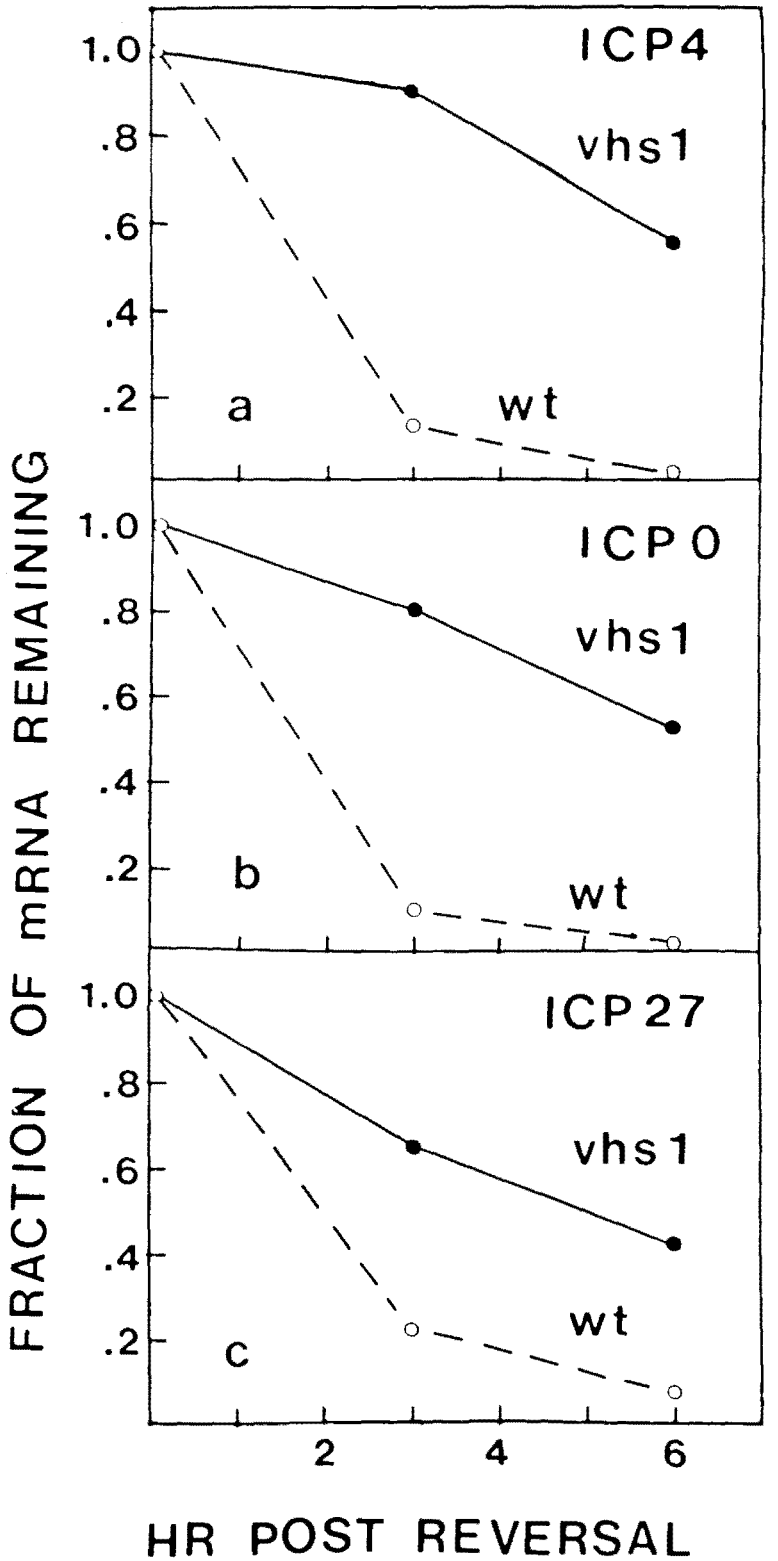

MOCK
WT
VHS 1
hr. post rev.
0 3 6 0 3 6 0 3 6
4
0
27
1 2 3 4 5 6 7 8 9

Figure 5 Quantitation of alpha mRNA decay. The autoradiogram shown in Figure 4 was scanned, and the areas under the peaks were integrated. For each virus, the amount of mRNA present at 0, 3, or 6 h is expressed as a fraction of the amount present at 0 h post cycloheximide reversal. The results are shown for mRNAs encoding ICP4, ICP0, and ICP27 (Oroskar and Read, 1987).


ICP4
vhs1
wt
a
ICP 0
vhs1
wt
b
ICP 27
vhs1
wt
c
1.0
.8
.6
.4
.2
2
4
6
FRACTION OF mRNA REMAINING
HR POST REVERSAL

sal. Although one cannot accurately determine the half lives of these mRNAs from just three points, these values for the half lives is consistent with those that we obtained through a systematic study done thereafter.

These results strongly suggested that the vhs-mediated posttranscriptional modulation of alpha mRNAs involved the functional and physical destabilization of alpha mRNAs. The similarity of modulation at the level of mRNA, further suggested that the same vhs component might be involved in regulating the stabilities of both the host and alpha mRNAs. Kwong and Frenkel (1987) subsequently published a report that essentially corroborated our results. Cells were infected with the vhs 1 mutant in the presence of cycloheximide. At six hours after infection, the cycloheximide block was reversed and the cells were superinfected with either the wild type or the vhs 1mutant virus in the presence of Actinomycin D. Northern blots of RNAs were analyzed 1, 3, and 4.5 h post superinfection. The autoradiograms showed that by 3 hr after cycloheximide reversal, only the superinfecting wild type virions destabilized the preexisting alpha mRNAs in vhs 1 infected cells. In comparison, alpha mRNAs were quite stable in cells superinfected with the vhs 1 mutant upto 4.5 hr after cycloheximide reversal. Use of gradient purified virions in this superinfection protocol further strengthened the assumption that the vhs component was indeed part of the virion and did not constitute virus factors from the inoculum which is generally prepared from freeze thawing total infected cell lysates.

We wished to further explore the nature of vhs mediated control of alpha mRNA stability. Alpha mRNA stability had been studied under

conditions of cycloheximide reversal. In presence of cycloheximide, alpha mRNAs are transcribed; when the cycloheximide is removed and Actinomycin-D added, alpha mRNAs can now be translated but all further transcription is inhibited. Under these conditions, the wild-type virus but not the vhs 1 mutant virus had regulated alpha mRNA stability. We therefore hypothesized that the wild-type vhs component played a key role in alpha mRNA stability and that since the vhs 1 mutant was defective in this virion function, alpha mRNAs were much more stable in vhs 1 infections. At that time, our laboratory was conducting studies that were aimed at mapping the vhs gene. The strategy involved a technique called marker rescue. HSV-1 EcoR1 restriction fragments were transfected along with the vhs 1 viral DNA and the recombinant progeny were tested for the wild type vhs functions. Through marker rescue Krikorian and Read (unpublished observations) found that the wild type EcoR1 "A" fragment was able to transfer the wild-type vhs function to the mutant vhs 1 virus. Furthermore, six out of six recombinant viruses that were wild-type for the host shutoff were also found to regulate the posttranscriptional status of alpha mRNAs. Thus, the rate of alpha polypeptide synthesis measured through the incorporation of ^{35}[S]-methionine in the presence of Actinomycin-D, was found to be downregulated only in infections with the wild type-recombinant but not with the vhs 1 mutant virus. Indeed, Kwong and colleagues (1988) have recently proved, again through marker rescue, that the same vhs component was involved in the regulation of host mRNA stability and the posttranscriptional regulation of alpha mRNAs.

However, it remained a distinct possibility that alpha polypep-

tides in conjunction with the wild-type vhs protein were responsible for alpha mRNA instability. This conclusion arises from the fact that posttranscriptional control of alpha mRNAs had always been studied under conditions of cycloheximide reversal, where alpha protein synthesis was also taking place. The logical way to test this hypothesis was to study alpha mRNA stability under conditions where only the wild-type vhs components were available but alpha polypeptides were not.

3.2 Requirement for alpha polypeptides in vhs mediated decay of alpha mRNAs.

The question of whether alpha polypeptides play a role in vhs mediated alpha mRNA decay was pursued through the superinfection protocol. The contribution of alpha polypeptides was controlled either by the presence or absence of cycloheximide which either inhibited or allowed the translation of preexisting alpha mRNAs. Cells were infected with the vhs 1 mutant in presence of cycloheximide. At 5 h post infection, the cultures were divided into two different sets. The cycloheximide block was reversed from one set but not from the other. At this time each of the two sets was superinfected with either mock lysates or wlid type or the vhs 1 mutant virus in presence of Actinomycin-D. Total cytoplasmic RNA was extracted at the time of superinfection, after 2.5, or after 5 hours. Northern blots indicated that alpha mRNAs were quite stable in cells which were superinfected with either mock lysates or the vhs 1 virions in the absence of cycloheximide. In contrast, by 5 hr post cycloheximide reversal, the preexisting alpha mRNAs encoded by the first vhs 1 virus had been significantly

degraded by the superinfecting wild-type virions (see Figure 6). These results confirm those published by Kwong and Frenkel (1987). In cells in which the contribution of alpha polypeptides was inhibited by cycloheximide, alpha mRNAs were stable under superinfection by either the wild-type or the vhs 1 virions or mock lysates (see Figure 7).

Several possibilities may explain these results. 1) Alpha polypeptides may cooperate with the wild-type vhs components to induce alpha mRNA degradation. 2) Alternatively, the lack of alpha mRNA decay could be due to stabilization of alpha mRNAs by cycloheximide. Precedences for cycloheximide mediated stabilization of eukaryotic mRNAs has been previously documented (Linial et al, 1985, and Shaw and Kamen, 1986). The case of alpha mRNAs does not appear analogous to the case of those protooncogenes whose decay is stabilized by cycloheximide. Several investigators showed HSV-1 virion host shutoff in the presence of cycloheximide, emetine, sodium flouride, puromycin (Strom and Frenkel (1987), and also in the presence of anisomycin (Schek and Bachenheimer (1975). 3) Another possibility is that vhs mediates mRNA decay through cellular factors which are very short lived. 4) Finally, double mutants deleted in any one of the alpha genes and the vhs gene could be used to study this problem. Primary infection with each of the double mutants deleted in single alpha gene, and superinfection with either the wild type or the vhs 1 mutant, would be a better strategy to determine the effects of alpha polypeptides in vhs mediated alpha mRNA decay.

Figure 6

Vhs-mediated alpha mRNA decay in the presence of alpha polypeptides. Vero cells were infected with 30 pfu/cell of vhs 1 in the presence of 50 μg of cycloheximide/ml. At 5 h post infection the cycloheximide-containing medium was replaced with medium containing 5 μg of Actinomycin-D/ml and the cells were mock superinfected (lanes 2 and 3), superinfected with 150 pfu/cell of wild-type virus (lanes 4 and 5) or with 150 pfu/cell of vhs 1 (lanes 6 and 7). Total cytoplasmic RNA was extracted at the same time as superinfection (lane 1) or 2.5 h (lanes 2, 4, and 6) or 5 h (lanes 3, 5, and 7) after superinfection. RNAs were analyzed by Northern blotting using nick-translated EcoR1 "EK" fragment as a hybridization probe. The mRNAs encoding the alpha polypeptides ICP4, ICP0, and ICP27 are indicated by their numbers.

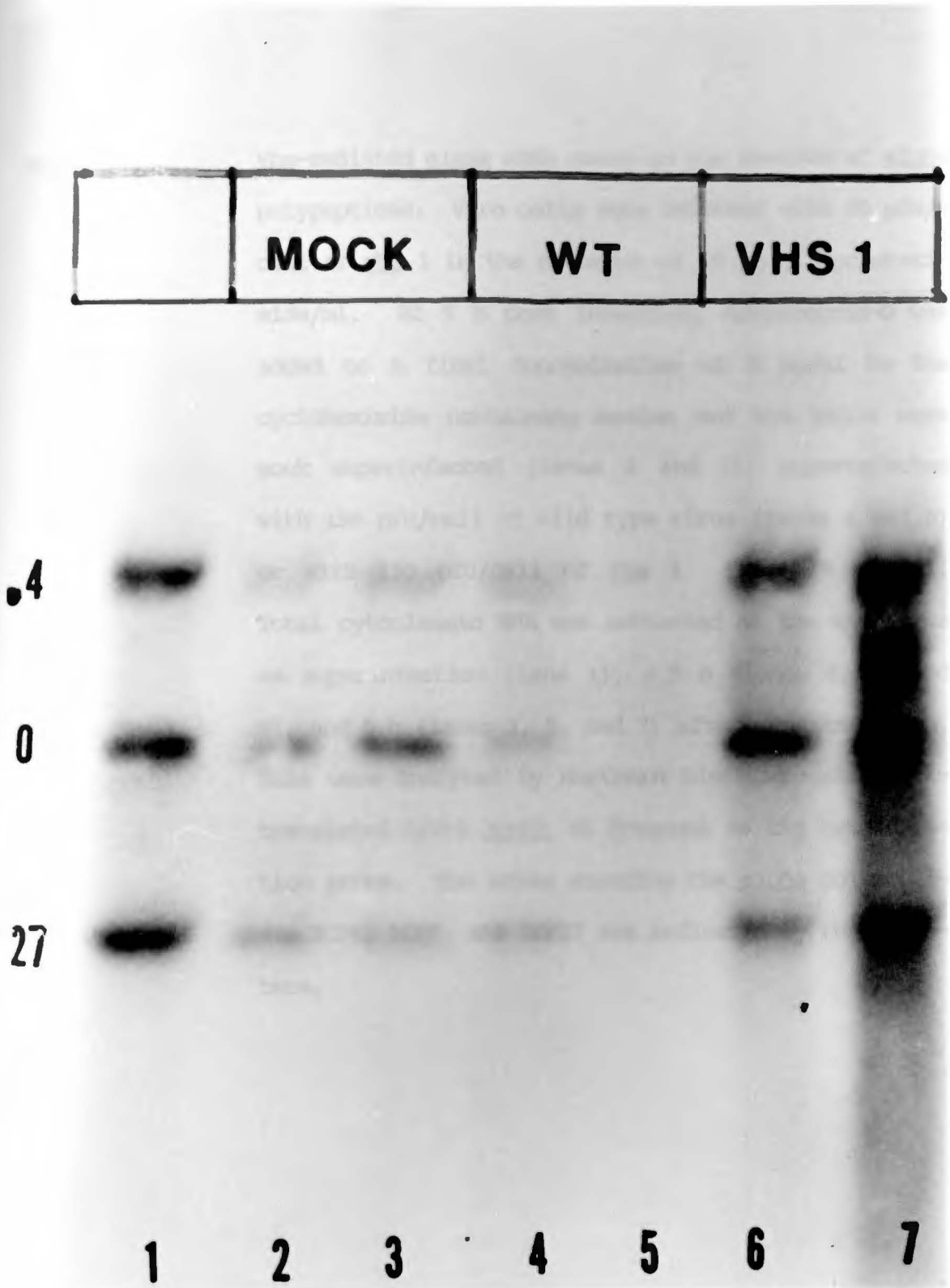
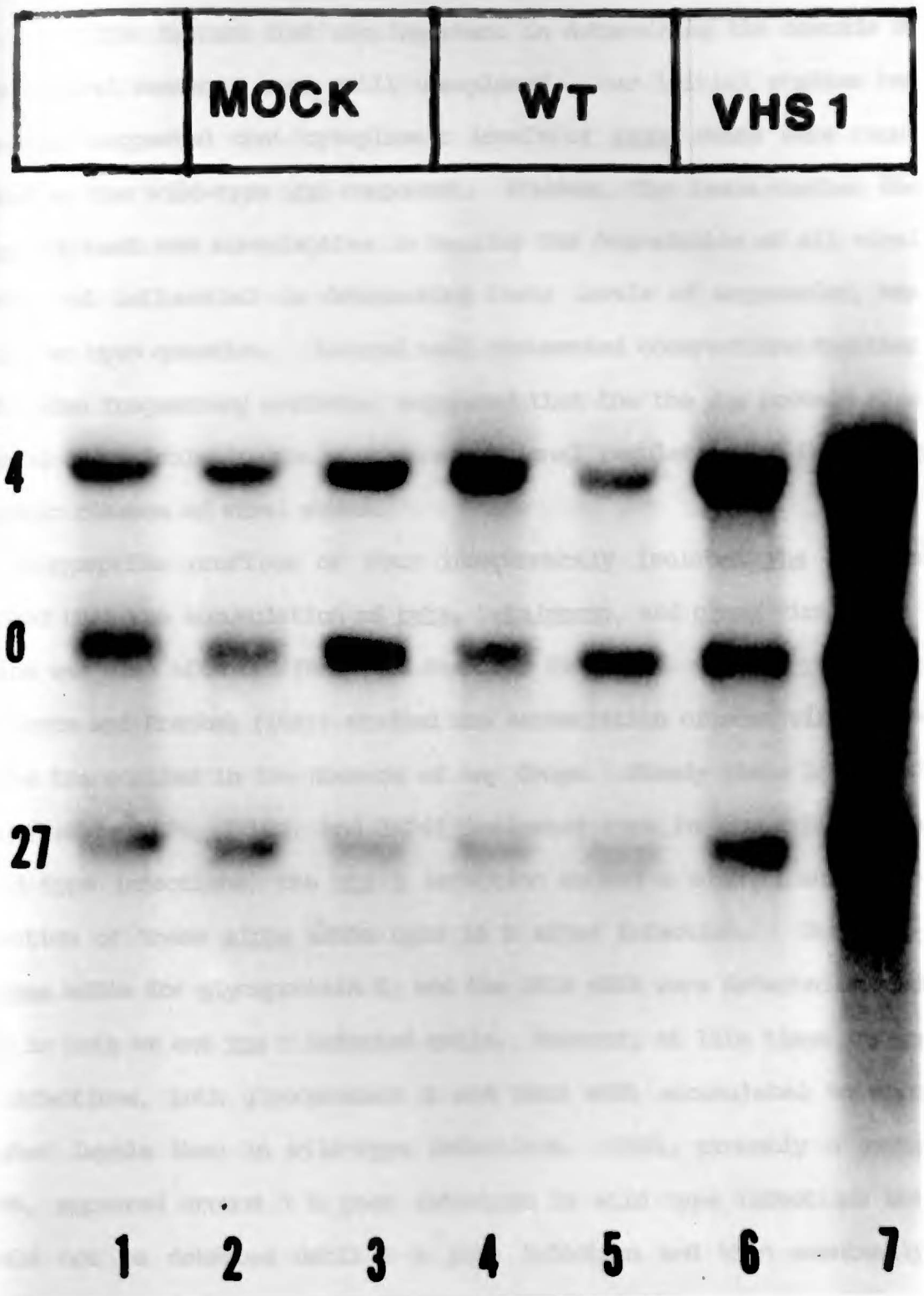

MOCK
WT
VHS 1
.4
0
27
1
2
3
4
5
6
7

Figure 7

Vhs-mediated alpha mRNA decay in the absence of alpha polypeptides. Vero cells were infected with 30 pfu/-cell of vhs 1 in the presence of 50 μg of cyclohexi-mide/ml. At 5 h post infection, Actinomycin-D was added to a final concentration of 5 μg/ml to the cycloheximide containing medium and the cells were mock superinfected (lanes 2 and 3), superinfected with 150 pfu/cell of wild type virus (lanes 4 and 5) or with 150 pfu/cell of vhs 1 (lanes 6 and 7). Total cytoplasmic RNA was extracted at the same time as superinfection (lane 1), 2.5 h (lanes 2, 4, and 6), and 5 h (lanes 3, 5, and 7) after superinfection. RNAs were analyzed by Northern blotting using nick-translated HSV-1 EcoR1 EK fragment as the hybridiza-tion probe. The mRNAs encoding the alpha polypepti-des ICP4, ICP0, and ICP27 are indicated by their num-bers.


MOCK
WT
VHS 1
4
0
27
1
2
3
4
5
6
7

3.3 Effect of the vhs 1 mutation upon the accumulation of viral mRNAs from different kinetic classes.

The factors that are important in determining the cascade of HSV-1 viral messages were still unexplored. Our initial studies had strongly suggested that cytoplasmic levels of alpha mRNAs were regulated by the wild-type vhs component. However, the issue whether the vhs component was nonselective in causing the degradation of all viral mRNAs and influential in determining their levels of expression, was still an open question. Several well documented observations together with some fragmentary evidence, suggested that the the vhs protein also played a key role in the posttranscriptional regulation of the other kinetic classes of viral mRNAs.

1. Polypeptide profiles of four independently isolated vhs mutants showed that the accumulation of beta, beta/gamma, and gamma viral proteins was also affected (Read and Frenkel, 1983).

2. Strom and Frenkel (1987) studied the accumulation of some viral messages transcribed in the absence of any drugs. Steady state levels of alpha mRNAs ICP4, ICP22, and ICP47 indicated that in comparison with wild type infections, the vhs 1 infection caused a significant accumulation of these alpha mRNAs upto 14 h after infection. The beta-/gamma mRNAs for glycoprotein E, and the US10 mRNA were detected around 5 h in both wt and vhs 1 infected cells. However, at late times in vhs 1 infections, both glycoprotein E and US10 mRNA accumulated to much higher levels than in wild-type infections. US11, probably a gamma mRNA, appeared around 7 h post infection in wild type infections but could not be detected until 9 h post infection and then eventually

accumulated to much higher levels in vhs 1 infected cells.

3. Kwong and Frenkel (1987) studied the rates of polypeptide synthesis from beta, beta/gamma, and gamma mRNAs in cells infected with either the the wild-type virus or the vhs 1 mutant. The viral infections were carried out for 7 h in the absence of drugs. At the end of 7 hours, Actinomycin D was added and the rate of protein synthesis was determined by pulse labeling the intact cells with ^{35}[S]-methionine at zero and six hours after the addition of Actinomycin-D. Once again, only in vhs 1 infected cells, beta ICP6 (ribonucleotide reductase), beta ICP8 (DNA-binding protein), and beta ICP36 continued to be synthesized upto 6 h after addition of Actinomycin D. In addition, many beta/gamma and gamma polypeptides continued to be synthesized, even after 6 h of Actinomycin D chase, only in vhs 1 infected cells.

Therefore, studies on the determination of target mRNA specificity in vhs-mediated viral mRNA decay were carried out by focussing on two aspects of viral mRNA metabolism. The first set of experiments was designed to determine whether the accumulation profiles of alpha, beta, beta/gamma, and gamma mRNAs were different in wild-type and vhs 1 infected cells. The second set of experiments was designed to determine the viral mRNA half lives in wild-type and vhs 1 infections beginnning at six hours after a normal drug free infection.

Our previous results enabled us to hypothesize that alpha mRNAs decayed more rapidly in cells infected with the wild-type HSV-1 than with the vhs 1 mutant, at least under conditions of cycloheximide reversal and presence of Actinomycin-D. Since viral mRNA stability in both the wild-type and vhs 1 infected cells was studied in the presence of

the same drug, it could be ruled out that the presence of Actinomycin-D had caused differential mRNA stabilities. In that case, vhs-mediated effects on viral mRNA stability should also be seen on the accumulation of viral mRNA in the absence of Actinomycin-D. To test this prediction, Vero cells were infected with 20 pfu/cell, of either wild-type virus or the vhs 1 mutant in the absence of any drugs. Total cytoplasmic RNA was extracted at 2 h intervals from 2.5 to 12.5 h or, in a second experiment from 2 to 16 h post infection. The RNA samples were then analyzed by Northern blotting and hybridization to specific DNA probes. X-ray films were exposed on blots for various lengths of time. The best exposure of the autoradiographic images for each viral message was scanned at 500 nm using a Gilford Response spectrophotometer. For reasons already mentioned about the phenotype of the vhs 1 mutant (Read and Frenkel, 1983), we also determined whether the vhs gene product had an effect on the accumulation of beta, beta/gamma, and gamma mRNAs. Figures 8, and 9 show composite pictures of the actual autoradiograms of accumulation patterns of different viral mRNAs. Figures 10, 11, 12, and 13 are the plots from integrated peak areas corresponding to the intensities of mRNA bands in the respective autoradiograms. The results indicate that the vhs 1 mutation affects the accumulation of viral mRNAs representing all four kinetic classes.

3.3-1 Alpha mRNAs

The accumulation kinetics of two of the alpha mRNAs varied slightly even within the wild-type infected cells (Figures 8, and 10). ICP27 mRNA was detected very early in infection; following this the

Figure 8

Accumulation of alpha, beta, and beta/gamma mRNAs in wild-type (WT) and vhs 1 infected cells. Vero cells were infected with 20 pfu/cell of either wild-type virus (lanes 1 to 6) or the vhs 1 mutant (lanes 7 to 12). Total cytoplasmic RNA was extracted at 2.5 h (lanes 1 and 7), 4.5 h (lanes 2 and 8), 6.5 h (lanes 3 and 9), 8.5 h (lanes 4 and 10), 10.5 h (lanes 5 and 11), and 12.5 hr (lanes 6 and 12). Five μg of total cytoplasmic RNA were analyzed by Northern blotting. Blots were probed with HSV-1 EcoR1 "EK" fragment to determine the levels of mRNAs encoding ICP0 and ICP27, and with the HSV-1 EcoR1 "N" fragment to determine the levels of mRNAs encoding thymidine kinase (TK) and glycoprotein H (gH).

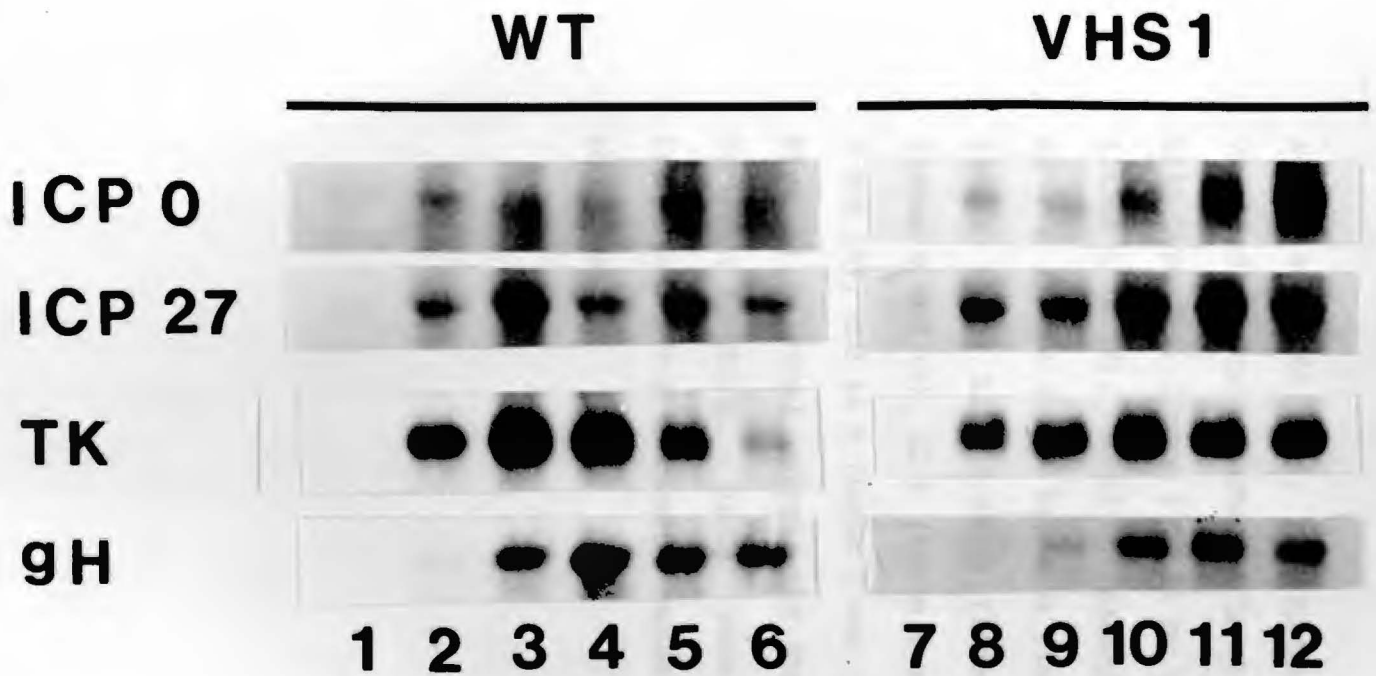

WT
VHS 1
ICP 0
ICP 27
TK
gH
1 2 3 4 5 6
7 8 9 10 11 12

Figure 9

Accumulation of beta, beta/gamma, and gamma mRNAs. Vero cells were infected with 20 pfu/cell of either wild-type (WT) (lanes 1 to 8) or the vhs mutant virus (lanes 9 to 16). Total cytoplasmic RNA was extracted at 2 h (lanes 1 and 9), 4 h (lanes 2 and 10), 6 h (lanes 3 and 11), 8.25 h (lanes 4 and 12), 10 h (lanes 5 and 13), 12 h (lanes 6 and 14), 14 h (lanes 7 and 15), and 16 h (lanes 7 and 16) after infection. Five μg of total cytoplasmic RNA were analyzed by Northern blotting. Blots were probed with nick-translated HSV-1 intragenic sequences cloned into M13 phage vectors.

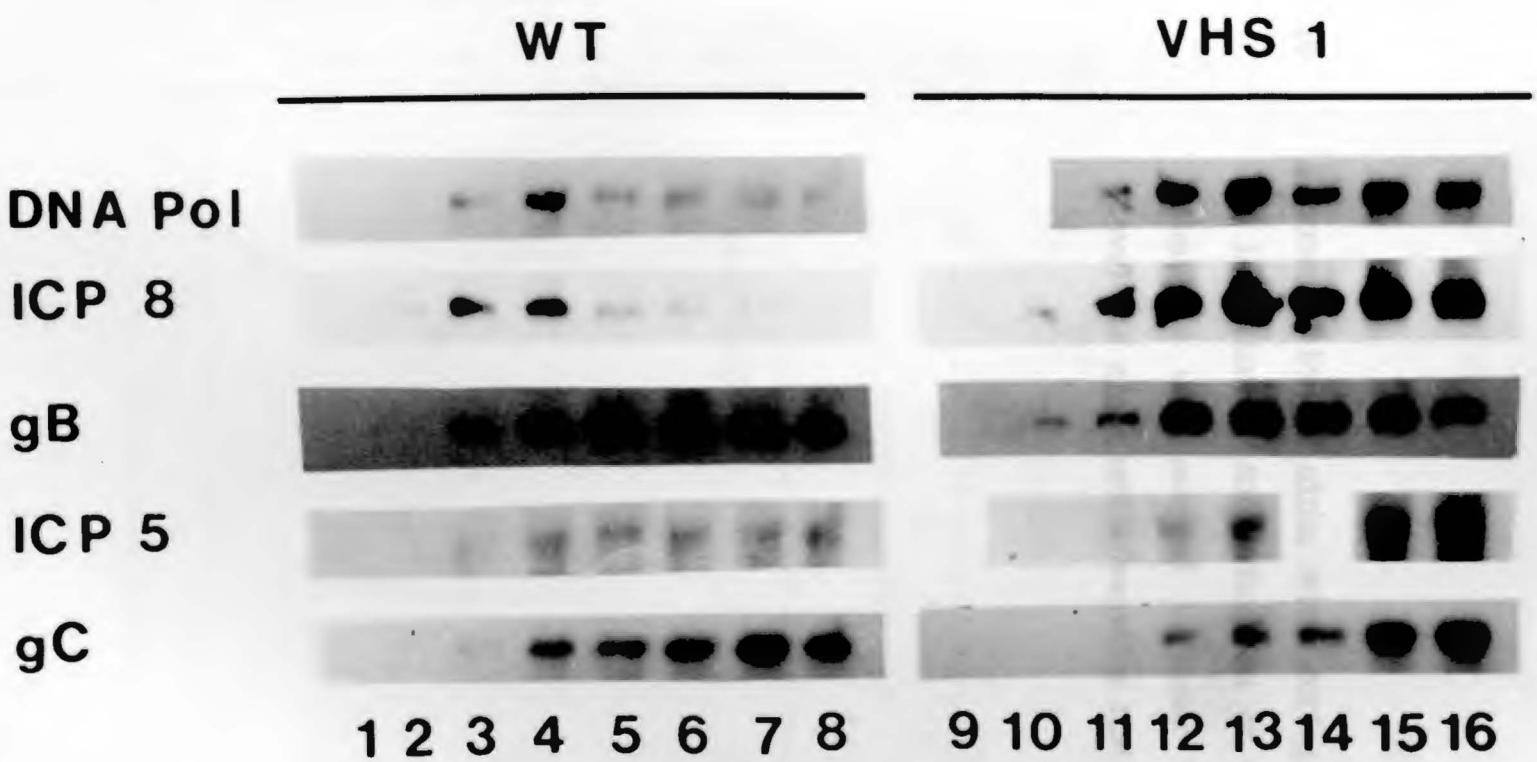
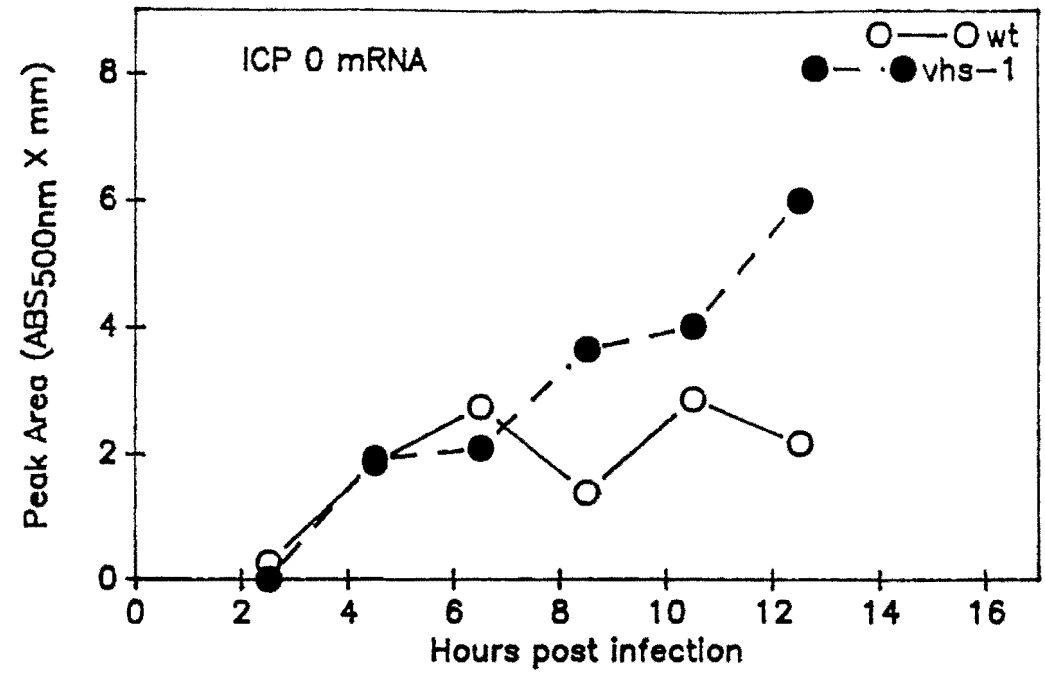

WT
VHS 1
DNA Pol
ICP 8
gB
ICP 5
gC
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16

Figure 10 Accumulation of alpha mRNAs during wild-type and vhs 1 infections. Autoradiograms of the gels shown in Figure 8 were scanned in order to quantitate the levels of alpha mRNAs encoding ICP0 and ICP27.


ICP 0 mRNA
wt
vhs-1
Peak Area (ABS500nm X mm)
Hours post infection
0
2
4
6
8
10
12
14
16

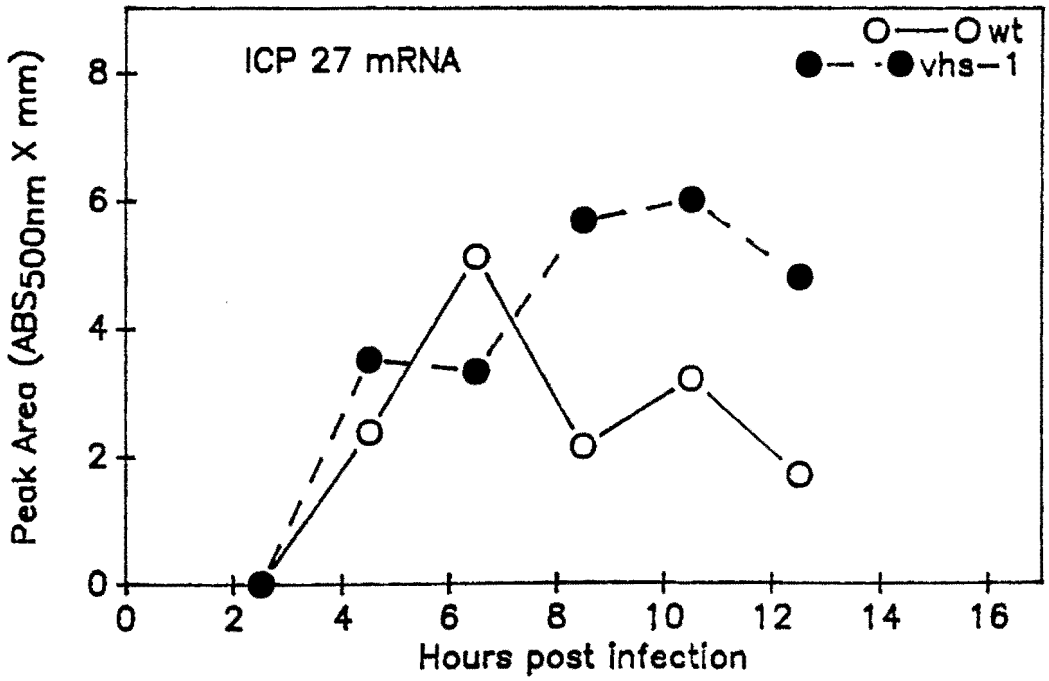

ICP 27 mRNA
wt
vhs-1
Peak Area (ABS500nm X mm)
Hours post infection
0
2
4
6
8
10
12
14
16

Figure 11 Accumulation of beta mRNAs during wild-type and vhs 1 infections. Autoradiograms of the gels shown in Figures 8 and 9 were scanned in order to quantitate the levels of beta mRNAs encoding thymidine kinase (TK), DNA-binding protein (ICP8), and DNA polymerase (DNA pol).

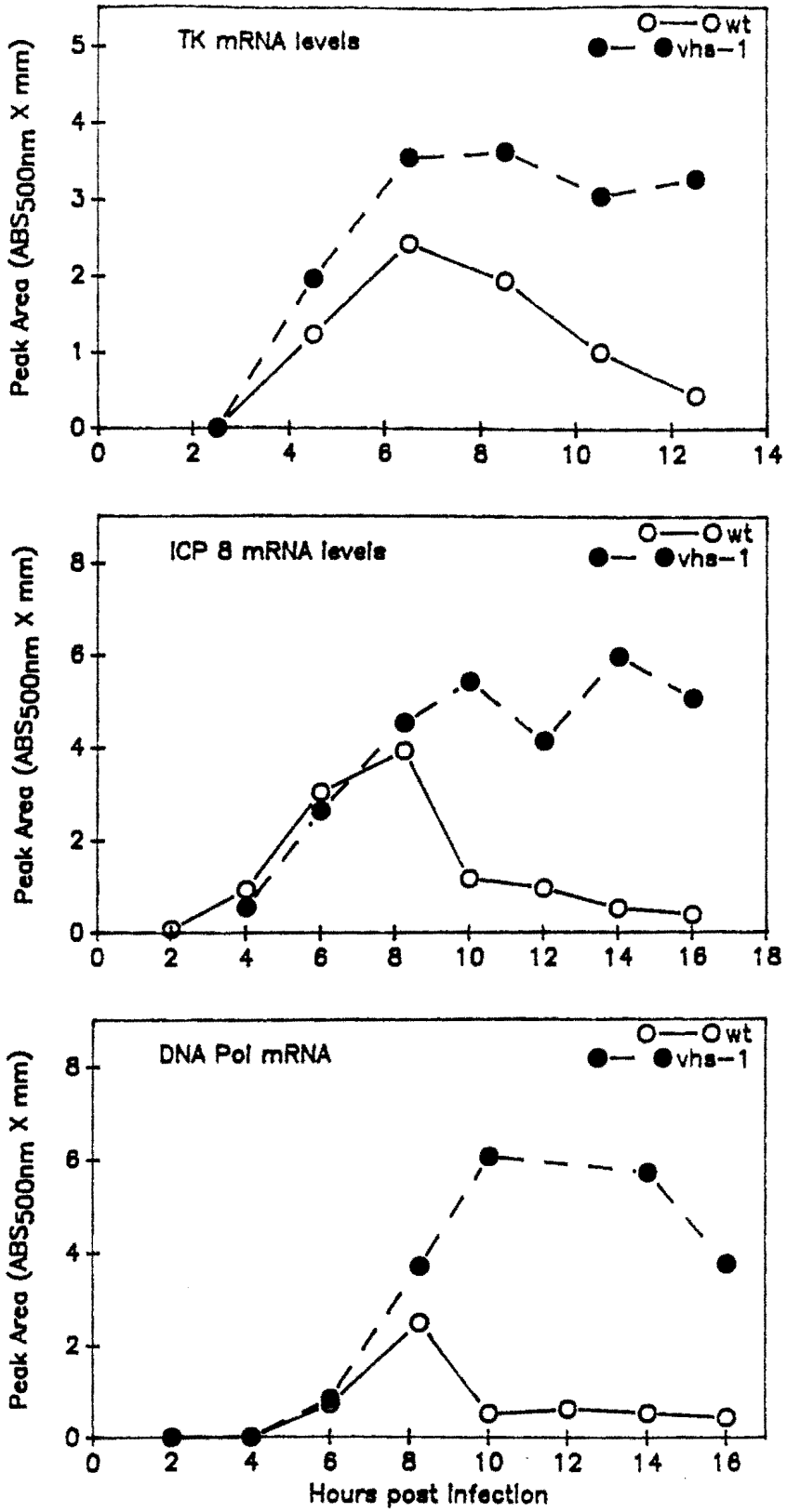

TK mRNA levels
wt
vhs-1
Peak Area (ABS500nm X mm)
0
1
2
3
4
5
0
2
4
6
8
10
12
14
ICP 8 mRNA levels
wt
vhs-1
Peak Area (ABS500nm X mm)
0
2
4
6
8
0
2
4
6
8
10
12
14
16
18
DNA Pol mRNA
wt
vhs-1
Peak Area (ABS500nm X mm)
0
2
4
6
8
0
2
4
6
8
10
12
14
16
Hours post infection

Figure 12 Accumulation of beta/gamma mRNAs during wild-type and vhs 1 infections. Autoradiograms of the gels shown in Figures 8 and 9 were scanned in order to quantitate the levels of beta/gamma mRNAs encoding the major capsid protein (ICP5), glycoprotein B (gB), and glycoprotein H (gH).

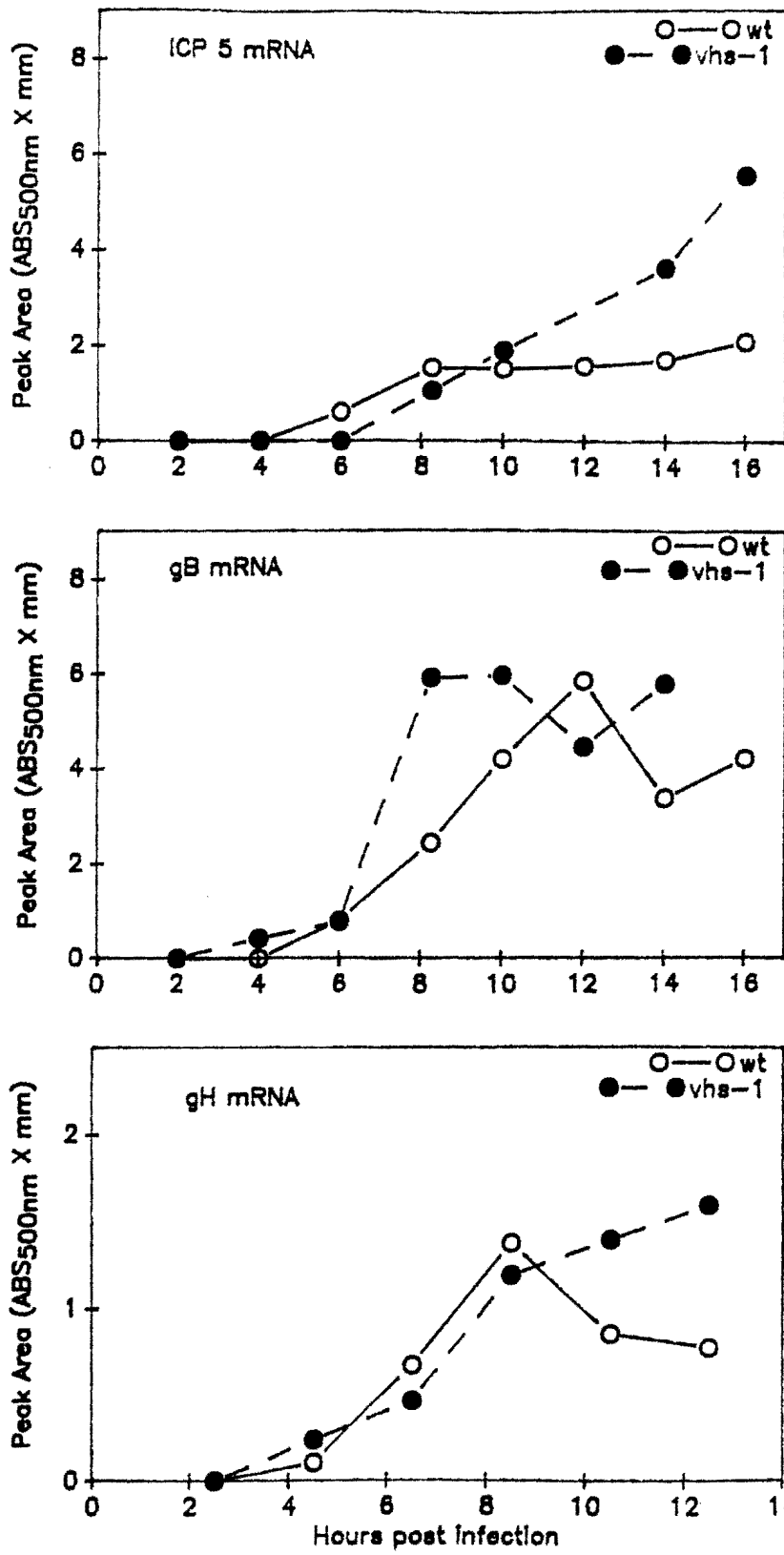
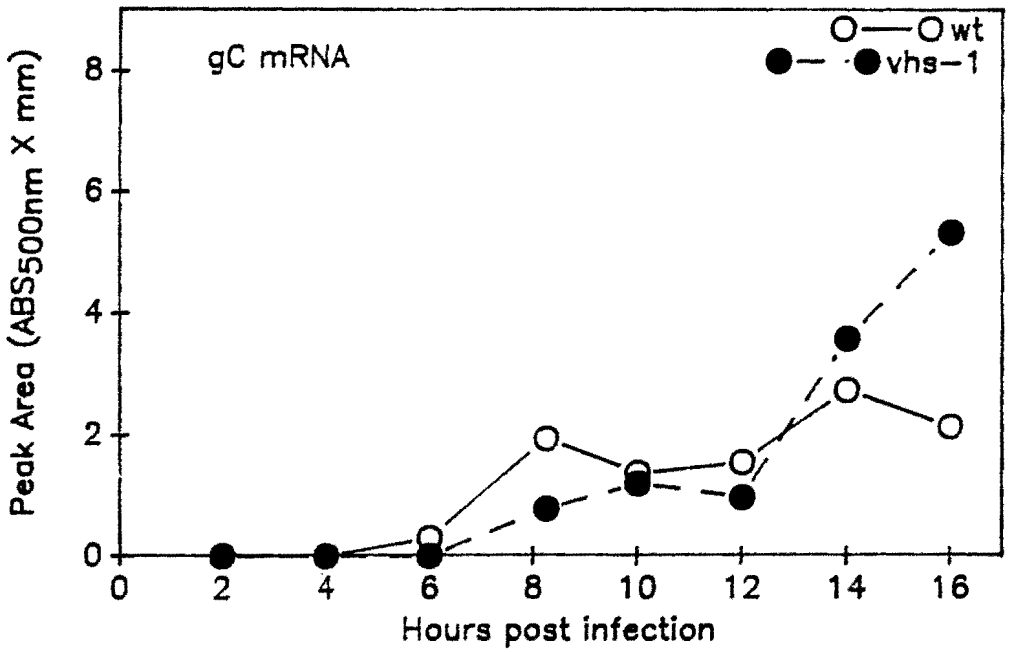

ICP 5 mRNA
wt
vhs-1
Peak Area (ABS500nm X mm)
gB mRNA
gH mRNA
Hours post infection

Figure 13 Accumulation of gamma mRNAs during wild-type and vhs 1 infections. Autoradiograms of the gels shown in Figure 9 were scanned in order to quantitate the levels of gamma mRNA encoding glycoprotein C (gC).


gC mRNA
wt
vhs-1
Peak Area (ABS500nm X mm)
0
2
4
6
8
0
2
4
6
8
10
12
14
16
Hours post infection

levels of ICP27 mRNA peaked at 6 h post infection and then declined. By late times in infection, only 40 to 50% of the peak ICP27 mRNA levels could be detected. In some respects, the accumulation profile of ICP0 mRNA was similar to that of the ICP27 mRNA. ICP0 mRNA appeared very early in infection and peaked to its maximal level by 6 h post infection. The accumulation profile of ICP0 mRNA was different from that of ICP27 mRNA in that the levels of ICP0 mRNA persisted at much higher levels at 12 h post infection. This effect could be explained by the presence of very diffusely migrating ICP0 mRNA transcripts late in infection. Although this characteristic of ICP0 mRNA has been reported by both Harris-Hamilton and Bachenehimer (1985), and Weinheimer and McKnight (1987), they found that ICP0 message persisted at low levels late in infection.

Not surprisingly, the vhs 1 mutation led to higher levels of accumulation for both the ICP0, and ICP27 mRNAs. In contrast to wild type infections, the level of ICP27 mRNA increased for 8 h post infection and then plateaued rather than declining. On the other hand, in vhs 1 mutant infections, ICP0 mRNA continued to increase throughout the first 12 hours of infection until, at late times, there was about three times as much message as in cells infected with the wild-type virus.

3.3-2 Beta mRNAs

The accumulation kinetics of thymidine kinase (TK), DNA polymerase (DNA pol), and ICP8 mRNAs were strikingly similar to each other in wild-type infected cells. Each appeared slightly later than the alpha messages and reached its maximum levels between 6 to 8 h after infec-

tion. Beta mRNA levels then sharply declined by 12 to 16 hours post infection and very little beta mRNA could be detected in the cytoplasm (see Figures 8, 9, and 11). In our hands, the decline in the abundance of beta mRNAs was more acute and complete than it was for either the ICP0 or ICP27 alpha messages. Furthermore, the pattern of beta mRNA accumulation seen here was similar to that observed in earlier studies (Harris-Hamilton and Bachenheimer, 1985, and Weinheimer, and McKnight, 1987).

The accumulation profiles of thymidine kinase, DNA polymerase, and ICP8 mRNAs in vhs 1 infected cells were significantly different from those seen in wild-type infected cells. Essentially, all of these mRNAs over accumulated in vhs 1 infected cells. In contrast to the wild-type infected cells, levels of thymidine kinase, DNA polymerase, and ICP8 mRNA in vhs 1 infected cells peaked between 10 to 12 h post infection, and remained at a plateau rather than declining (see Figures 8, 9, and 11). Thus the vhs function plays an important role in the control of beta gene expression. The defective vhs protein present in the vhs 1 mutant caused an imbalance in the abundance control of cytoplasmic beta mRNAs (see Figures 8, 9, and 11)

3.3-3 Beta/gamma mRNAs

In wild-type infected cells, beta/gamma mRNAs encoding the major capsid protein, ICP5, glycoprotein B, and glycoprotein H were first detected in the cytoplasm about the same time as beta messages. The accumulation profiles for beta/gamma mRNAs however, were significantly different from those of the beta mRNAs. First, the maximal levels of

the beta/gamma mRNAs were found between 8 to 12 h post infection, in contrast to the beta mRNAs where the maximal levels were detected between 6 to 8 h post infection. Second, in contrast to the precipitous decline seen in beta mRNA levels, the beta/gamma mRNA levels did not decline but instead stayed significantly high even at 16 hr post infection (see Figures 8, 9, and 12). The accumulation profiles of beta/-gamma and gamma mRNAs in wild-type infections were also studied by Weinheimer and McKnight (1987). Consistent with our results, they found that ICP5, glycoprotein B, and glycoprotein H mRNAs first appeared in the cytoplasm at 4 hr post infection; then rose to peak levels around 8 hr, following which they either stayed at the same levels or increased until 12 h post infection, depending upon the gene.

The vhs 1 mutation however did not cause a dramatic overaccumulation of the beta/gamma mRNAs. In comparison to their levels in wild type infections, the ICP5, glycoprotein B, and glycoprotein H overaccumulated to variable extents in vhs 1 infections. Within the beta/-gamma group, the overaccumulation of glycoprotein B was minimal, whereas that of ICP5 was the maximal (see Figures 8, 9, and 12).

3.3-4 Gamma mRNAs

In many respects, the accumulation profile of the gamma mRNA for glycoprotein C was similar to that of the beta/gamma mRNAs. In wild-type infected cells, glycoprotein C mRNA first appeared in the cytoplasm at 4 h post infection and increased to high levels by 8 hr post infection. By late times in infection however, the levels of glycoprotein C mRNA showed very little decline. Thus, very high levels of

glycoprotein C mRNA were present upto 16 hr post infection (see Figures 8 and 12). Weinheimer and McKnight (1987) also found that gC mRNA first appeared in the cytoplasm at 6 h post infection and continued to increase up to 12 h in wild type infected cells.

The vhs 1 mutation once again did not lead to a dramatic overaccumulation of the glycoprotein C mRNA. Apart from the accumulation levels, there seemed to be some differences in the kinetics of glycoprotein C mRNA accumulation between wild-type and vhs 1 infected cells. In vhs 1 infected cells, glycoprotein C mRNA did not appear in appreciable quantities until 8 to 12 h post infection. However, subsequent to this initial lag, by 16 h post infection, levels of glycoprotein C mRNA were twice as high as those seen in wild-type infected cells (see Figures 9, and 13). The lag in the appearance of glycoprotein C mRNA in vhs 1 mutant infected cells, may be due to indirect effects of the vhs 1 phenotype on the cascade of viral mRNA expresssion. Some of these indirect effects of the mutant vhs 1 gene product will be elaborated upon in the discussion section.

3.4 Measurement of mRNA half-lives under conditions of Actinomycin-D chase

The accumulation profiles are a reflection of the steady state levels of mRNA in the cytoplasm. The balance achieved between the rates of mRNA synthesis, its transport and cytoplasmic decay, determine the steady state levels. A comparison of the accumulation kinetics of beta/gamma and gamma mRNAs with beta mRNAs are consistent with a model of some selectivity in the vhs mediated destabilization of viral messa-

ges. Thymidine kinase, DNA polymerase, and ICP8 mRNAs, showed similar accumulation profiles (i.e. early appearance, followed by peak levels and then a sharp decline in their levels late in infection). On the other hand, ICP5, glycoprotein B, glycoprotein H, and glycoprotein C mRNA levels did not decline, but rather persisted at high levels. One possible explanation was that beta/gamma and gamma messages are intrinsically more resistant to the effects of the vhs gene product than are alpha and beta messages. Furthermore, when comparing the accumulation profiles within the alpha and beta mRNA classes it appeared that, in contrast to the alpha class, the sensitivity to vhs regulation spanned the entire class of beta messages. Thus, in comparison to their maximal levels, small quantities of thymidine kinase, DNA-binding protein, and ICP8 mRNAs were found at late times of infection whereas comparatively higher levels of alpha ICP27 and alpha ICP0 mRNAs were still persistent at 12 h post infection. Alternatively, all viral mRNAs may be equally sensitive to the vhs protein but some may persist at high levels because they are continually replenished by new transcription and transport to the cytoplasm, while transcription of other genes may be turned off or turned down. My subsequent experiments were therefore designed to determine whether there was a target selectivity involved in vhs mediated degradation of viral mRNAs.

To resolve this question, the half lives of ten different mRNAs, representative of all four kinetic classes, were studied at 6 h post infection. Although mRNA levels from each kinetic class peak at different times, the most logical means of studying differential target selectivity was to measure the half-lives of different mRNAs exposed to

the same cytoplasmic factors at the same time. At six hours after infection, mRNAs belonging to all of the different kinetic classes are present at appreciable levels. I therefore chose six hours after infection as the time at which half-lives of all ten mRNAs would be determined.

Cells were infected with 20 pfu/cell of either the wild-type virus or the vhs 1 mutant in the absence of any drugs. Actinomycin-D was added at 6 hr post infection and total cytoplasmic RNA was extracted at 45 min intervals upto 3 hr post Actinomycin D addition. The levels of different viral mRNAs were analyzed by Northern blotting and quantitated by densitometric scanning. After satisfactory exposures for each viral mRNA had been obtained, the blots were stripped and reprobed with a genomic clone of Xenopus laevis encoding the 28S ribosomal RNA. The amount of total RNA loaded into each lane was quantitated in two different ways. First, the optical density at 260 nm for each RNA sample was measured in the Gilford Spectrophotometer. Based on the optical density at 260 nm, 5 μg of each RNA sample was loaded onto each lane prior to electrophoresis. Second, the autoradiographic images of the 28S rRNA from each lane were scanned at 500 nm in a Gilford Response Spectrophotometer. This allowed us to normalize the rRNA contents of each lane representing cells exposed to Actinomycin D, to the rRNA content of the lane representing cells that were incubated in the absence of drugs for 6 hr after infection. Finally, viral RNA bands representing both the wild-type and the vhs 1 mutant Northern blots, were normalized with respect to their respective rRNA contents. Thus, normalized values for each RNA band were used for plotting the

decay curves for viral mRNAs. We were thus able to control for small differences in the amounts of cytoplasmic RNA that was loaded onto different lanes of the gels.

The decay of viral mRNAs under the influence of the wild-type vhs gene product and mutant vhs 1 gene product are summarized in Figures 14 through 17. A second experiment in which the Actinomycin-D chase was carried out for longer time periods is plotted in Figures 18 and 19. The half-lives of viral mRNAs from all four kinetic classes were calculated from the regression curves in Figures 14 through 19 and are shown in Table 1. The half-life values are based on the output from the Sigma-Plot program outlined in the Materials and Methods. The following conclusions can be drawn from the decay curves of viral mRNAs.

1. Viral mRNAs from every kinetic class were significantly stabilized in cells infected with the vhs 1 mutant. When compared to that in wild-type infected cells, the half-life of each message in vhs 1 infected cells was increased several fold.

2. In wild-type infected cells the half-lives of viral mRNAs belonging to all kinetic classes ranged from 1 to 2.5 hours. It is possible that small differences in the half-lives of the mRNAs may exist. However, two separate determinations of viral mRNA decay under Actinomycin D chase for 3 to 6 h showed strong similarities in the half-life values.

The data suggest that little, if any, target mRNA selectivity is involved in the vhs-mediated degradation of viral mRNAs belonging to different kinetic classes. Thus, the differential accumulation kinetics of beta/gamma amd gamma mRNAs on the one hand and beta mRNAs on the

Figure 14 Decay of alpha mRNAs. Vero cells were infected with 20 pfu/cell of either the wild-type or the vhs 1 mutant virus. At six hours post infection Actinomycin-D was added to a final concentration of 5μg/ml and the incubation continued. Total cytoplasmic RNAs were isolated at the time of Actinomycin-D addition and at 45 min intervals up to 3 h after the start of chase. Five μg RNA samples were analyzed by Northern blotting. The blots were probed for viral mRNAs and for 28S rRNA. The autoradiograms were scanned at 500 nm in a Gilford Response Spectrophotometer and the integrated peak areas, each from the wild-type and the vhs 1 mutant were used to normalize the amount of RNA with respect to their rRNA contents respectively. This normalization enabled us to correct for errors in loading equal amounts of cytoplasmic RNA onto each lane of the gel. The fraction of mRNA remaining at time 't' was then plotted as a logarithmic plot with linear regression using the SigmaPlot program as described in the section on statistical analyses in Materials and Methods. Autoradiograms of the resulting gels were scanned in order to quantitate the levels of alpha mRNAs encoding ICP4, ICP0, and ICP27.

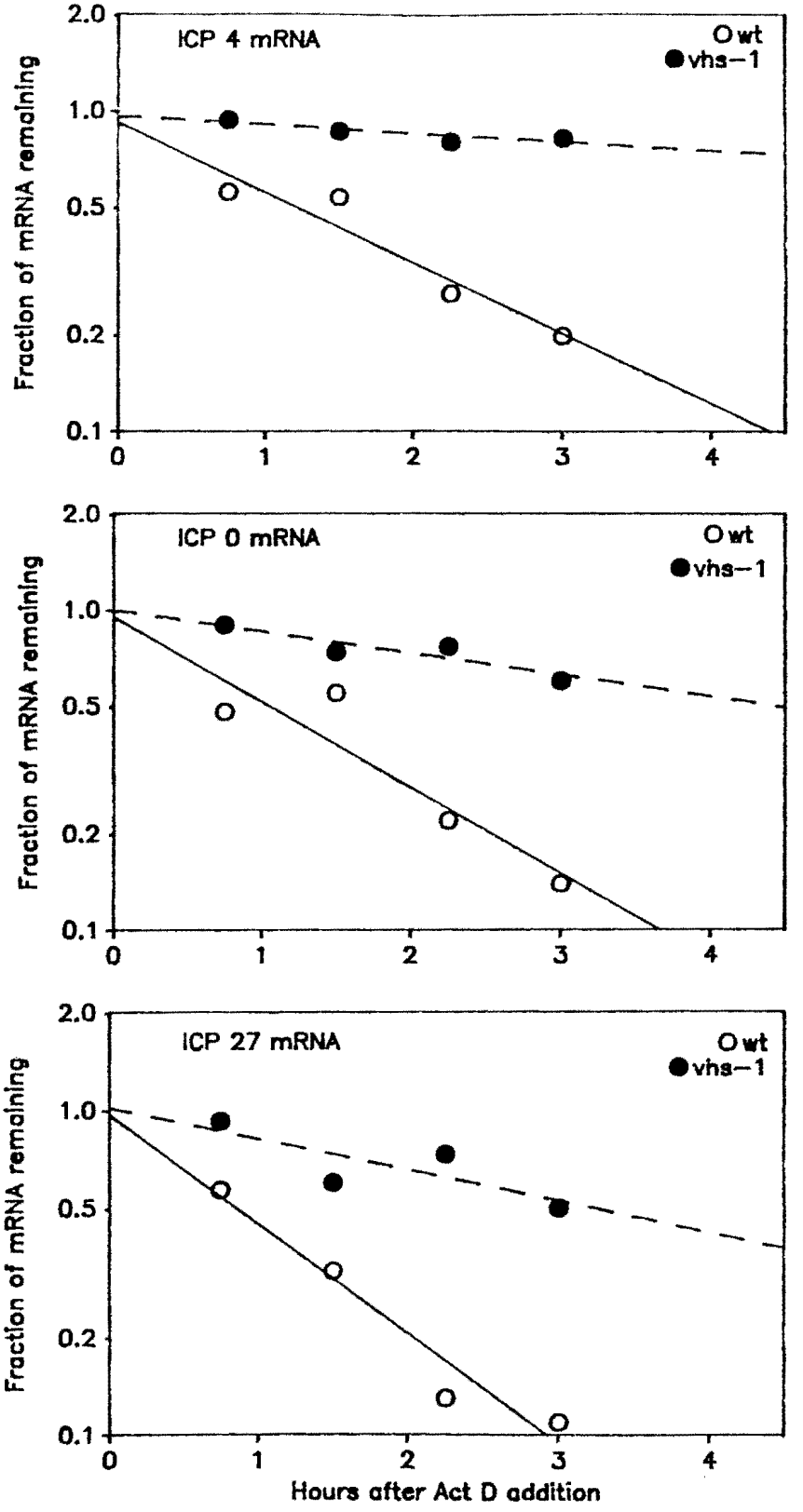

ICP 4 mRNA
O wt
● vhs−1
Fraction of mRNA remaining
2.0
1.0
0.5
0.2
0.1
0
1
2
3
4
ICP 0 mRNA
O wt
● vhs−1
Fraction of mRNA remaining
2.0
1.0
0.5
0.2
0.1
0
1
2
3
4
ICP 27 mRNA
O wt
● vhs−1
Fraction of mRNA remaining
2.0
1.0
0.5
0.2
0.1
0
1
2
3
4
Hours after Act D addition

Figure 15 Decay of beta mRNAs. Vero cells were infected with 20 pfu/cell of either the wild-type or the vhs 1 mutant virus. At six hours post infection Actinomycin-D was added to a final concentration of 5μg/ml and the incubation continued. Total cytoplasmic RNAs were isolated at the time of Actinomycin-D addition and at 45 min intervals up to 3 h after the start of chase. Five μg RNA samples were analyzed by Northern blotting. The blots were probed for viral mRNAs and for 28S rRNA. The autoradiograms were scanned at 500 nm in a Gilford Response Spectrophotometer and the integrated peak areas, each from the wild-type and the vhs 1 mutant were used to normalize the amount of RNA with respect to their rRNA contents respectively. This normalization enabled us to correct for errors in loading equal amounts of cytoplasmic RNA onto each lane of the gel. The fraction of mRNA remaining at time 't' was then plotted as a logarithmic plot with linear regression using the SigmaPlot program as described in the section on statistical analyses in Materials and Methods. Autoradiograms of the resulting gels were scanned in order to quantitate the levels of beta mRNAs encoding thymidine kinase (TK), DNA-binding protein (ICP8), and DNA polymerase (DNA pol).

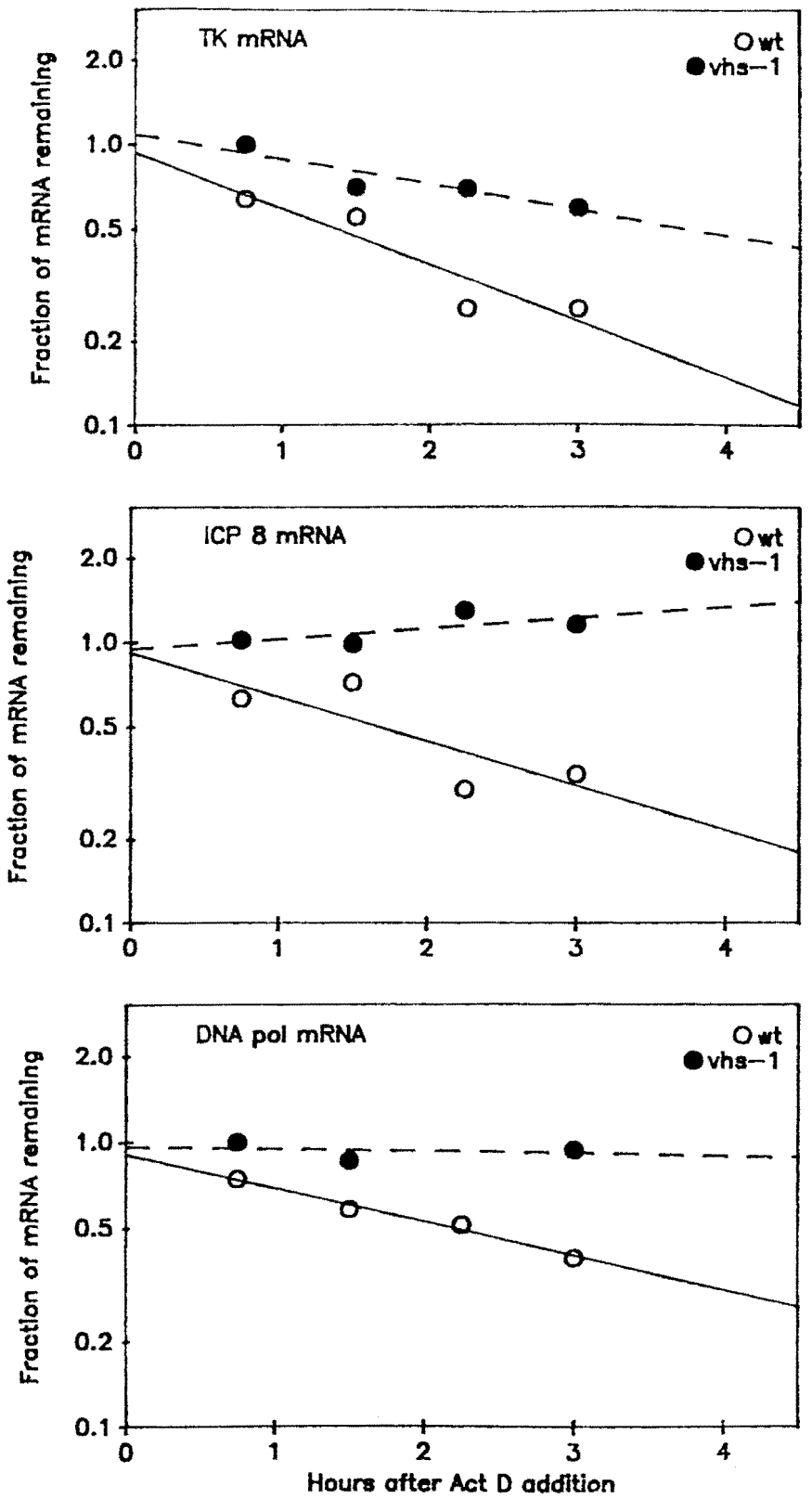

TK mRNA
wt
vhs−1
Fraction of mRNA remaining
2.0
1.0
0.5
0.2
0.1
0
1
2
3
4
ICP 8 mRNA
wt
vhs−1
Fraction of mRNA remaining
2.0
1.0
0.5
0.2
0.1
0
1
2
3
4
DNA pol mRNA
wt
vhs−1
Fraction of mRNA remaining
2.0
1.0
0.5
0.2
0.1
0
1
2
3
4
Hours after Act D addition

Figure 16 Decay of beta/gamma mRNAs. Vero cells were infected with 20 pfu/cell of either the wild type or the vhs 1 mutant virus. At six hours post infection Actinomycin-D was added to a final concentration of 5μg/ml and the incubation continued. Total cytoplasmic RNAs were isolated at the time of Actinomycin-D addition and at 45 min intervals up to 3 h after the start of chase. Five μg RNA samples were analyzed by Northern blotting. The blots were probed for viral mRNAs and for 28S rRNA. The autoradiograms were scanned at 500 nm in a Gilford Response Spectrophotometer and the integrated peak areas, each from the wild-type and the vhs 1 mutant were used to normalize the amount of RNA with respect to their rRNA contents respectively. This normalization enabled us to correct for errors in loading equal amounts of cytoplasmic RNA onto each lane of the gel. The fraction of mRNA remaining at time 't' was then plotted as a logarithmic plot with linear regression using the SigmaPlot program as described in the section on statistical analyses in Materials and Methods. Autoradiograms of the resulting gels were scanned in order to quantitate the levels of beta/gamma mRNAs encoding the major capsid protein (ICP5), glycoprotein B (gB), and glycoprotein H (gH).

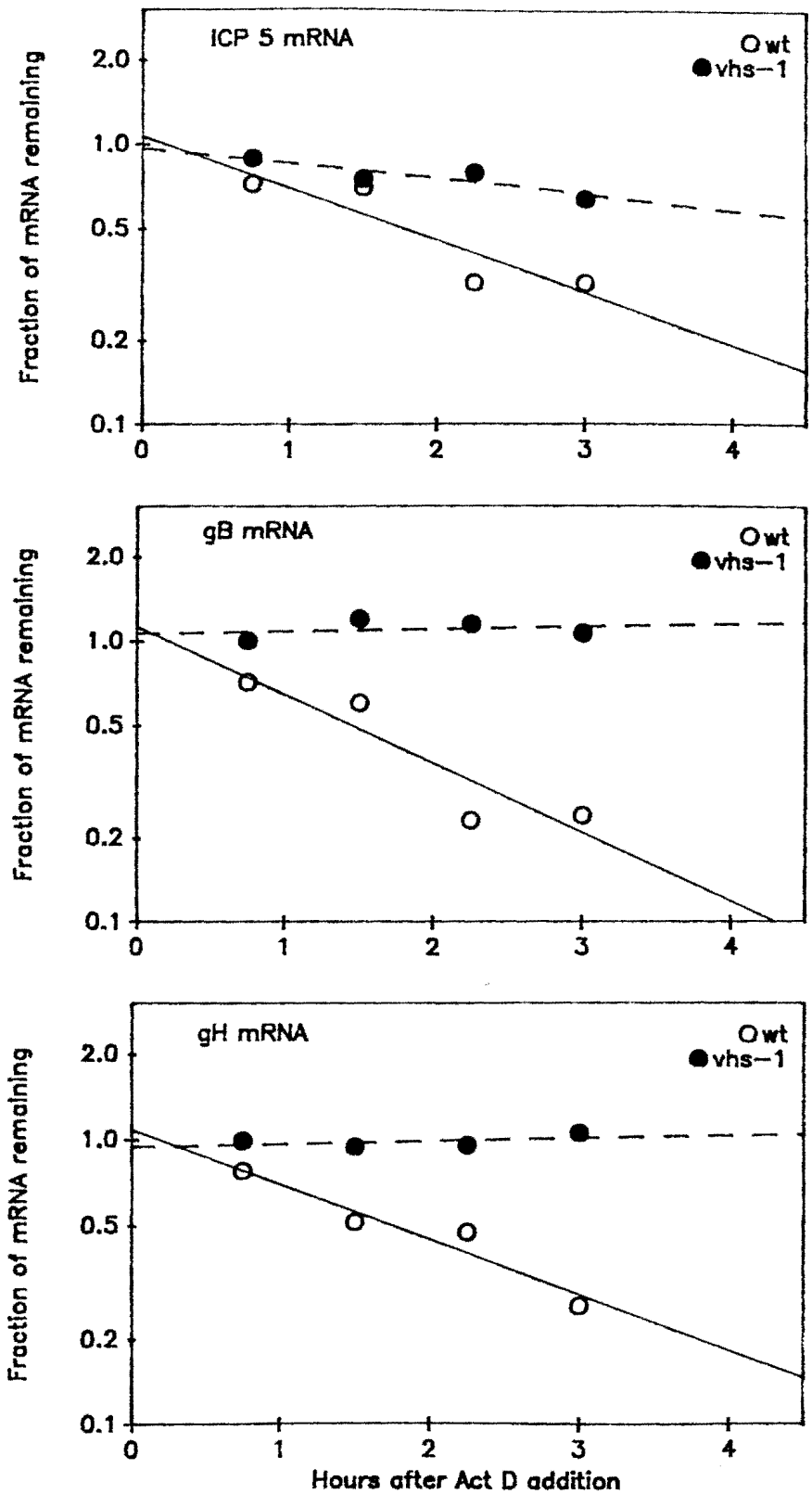

ICP 5 mRNA
gB mRNA
gH mRNA
O wt
● vhs−1
Fraction of mRNA remaining
2.0
1.0
0.5
0.2
0.1
0
1
2
3
4
Hours after Act D addition

Figure 17 Decay of gamma mRNAs. Vero cells were infected with 20 pfu/cell of either the wild type or the vhs 1 mutant virus. At six hours post infection Actinomycin-D was added to a final concentration of 5μg/ml and the incubation continued. Total cytoplasmic RNAs were isolated at the time of Actinomycin-D addition and at 45 min intervals up to 3 h after the start of chase. Five μg RNA samples were analyzed by Northern blotting. The blots were probed for viral mRNAs and for 28S rRNA. The autoradiograms were scanned at 500 nm in a Gilford Response Spectrophotometer and the integrated peak areas, each from the wild-type and the vhs 1 mutant were used to normalize the amount of RNA with respect to their rRNA contents respectively. This normalization enabled us to correct for errors in loading equal amounts of cytoplasmic RNA onto each lane of the gel. The fraction of mRNA remaining at time 't' was then plotted as a logarithmic plot with linear regression using the SigmaPlot program as described in the section on statistical analyses in Materials and Methods. Autoradiograms of the resulting gels were scanned in order to quantitate the levels of gamma mRNAs encoding glycoprotein C (gC).

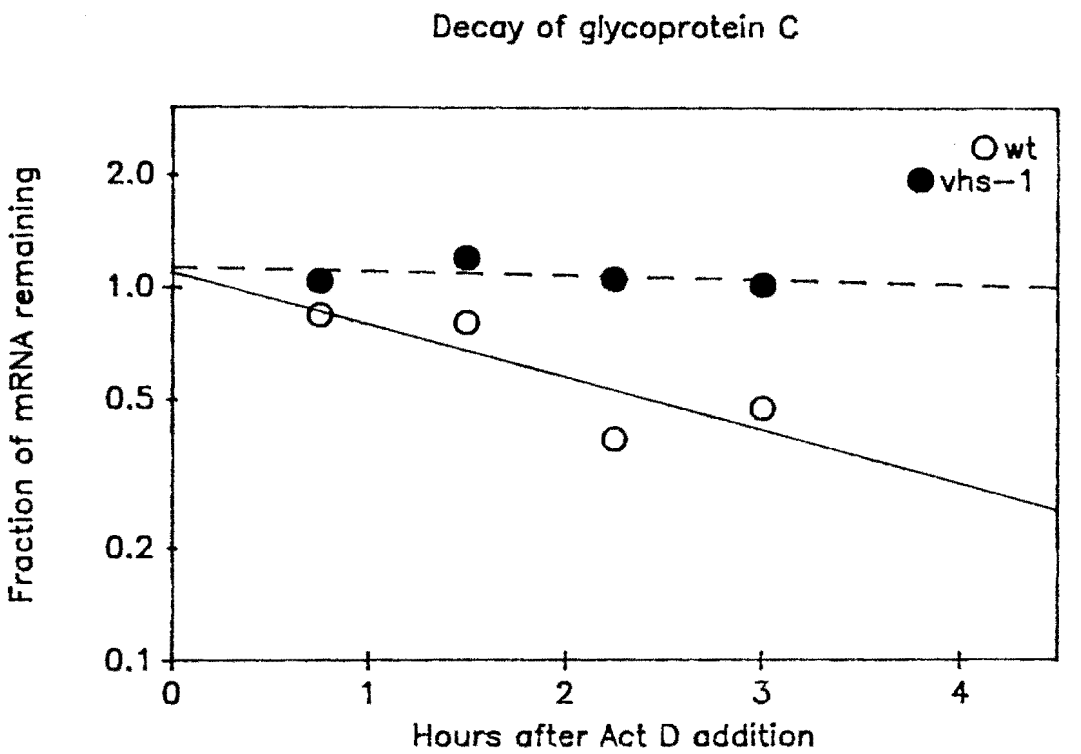
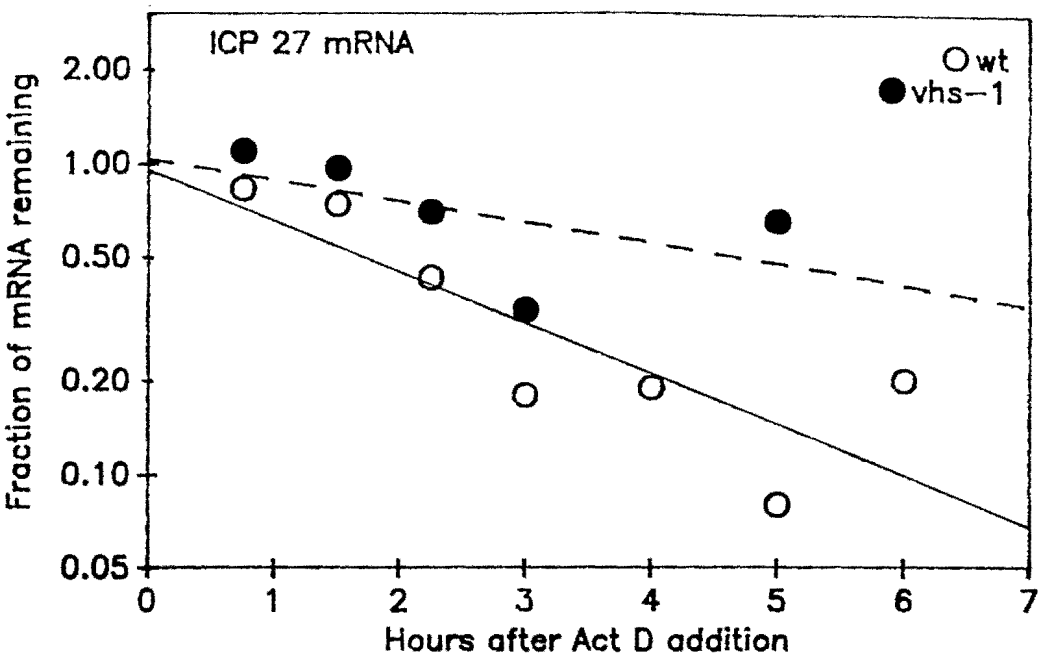

Decay of glycoprotein C
Fraction of mRNA remaining
2.0
1.0
0.5
0.2
0.1
0
1
2
3
4
Hours after Act D addition
O wt
● vhs−1

Figure 18 Decay of alpha and beta mRNAs. Experiments were repeated to determine the viral mRNA half lives in the presence of Actinomycin-D. The experiment was performed exactly as in Figs. 14 and 15, except that total cytoplasmic RNA was extracted for a period up to 6 h after the addition of Actinomycin-D. Autoradiograms of the resulting gels were scanned in order to quantitate the levels of mRNAs encoding ICP27 and thymidine kinase (TK).


ICP 27 mRNA
O wt
● vhs−1
Fraction of mRNA remaining
2.00
1.00
0.50
0.20
0.10
0.05
0
1
2
3
4
5
6
7
Hours after Act D addition

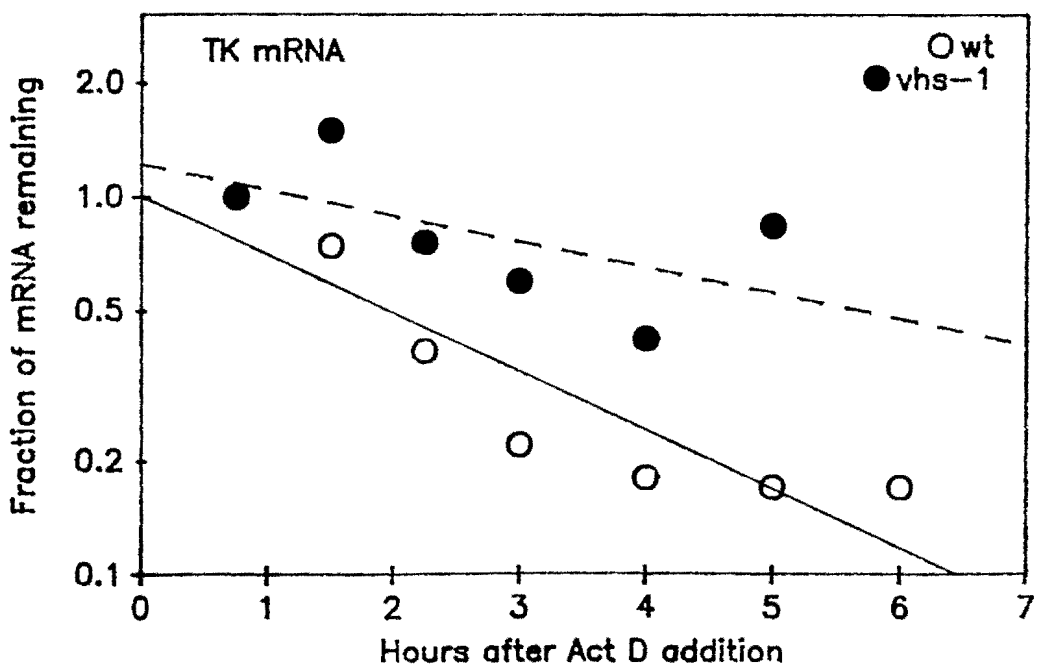

TK mRNA
O wt
● vhs−1
Fraction of mRNA remaining
2.0
1.0
0.5
0.2
0.1
0
1
2
3
4
5
6
7
Hours after Act D addition

Figure 19 Decay of beta/gamma and gamma mRNAs. Experiments were repeated to determine viral mRNA half lives in the presence of Actinomycin-D. The experiment was performed exactly as in Figs. 16 and 17, except that total cyoplasmic RNA was extracted for a period up to 6 hr after the addition of Actinomycin-D. Autoradiograms of the resulting gels were scanned in order to quantitate the levels of mRNAs encoding ICP27 and thymidine kinase (TK).

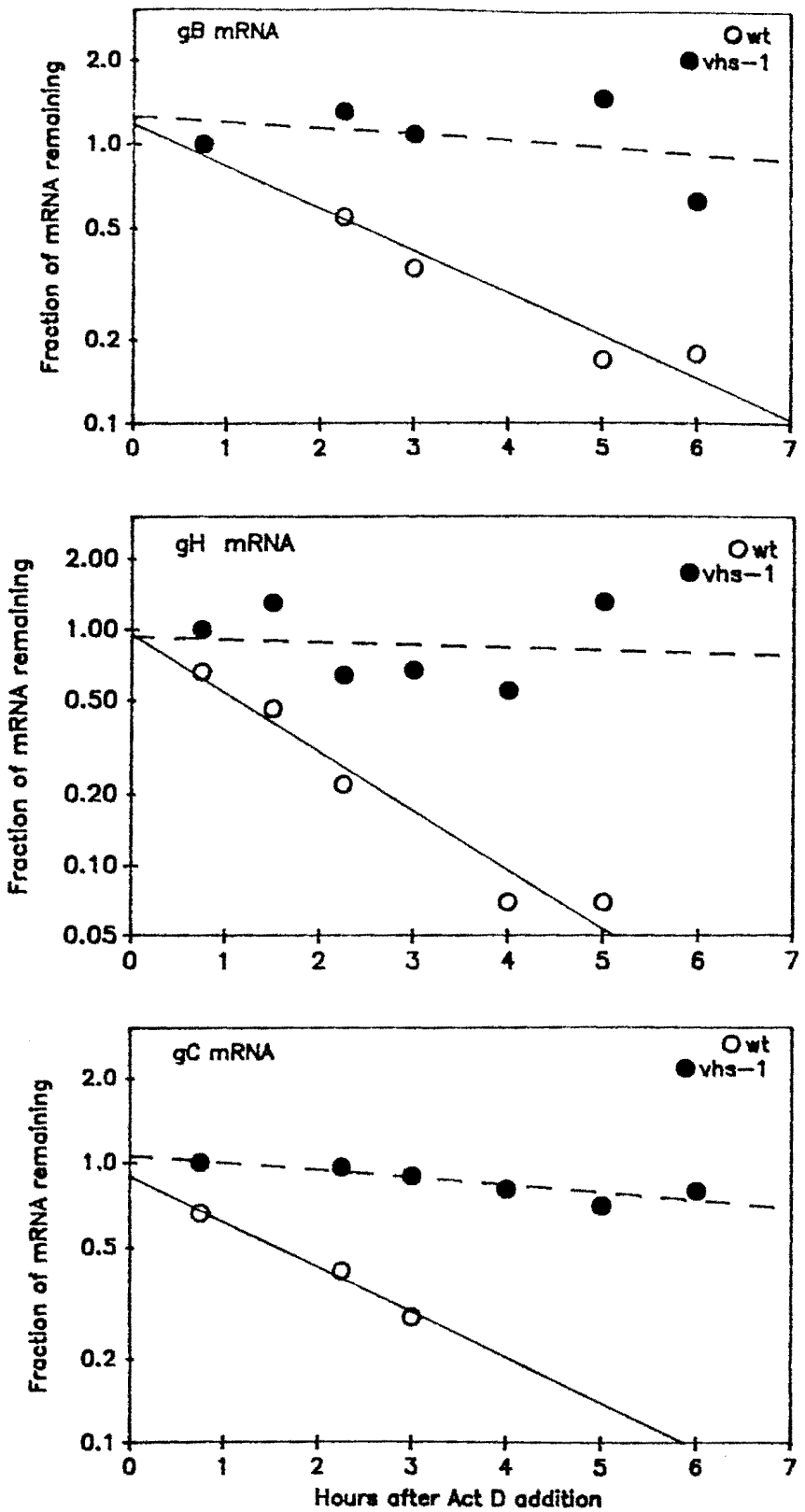

gB mRNA
wt
vhs−1
Fraction of mRNA remaining
2.0
1.0
0.5
0.2
0.1
0
1
2
3
4
5
6
7
gH mRNA
wt
vhs−1
Fraction of mRNA remaining
2.00
1.00
0.50
0.20
0.10
0.05
0
1
2
3
4
5
6
7
gC mRNA
wt
vhs−1
Fraction of mRNA remaining
2.0
1.0
0.5
0.2
0.1
0
1
2
3
4
5
6
7
Hours after Act D addition

TABLE 1

HALF-LIVES OF HSV-1 VIRAL mRNAs IN THE PRESENCE OF ACTINOMYCIN-D[a]

mRNA	Half-life (h)			
	Wild-type virus		vhs 1	
	Expt 1	Expt 2	Expt 1	Expt 2
ICP4	1.4		S[b]	
ICP0	1.1		4.4	
ICP27	0.9	1.8	3.3	4.4
TK	1.5	1.9	4.1	4.4
DNA pol	2.5		S	
ICP8	2.0		S	
ICP5	1.6		5.5	
gB	1.3	1.9	S	S
gH	1.6	1.2	S	S
gC	2.1	1.8	S	S

[a] Determined from data shown in Fig. 14 through 19, using the formula described in Materials and Methods.

[b] S, Half-life greater than 8 h.

other, cannot be attributed to differential sensitivity of the mRNAs to vhs-mediated degradation. If, as our studies indicate, the vhs gene product degrades all viral mRNAs without regard to their class affiliation, then the accumulation profiles of beta/gamma, and gamma mRNAs suggests that continuing mRNA transcription and transport of these mRNAs contributes significantly to their cytoplasmic steady state levels at late times after infection.

3.5 Determination of proportionality between length of film exposure and optical density

Half lives of some of the viral messages were determined from two separate experiments. On both occasions, the viral messages followed a similar kinetics of decay after the addition of Actinomycin D. Nevertheless, we wished to check the reproducibility of half life determinations by studying other variables that could potentially have a significant effect on our conclusions.

The first question addressed was whether the DNA probes were in sufficient excess to drive the hybridization of messages to completion.

The following assumptions were made:

1. At six hours post HSV-1 infection about 70% of total polyribosome associated mRNA is HSV specific. This RNA is made up of both polyadenylated and nonpolyadenylated RNA. Furthermore, the proportion of nonpolyadenylated RNA is invariant between the monosome and polyribosome fractions. One can therefore assume that in cells infected for 6 hr, the amount of viral RNA under vhs modulation is about 70% of total cellular mRNA (Stringer et al., 1977).

2. All of the possible 70 genes (McGeoch et al., 1988) encoded by the HSV-1 are transcribed in sufficient amounts contributing to a pool of at least 70 different viral mRNAs at six hours post infection.

3. About 1% of the total cytoplasmic RNA is messenger RNA.

One can then calculate that of the 50 ng of cellular mRNA (about 1% of 5000 ng of total cellular RNA per lane), the total amount of virus specific mRNA would be about 35 ng (70% of 50 ng). With 70 different viral genes transcribed, about 0.5 ng mRNA (35 ng/ 70 mRNA types) is contributed by each type of viral mRNA. Since each blot contained a total of 5 lanes representing viral mRNA from either the wild-type or the vhs 1 infected cells, each blot contained a total of 0.5 X 5 or 2.5 ng of each specific viral RNA. The probe or the DNA inserts averaged about 1/7 or (1 kb of 7.2 kb M13mp vectors) of the radioactive probe. Since, 1 μg of that DNA was nick translated, then 143 ng of it was HSV specific. Thus 143 ng of viral DNA specific probe was added to each blot containing a total of 2.5 ng of each specific viral message. Thus, when a blot was probed, about 57 fold of excess DNA probe specific for each viral gene was present.

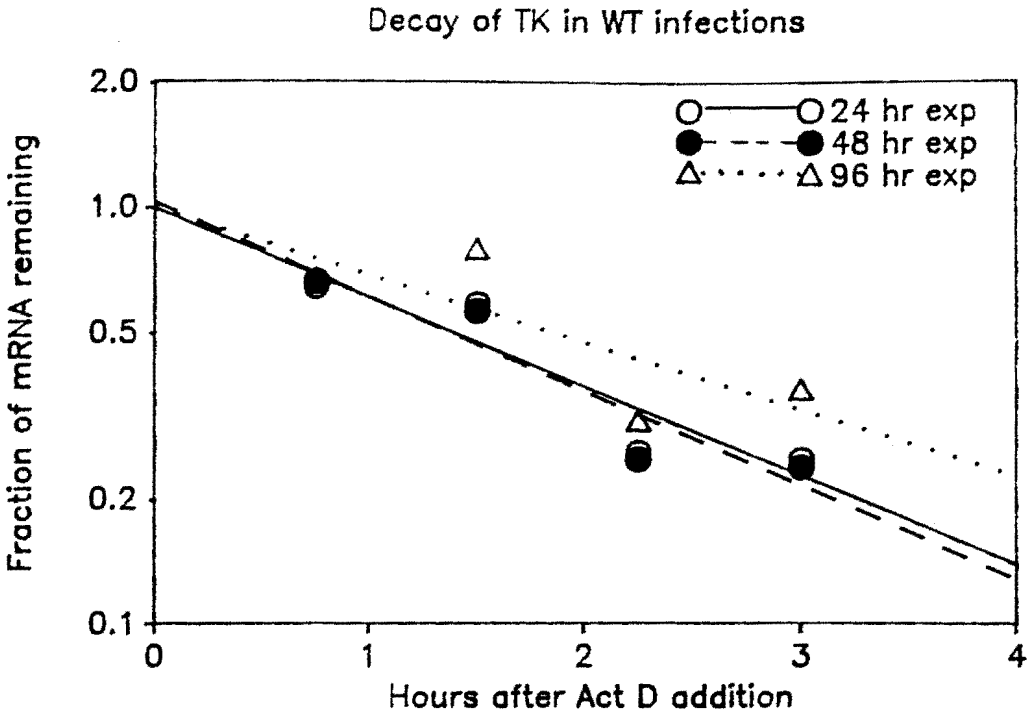
Finally, the two separate determinations of viral mRNA half-lives were performed with probes nick translated on different ocassions. It is therefore possible that the two different ^{32}[P]-labeled probe preparations could have slightly different specific activities. If the specific activity of the probe was so critical, then it would have given rise to different decay kinetics in repeat measurements. It is obvious that our results have been consistent even when the determinations were made on different RNA preparations and hybridized with pro-

bes of probably variable specific activities. We can therefore conclude that the complementary DNA probes were present in sufficient excess to allow the hybridation reaction to go to completion.

A second variable that could potentially affect our results was the linear response range of the X-ray sensitive film. We addressed this aspect by determining if the autoradiograms were indeed scanned in the linear response range of the X-ray film. In this range, the intensity of exposed silver grains is directly proportional to the length of its exposure to irradiation. Representative scans of multiple exposures for the decay of thymidine kinase and DNA polymerase mRNA from wild-type infections and the glycoprotein H mRNA from vhs 1 infection are shown in Figures 20 and 21. All of these measurements were carried out on autoradiographic exposures from the first experiment. In the case of thymidine kinase mRNA in wild type infections, the half-lives from 24 h, 48 h, and 96 h exposures corresponded to 1.5, 1.3, and 1.9 h respectively. Values from 24 h exposures have beeen used. With the DNA pol mRNA in wild-type infections, the half lives from 6 h, 17 h, 24 h, and 48 h exposures were 2.8 h, 2.5 h, 2.7 h, and 8.2 h respectively. Obviously, in this case the 48 h exposure denotes an overexposed film. Therefore, half-life values from the 17 h exposure were used for DNA polymerase mRNA decay in wild type infections.

Finally, glycoprotein H mRNA half-lives in vhs 1 infections ranged from 22 h, 25 h, and 52 hr in films exposed for 24, 48, and 96 h respectively. The half life for glycoprotein H from the 48 h exposures was used in the vhs 1 infected group. Thus, all representative plots provide sufficient evidence to our claim that half-lives of each viral

Figure 20 Linear response range of the X-ray film for autoradiographic exposures for viral mRNA decay in wild-type infections. Northern blots of experiments performed in Figure 15 were exposed for varying lengths of time on X-ray films at -90°C. For viral RNAs from each autoradiographic image, the normalization was performed exactly as in Figure 15. The fraction of viral mRNA remaining was then plotted as regression curves on the Sigma plot program written for the IBM PC. Autoradiograms of blots from wild-type infected cells were scanned to quantitate the levels of thymidine kinase (TK) and DNA polymerase (DNA pol) mRNAs.


Decay of TK in WT infections
Fraction of mRNA remaining
2.0
1.0
0.5
0.2
0.1
0
1
2
3
4
Hours after Act D addition
24 hr exp
48 hr exp
96 hr exp

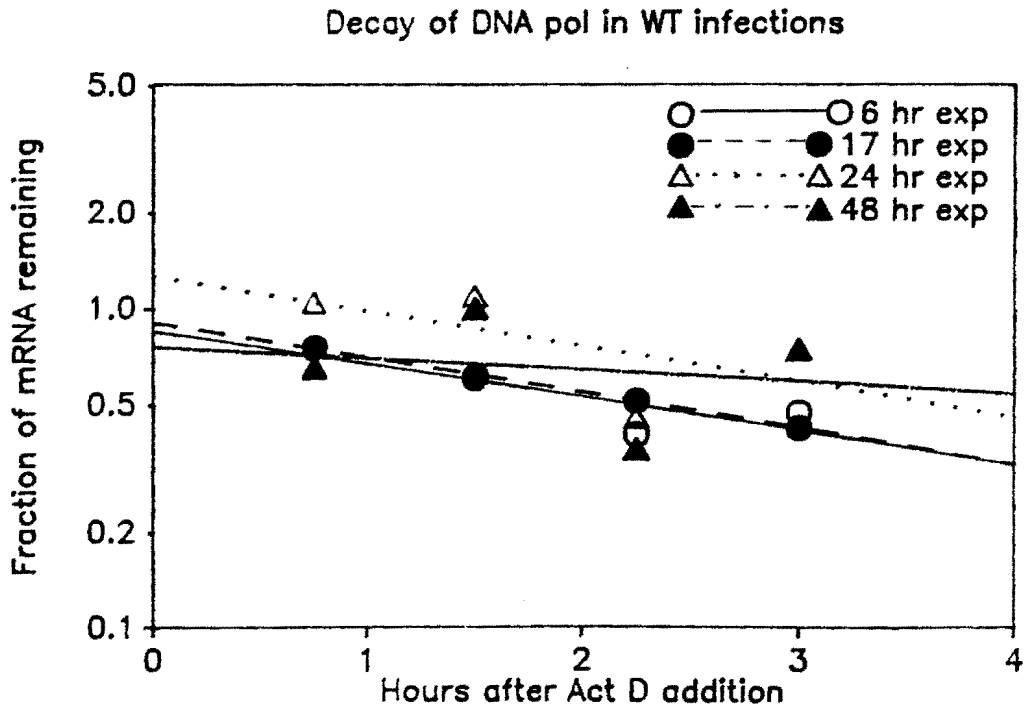
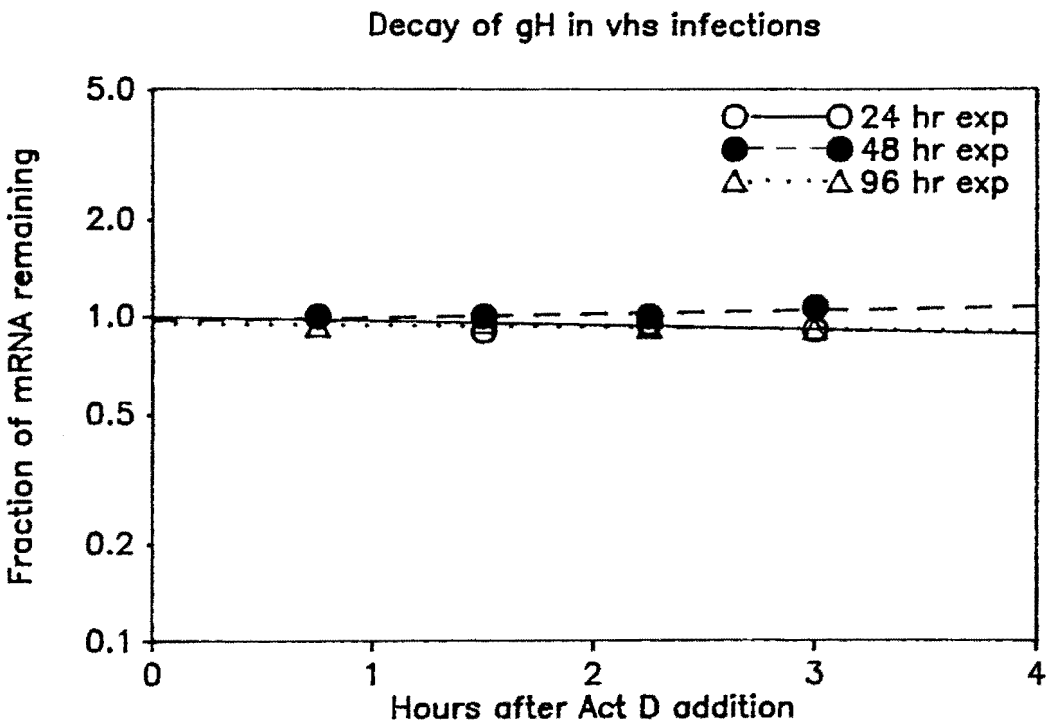

Decay of DNA pol in WT infections
Fraction of mRNA remaining
5.0
2.0
1.0
0.5
0.2
0.1
0
1
2
3
4
Hours after Act D addition
6 hr exp
17 hr exp
24 hr exp
48 hr exp

Figure 21 Linear response range of the X-ray film for autoradiographic exposures for viral mRNA decay in vhs 1 infections. Northern blots of experiments performed in Figs. 15 and 16 were exposed for varying lengths of time on X-ray films at -90°C. For viral RNAs from each autoradiographic image, the normalization was performed exactly as in Figure 15. Viral RNAs were normalized to the content of 28S rRNA within that sample. The fraction of viral mRNA remaining was then plotted as regression curves on the Sigma plot program written for the IBM PC. Autoradiograms of blots from vhs 1 infected cells were scanned to quantitate the levels of glycoprotein H (gH) mRNA.


Decay of gH in vhs infections
Fraction of mRNA remaining
5.0
2.0
1.0
0.5
0.2
0.1
0
1
2
3
4
Hours after Act D addition
24 hr exp
48 hr exp
96 hr exp

mRNA have been correctly read in the linear response range of the film.

We next determined whether the rest of the autoradiographic images were also read in the linear response range of the film. Table 2 shows the highest values of actual peak areas for mRNA bands scanned at 500 nm in the Gilford spectrophotometer Response model. From the analysis of half lives obtained from Figure 20 and 21 we concluded that all the peak areas determined at 500 nm between the range of 0.1 (lowest peak area for 6 h exposure of DNA polymerase mRNA from wild type infections) to 5.9 (highest peak area for 48 h exposure of thymidine kinase mRNA from wild type infections) fell within the linear response range of the X-ray film. Our line of reasoning is as follows. In the case of the DNA polymerase mRNA from wild type infections, the decay curve obtained from the peak areas of 6 h, 17 h, and 24 h exposures, each gave similar half lives ranging from 2.8 h, 2.5 h, and 2.7 h. Furthermore, as the lowest peak area obtained from 6 h exposed film was 0.1, we can assume that an area of 0.1 is in the linear response range of a properly exposed film. In the case of the thymidine kinase mRNA from wild-type infections, the decay curves obtained with 24 h and 48 h exposures, each gave similar half lives of 1.5 and 1.3 h respectively. Again, since the highest peak area obtained with the thymidine kinase mRNA bands from 48 h exposed films was 5.9, we can conclude that a peak area of 5.9 also falls within the linear response range of an appropriately exposed film. Thus peak areas of less than 0.1 may represent films that were underexposed, and those over 5.9 may indicate that the film was overexposed. Table 2 has been compiled to show the minimum and the maximum absorbance peak areas determined at 500 nm, for RNAs

TABLE 2

RANGE OF PEAK AREAS OF AUTORADIOGRAPHIC IMAGES* SCANNED AT 500 nm

	Wild type		vhs 1	
	Minimum	Maximum	Minimum	Maximum
ICP4	0.64	1.1	0.9	1.7
ICP0	0.15	0.4	0.53	0.9
ICP27	0.17	0.7	1.0	1.8
TK				
24 hr exp	1.4	2.8	2.2	3.1
48 hr exp	2.8	5.9		
96 hr exp	7.1	11.5		
DNA pol				
6 hr exp	0.09	0.12		
17 hr exp	0.27	0.4	0.37	0.6
24 hr exp	0.39	0.79		
48 hr exp	2.5	11.5		
ICP8	0.8	1.2	0.4	0.7
ICP5	0.36	0.6	1.8	3.4
gH				
24 hr exp			0.18	0.3
48 hr exp			0.4	0.65
96 hr exp	0.32	0.7	0.84	1.5
gB	0.7	1.7	0.15	0.3
gC	1.1	1.9	0.8	1.6

* From Fig. 8 and 9

from both the wild type and vhs 1 infected cells. Whenever appropriate the length of the film exposure has been indicated in the table. In each case, the autoradiographic images represent only those films that have been used to plot viral mRNA decay curves. It is thus quite evident that the exposures and peak areas used in calculating half-lives from autoradiographic images of other viral mRNAs also fall within the linear response range of the film. We conclude that the half-lives of all viral mRNAs analyzed have therefore been properly calculated.

3.6 Measurement of mRNA half-lives using a pulse chase protocol

There are several general methods that attempt to assay the rate of turnover of mRNAs. Half-life measurements under Actinomycin D chase have produced meaningful results in several viral and nonviral systems. However, there is never an assurance that mRNA turnover goes on normally in the face of complete inhibition of transcription. If similar results can be obtained with an Actinomycin D chase and some other method, this tends to validate both techniques. We therefore chose to study viral mRNA stability by the pulse chase protocol.

HeLa cells were infected with 20 pfu/cell of wild type-virus for about 5.5 hours. The cells were then pulsed with ^{3}[H]-uridine for 30 minutes. Following the pulse, the label was removed, the cells washed and then overlaid with chase medium containing an excess of cold uridine, cold cytidine, and glucosamine. Total cytoplasmic RNA was extracted immediately and at 30 min intervals up to the first hour and then at hourly intervals for 6 h after the start of chase. The amounts of label specific for the alpha ICP0 and beta thymidine kinase mRNAs were

measured by hybridization of total cytoplasmic RNA to specific DNA probes fixed onto nitrocellulose filter disks. The background counts obtained by hybridization with the chicken thymidine kinase probe were subtracted from the counts obtained by specific hybridization to the two viral DNA probes specific for ICP0 and the thymidine kinase mRNAs. These values were then plotted as regression curves on the Sigma plot program of the IBM-PC. Plots of the decay curves are shown in Figure 22 and the mRNA half lives calculated from both the SigmaPlot and the Expofit programs are shown in Table 3. ICP0 and TK mRNA half-lives were comparable when analyzed both by the SigmaPlot and the Expofit programs. Also, the half lives of viral mRNAs representing the alpha and beta mRNA classes as determined through pulse chase protocols closely matched the values obtained with Actinomycin-D chase procedure (compare Tables 1 and 3). Despite the pros and cons peculiar to each methodology, the estimates of half-lives calculated were surprisingly similar. These results strongly suggest that the vhs function acts to regulate cytoplasmic levels of many, if not all, viral mRNAs by degrading them apparently in a nonselective fashion.

Figure 22 Measurement of mRNA half lives by the pulse-chase protocol. HeLa cells were infected with 20 pfu/cell of the wild-type virus. At 5.5 h post infection cells were pulse labeled with ^{3}[H]-uridine for 30 minutes. At the end of the labeling period, the label was washed off and the medium was replaced with one containing excess cold uridine, cytidine, and glucosamine. Total cytoplasmic RNA was extracted at the start of chase and at approximately 1 h intervals after the start of chase. RNA was then hybridized to excess HSV-1 DNA immobilized onto nitrocellulose filter disks. The background counts obtained by hybridization of the labeled RNA with the chicken thymidine kinase were subtracted from the counts obtained by specific hybridization of the labeled RNA to each of the two viral DNA probes. Figure 22 shows the decrease in labeled mRNA encoding ICP0 and thymidine kinase (TK) measured for a period of 6 h after the start of chase.

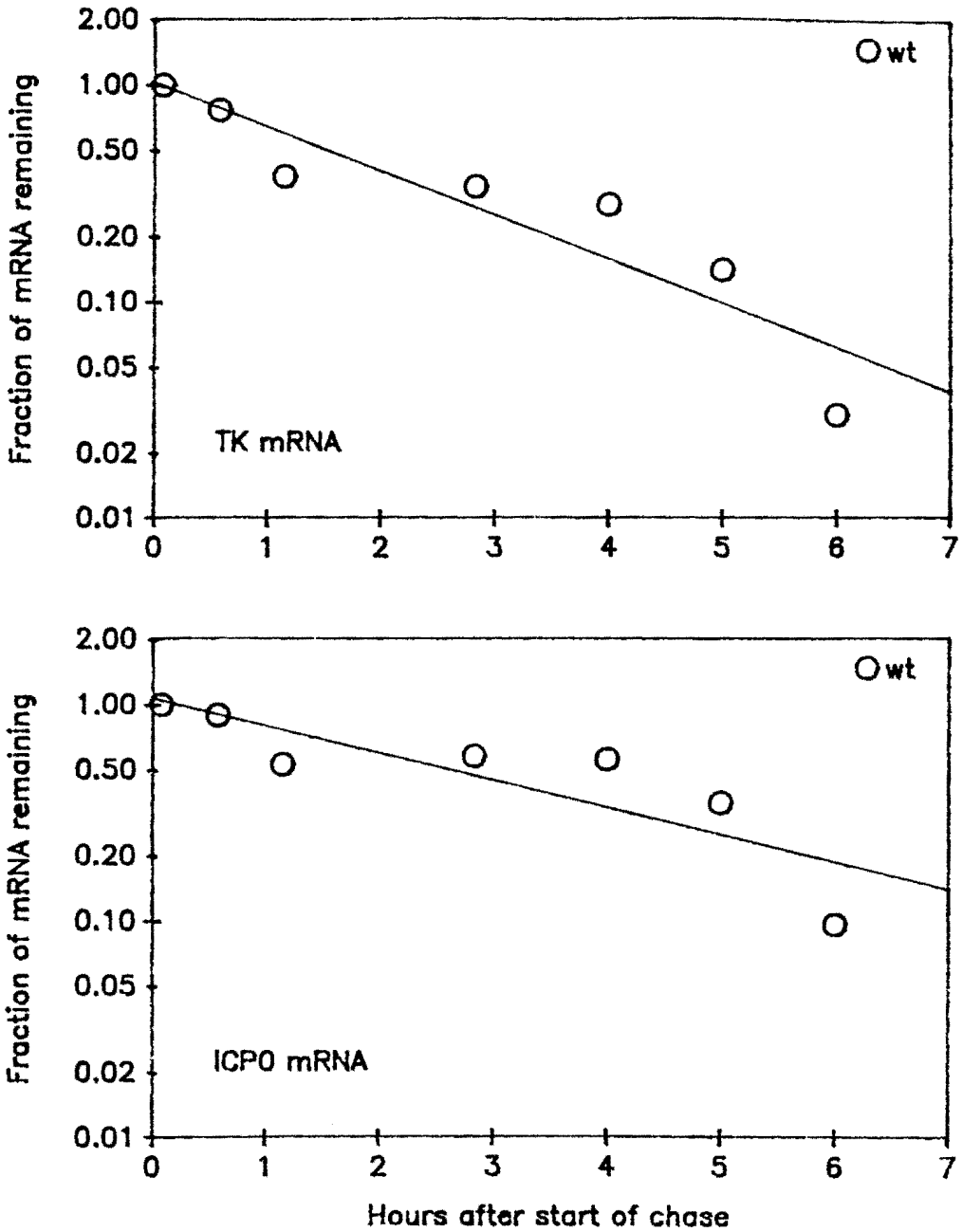

2.00
1.00
0.50
0.20
0.10
0.05
0.02
0.01
Fraction of mRNA remaining
wt
TK mRNA
0
1
2
3
4
5
6
7
2.00
1.00
0.50
0.20
0.10
0.05
0.02
0.01
Fraction of mRNA remaining
wt
ICP0 mRNA
0
1
2
3
4
5
6
7
Hours after start of chase

TABLE 3

HALF-LIVES OF HSV-1 VIRAL mRNAs MEASURED THROUGH PULSE CHASE

mRNA	Half-lives (h) in wild-type infections	
ICP0	2.4[a]	3.1[b]
TK	1.5[a]	1.6[b]

[a] Determined by linear regression analyses of the logarithmic plots in Fig. 22

[b] Determined by fitting mRNA levels to an exponential decay curve using Expofit (Elsevier-Biosoft, Elsevier Science Publishers, Amsterdam).

DISCUSSION

One of the major objectives of this study was to determine whether the HSV-1 wild type vhs gene product showed selectivity in inducing degradation of messenger ribonucleic acid. The experiments reported here demonstrate that the vhs gene product affected the stabilities of viral mRNAs belonging to all kinetic classes. This study involved a systematic measurement of the half-lives of 10 different viral mRNAs belonging to different kinetic classes. Ours is the first such report that has attempted to determine the half lives of viral mRNAs from either the wild type or vhs 1 infected cells. We found that the wild type vhs protein affected the stability of every viral mRNA in a manner such that when measured beginning at 6 hours post infection, every viral mRNA had a similar half life. Furthermore, the vhs 1 mutation dramatically increased the stabilities of all viral mRNAs thereby increasing their half lives several fold.

Immediately after infection, the vhs protein enters the cell as a component of the infecting virion and causes degradation of a variety of preexisting cellular mRNAs (Fenwick and McMenamin, 1984, Schek and Bachenheimer, 1985, and Strom and Frenkel, 1987). The conclusion that the vhs gene product is nonselective, is further strengthened by a

report from Smiley and coworkers (1987). They found that when a rabbit beta globin gene was inserted into the HSV-1 viral genome, the accumulation kinetics of the resulting beta globin mRNA were similar to those of beta viral messages. It can be said with little reservation that in an appropriate virus-cell combination, the vhs gene product negatively regulates the half lives of almost all mRNAs with little, if any, target selectivity. Studies on the accumulation profiles of mRNAs showed that the vhs 1 mutation caused an overaccumulation of a number of viral mRNAs. This effect was most dramatic for the alpha and beta mRNAs studied. On the other hand, the overaccumulation was less dramatic for the beta/gamma and the gamma class of messages. This was true despite the apparent lack in target selectivity of vhs-mediated decay of alpha, beta, beta/gamma, and gamma mRNAs. The results of the accumulation profiles and half-lives of viral mRNAs together suggest that the vhs protein is important in determining the cytoplasmic levels of alpha and beta mRNAs and therefore plays an important role in down regulation at late times in alpha and beta gene expression.

Role of the vhs gene product in the accumulation kinetics of viral mRNAs.

The steady state levels of mRNA reflect a balance between biosynthesis and degradation. If either the mRNA synthesis or its stability is modified, it would affect its accumulation. It follows that if the vhs 1 mutation had a striking effect on viral mRNA stability then the accumulation patterns should be drastically modified. We therefore determined the accumulation profiles of mRNAs belonging to all four

kinetic classes. Our studies brought forth interesting results that will ultimately help in understanding the multifaceted regulation of particular viral genes, and enable the comparison of expression of genes within a class, and between genes of different classes.

Accumulation profiles of alpha and beta mRNA

The accumulation profiles of alpha ICP0 and ICP27 mRNAs in wild type infections were quite dissimilar, despite their traditional classification as members of the same class of viral genes. Although, the levels of both mRNAs were detected very early, and increased to the maximum around 6 h post infection, the levels of ICP27 mRNA eventually declined whereas those of ICP0 mRNA persisted at high levels even after 12 h of infection. In fact, the alpha ICP27 mRNA profile appeared much like those of the beta mRNAs, except that its decline was not as complete or abrupt. Our results on alpha ICP0 and ICP27 are in close agreement with those already published by Harris-Hamilton and Bachenheimer (1985), and Weinheimer and McKnight (1987). With regard to the accumulation profiles of alpha ICP4 mRNA, both Harris-Hamilton and Bachenheimer (1985), and Weinheimer and McKnight (1987), reported that in wild-type infections, accumulation of alpha ICP0 and ICP4 mRNAs was similar at earlier times in infection. However, at late times of infection, the mRNA for ICP4 persisted at lower levels and the mRNA for ICP0 persisted at fairly high levels as compared to their peak levels of accumulation. In addition, Weinheimer and McKnight (1987), discovered that the temporal pattern of alpha ICP22/47 mRNA accumulation was not similar to that of the ICP0 mRNA. The ICP22/47 transcripts

steadily declined in abundance between 6 to 10 h post infection and were undetectable by 12 h post infection.

The temporal pattern of alpha mRNA transcription in wild-type infections has been studied by Weinheimer and McKnight (1987). Of the three alpha mRNAs examined in the nuclear runoff transcription assays, two strikingly different patterns of synthesis were seen. The ICP0 and ICP22/47 genes were transcribed at increasing rates from 1 to 6 h post infection. In contrast, transcription of the alpha ICP4 gene appeared to be repressed after 1 h post infection, and then reactivated 4 to 6 h post infection. It is worth mentioning that between 4 to 6 h post infection, the nuclear runoff transcription assay showed nonspecific transcription of HSV-1 genes. Godowski and Knipe (1986), had also seen symmetric transciption of the coding and noncoding DNA strands of the alpha ICP4 gene during this time interval. Several possibilities could account for this effect. It is possible that at late times of infection, termination of transcription may not be a tightly regulated process. Thus read through transcription may occur. Furthermore, it is also conceivable that cryptic promoters may compete giving transcripts with different start sites. Thus, fidelity of the transcription machinery may be affected after 4 to 6 hours of infection, if studied in vitro. All results after this cutoff interval therefore, should be interpreted with caution. Nevertheless, when taken together, these differences may indicate different modes of regulation even within the alpha class and warrant their grouping into two or more subsets of differentially expressed alpha genes.

Our studies on the mutant showed that the vhs 1 mutation had a

significant effect on the alpha mRNA accumulation profile. With both ICP0 and ICP27, the mRNA levels were in excess of their wild-type counterparts at almost every point late in infection. The levels of both ICP0 and ICP27 mRNAs increased up to 8 hours post infection. Following this rise, levels of ICP27 mRNA plateaued at approximately twice the levels seen in wild-type infections, whereas ICP0 mRNA accumulated to three times the levels from wild type infections.

The accumulation profile of beta mRNAs from wild type infections followed a strikingly consistent pattern within that class. Consistent with previous reports (Harris-Hamilton 1985, Sharp et al, 1983, and Weinheimer and McKnight, 1987), Thymidine kinase, DNA-binding protein (ICP8), and DNA polymerase beta mRNAs appeared only slightly later than alpha mRNAs, peaked in abundance between 6 and 8 h post infection and declined very sharply at late times of infection. According to studies by Weinheimer and McKnight (1987), the transcription rates of thymidine kinase, ICP8, and DNA polymerase mRNAs showed a steady increase from 2 to 6 h post infection. Because of the symmetric transcription of genes that was seen in the in vitro nuclear runoff transcription assay, no firm conclusions about the transcription rates of beta mRNAs could be made in the interval between 4 and 12 h post infection.

Our studies of the vhs 1 mutant showed that the cytoplasmic steady state levels of beta mRNAs were significantly affected in vhs 1 infections. Compared to that of wild-type infections, the vhs 1 infected cells showed thymidine kinase, ICP8, and DNA polymerase mRNAs to overaccumulate considerably. Eventually, these mRNA levels plateaued instead of declining. Furthermore in each case at 12 to 16 h post

infection, the final steady state levels of beta mRNAs from vhs 1 infections was significantly higher than that seen in wild-type infected cells. The overaccumulation was particulary striking since, in wild-type infections since, after reaching maximal cytoplasmic levels, thymidine kinase, ICP8, and DNA polymerase mRNAs declined very abruptly and stayed down. In contrast, after reaching peak levels, thymidine kinase, ICP8, and DNA polymerase mRNAs just plateaued instead of declinig in vhs 1 infected cells.

An attractive hypothesis to explain the significant differences of alpha and beta mRNA accumulation profiles between wild-type and vhs 1 infections is that as long as transcription rates of alpha and beta genes are high the levels of mRNA increase. Following a decline in the transcription rates, the levels of alpha and beta mRNAs get depleted in wild type infected cells as a result of vhs induced message turnover. Our data is also consistent with the hypothesis that the transcription rates of alpha and beta genes were similar in wild-type and vhs 1 infected cells and that the differences in their accumulation reflect differences in their stabilities under the wild-type and mutant vhs 1 regulation of mRNA half-lives. These results suggest that the vhs protein plays an important role in determining the half-lives of viral mRNAs belonging to all kinetic classes and in so doing is important in the normal down regulation at late times of alpha and beta gene expression.

Accumulation profiles of beta/gamma and gamma mRNAs

The patterns of accumulation of beta/gamma and gamma messages

from wild-type infected cells were very similar to those reported in the literature (Harris-Hamilton and Bachenheimer, 1985, and Weinheimer and McKnight, 1987). The beta/gamma mRNAs for the major capsid protein, ICP5, glycoprotein B, and glycoprotein H could be first detected around 4 h post infection; following this they increased steadily in abundance until 8 to 10 h post infection and then remained present in significant amounts upto atleast 16 h after infection. Glycoprotein C, a true gamma mRNA appeared around 6 h post infection and persisted at high levels until late times in infection. Published reports on the temporal pattern of beta/gamma and gamma gene transcription showed that these transcripts were first detected at 3 h post infection. and increased steadily upto 6 h post infection (Weinheimer and McKnight, 1987). Weinheimer and McKnight (1987) also found that the accumulation profile of beta/gamma mRNAs therefore compares well with their transcription rates. The differences that were observed in wild-type infections between the accumulation patterns of the beta mRNAs on the one hand and the beta/gamma and gamma mRNAs on the other were particularly striking in view of the fact that the half-lives were very similar. The data therefore indicate that these differences in accumulation kinetics were primarily due to controls at the levels of transcription, processing, or mRNA transport rather than differential mRNA stabilities.

Our studies on the steady state levels of the major capsid protein, ICP5, glycoprotein B, glycoprotein H and glycoprotein C mRNAs in vhs 1 infected cells showed only slight differences from the profiles seen in wild-type infections. The overaccumulation of beta/gamma and

gamma mRNAs however was not as dramatic as that seen for the alpha and beta mRNAs. These accumulation kinetics are particularly interesting against the observation that in comparison with the wild-type infections, there was a significant decrease in the decay rates of ICP5, glycoprotein B, glycoprotein H, and glycoprotein C mRNAs in vhs 1 infected cells. Despite this increase in the stabilities of beta/gamma and gamma mRNAs, their mRNA levels never exceed two to three times those in wild-type infections even by 8 to 14 h post infection. Since increases in viral mRNA stability did not lead to significant increase in the steady state levels of these messages, it would suggest that the vhs 1 mutation may have indirectly affceted the rates of transcription, processing, or transport of the beta/gamma and gamma messages. In fact, the accumulation kinetics of glycoprotein C in vhs 1 infected cells, are supportive of this hypothesis. In vhs 1 infected cells, accumulation of gC message actually lagged behind that observed in wild-type infections for upto 12 h post infection. After that the glycoprotein C mRNA levels in vhs 1 infected cells actually surpassed those seen in wild-type infected cells. In vhs 1 infected cells, such a lag was also noticeable in the accumulation profile of US11, a gamma mRNA (Strom and Frenkel, 1987). One can speculate that an over expression of one or more earlier gene products may delay or reduce the transcription of genes that are expressed late in the viral replication cycle. Direct proof of these adverse effects of the vhs 1 mutation on transcription rates of beta/gamma and gamma genes can only be obtained through studies on their rates of transcription.

Role of the vhs gene product in the stability of viral mRNAs

Alpha mRNA stability

The stability of alpha mRNAs was found to be regulated by the wild type vhs components under three different protocols. First, under conditions of cycloheximide reversal and in the presence of Actinomycin-D, only the wild type vhs protein was capable of causing functional and physical destabilization of alpha mRNAs (Oroskar and Read, 1987). Second, measurement of alpha mRNA stability beginning at 6 hr after infection and in the absence of further transcription, again showed that only the wild type vhs protein could degrade alpha messages. Third, alpha mRNA half-lives obtained through pulse-chase starting at 6 hr post infection, were similar to the alpha mRNA half-lives obtained under Actinomycin-D chase beginning at 6 hr post infection.

The data indicate that the vhs protein regulates alpha mRNA stability by modifying both the functional and physical stabilities of alpha mRNAs. In cells infected with the wild type virus, alpha mRNAs transcribed in the presence of cycloheximide were physically intact and translatable in vitro, only immediately after cycloheximide reversal. Five hours after cycloheximide reversal and in the presence of Actinomycin-D however, the alpha mRNAs from wild type infections had lost their in vitro translatability and were also physically degraded. The vhs 1 mutant encodes a defective vhs protein. This resulted in extending the physical stability and in vitro translatability of alpha mRNAs even five hours after cycloheximide reversal in the presence of Act-D, in vhs 1 infected cells (Oroskar and Read, 1987).

Our attempts at delineating the role of alpha polypeptides in vhs

mediated alpha mRNA decay brought mixed results. We found that under conditions where cycloheximide was removed and Actinomycin-D added, preexisting alpha mRNAs in vhs 1 infected cells were completely destabilized by superinfecting wild type virions but not by the vhs 1 mutant virions. Our results confirmed those of Kwong and Frenkel (1987), that vhs components were indeed part of the incoming virion rather than the contaminating infected cell extracts. However, when the contribution of alpha polypeptides was inhibited by the addition of cycloheximide, neither the wild type nor the vhs 1 superinfecting virions caused alpha mRNA decay in the presence of Actinomycin-D. Several possibilities could give these results:

a) Alpha polypeptides cooperate with the vhs protein in vhs mediated degradation of alpha mRNAs.

b) vhs induced alpha mRNA decay is prevented under conditions where protein synthesis is inhibited, because a very labile cellular protein is needed for vhs mediated mRNA decay. This hypothesis is contradictory to the results of several investigators. Schek and Bachenheimer (1985) found that in wild-type HSV-1 infected cells, the vhs virion protein mediated the degradation of actin mRNA even in the presence of Anisomycin. Also, results from Strom and Frenkel (1987), clearly showed that tubulin mRNA is degraded in wild-type HSV-1 infections in the presence of cycloheximide, emetine, sodium fluoride, and puromycin, drugs that inhibit various stages of polypeptide synthesis. Finally, Fenwick and McMenamin (1984), studied the in vitro translatability of cellular mRNAs from wild-type infections. They found that in presence of cycloheximide, the HSV-1 virion components affected the in

vitro translatability of cellular mRNAs. Therefore, it appears that atleast in the case of HSV-1, there is no evidence that cycloheximide stabilized cellular transcripts, unlike the situation with some protooncogenes and growth factors (Linial et al, 1985, and, Shaw and Kamen, 1986, Wilson and Treisman, 1988).

c) That vhs induces decay of alpha mRNAs only when they are present in the nonpolysomal mRNA fraction. Again, data from Strom and Frenkel (1987) argues against this theory. Cellular mRNAs were equally susceptible to vhs induced decay in the presence of translation inhibitors that lead to dissociation of polyribosomes. Thus, sodium fluoride which inhibits translation initiation and puromycin, which causes premature termination of translation, did not adversely affect the vhs mediated degradation of cellular messages.

d) Finally, it is possible that cycloheximide which was actually used to inhibit alpha polypeptide synthesis, may have inadvertently inhibited alpha mRNA turnover. This hypothesis stems from the studies done by Darnell's group (Sheiness et al, 1975) on the size of the polyadenylated (poly(A)) tract in mRNAs under steady state conditions in HeLa cells. Darnell's group found that although the original size of poly(A) in mRNAs was about 200 nucleotides, most of the mRNAs contained less than 50 nucleotides at steady state. They further found that emetine, an inhibitor of translation, not only inhibited poly(A) shortening but also inhibited mRNA turnover in the presence of Actinomycin-D. Darnell concluded that since emetine retarded both poly(A) shortening and mRNA turnover, it was possible that poly(A) shortening and mRNA turnover processes were interrelated. It is not known whether

cycloheximide treatment affects the poly(A) tract or induces other structural changes in the mRNAs. If so, alpha mRNA turnover after the addition of Actinomycin-D, may indeed be affected by the presence of cycloheximide.

The possible requirement for alpha polypeptides in vhs mediated alpha mRNA decay could be studied through several double mutants defective in one of the alpha genes and the gene encoding the vhs component. Deletion mutants deleted in alpha ICP4 (DeLuca et al, 1985), alpha ICP0 (Sacks and Schaffer, 1987), alpha ICP27 (McCarthy et al, 1989), alpha ICP22 (Post and Roizman, 1981), and alpha ICP47 (Mavromara-Nazos et al, 1986) are available and could be each used to study the requirement of the respective alpha polypeptide in vhs mediated alpha mRNA decay. The double mutant will have to be created by recombining into the alpha deletion mutant, a fragment containing a deletion of the vhs gene but having flanking wild type genes. Use of each of the double deletion mutants, one at a time, as the primary infecting virus will allow determination of the requirement of each of the alpha polypeptides in vhs mediated alpha mRNA regulation. The combined effects of the alpha polypeptides and the vhs gene product on alpha mRNA decay can be studied through the use of the superinfecting protocol. This protocol when used in combination with Actinomycin-D, allows one to study the effects of the virion components of the superinfecting virus on alpha mRNA decay. Cells should first be infected with the alpha ICP4 vhs double mutant in the presence of cyloheximide. After five hours of infection, cycloheximide should be removed and the cells superinfected with the wild-type HSV-1 virions or the vhs 1 mutant in the presence of

Actinomycin-D. Total cytoplasmic RNA extracted at say, 3 and 6 h post cycloheximide reversal, can be analyzed for the physical stability of alpha ICP0, -22, -27, and -47 with specific DNA probes. This experiment will permit the evaluation of the role of each of the alpha polypeptides in combination with either the wild-type or the vhs 1 mutant gene products on the decay of the remaining four alpha messages transcribed from the primary infecting virus.

Another classical approach to studying protein-protein interactions is through the use of second-site revertants. If alpha polypeptides indeed cooperate with the vhs protein in mediating alpha mRNA decay, then a vhs 1 mutant with additional mutation in one of the alpha genes would still be competent in regulating the stability of alpha mRNAs. In fact, if the results from the genetic experiments support the earlier observations where alpha polypeptides were apparently required for vhs mediated alpha mRNA decay, then one could envisage specific pathways operating in regulating cellular and viral mRNA stability in HSV infected cells. Each degradative pathway would involve additional factors while still utilizing the vhs gene product as a key player in the degradation of mRNAs.

Reports from the literature and our own findings have brought out differences in the alpha mRNA accumulation profiles of different alpha genes. On the other hand however, alpha ICP4, alpha ICP0, and alpha ICP27 mRNAs were found to be equally susceptibile to vhs mediated decay as determined with the cycloheximide reversal protocol (Oroskar and Read, 1987). To be able to reconcile the two results we saw it fit to carry out further studies on alpha mRNA decay under conditions of a

normal infection. Alpha mRNA stability was therefore analyzed using the Actinomycin-D chase protocol beginning at 6 h post infection. In wild-type infected cells, alpha mRNAs encoding ICP0, ICP4, and ICP27 showed a 50% decay between 1 to 2 h after addition of Actinomycin-D. The vhs 1 mutant on the other hand was deficient in this function and led to a significant increase in alpha mRNA half-lives. Thus, the vhs mediated decay of alpha mRNAs was evident even under conditions where the initial infection was carried out in the absence of cycloheximide.

It has been suggested that metabolism of mRNA may be affected under conditions where transcription is inhibited. Alpha mRNA stability was therefore also assayed by the pulse-chase protocol. With this technique there always remains the possibility that a small number of labeled nuclear molecules can both be continually transported to, and also decay in the cytoplasm; this will continue to feed label into newly synthesized mRNA during the chase. Despite this disadvantage, the decline in the labeled ICP0 mRNA during pulse chase, still showed the half-life of ICP0 mRNA to be 2.4 h in cells infected with the wild type virus.

Both the cycloheximide reversal and the superinfection protocols test the vhs function in the form of a virion component. However, the stability of alpha mRNA through the Actinomycin-D and the pulse chase protocols measure the vhs function of the vhs protein which comes packaged as a virion component as well as those vhs copies that are synthesized in HSV infected cells but not packaged into virions. Since the decay of alpha mRNAs apparently followed a similar pattern when tested through the cycloheximide reversal protocol and the Actinomycin-

D chase and the pulse chase protocols, this suggests that the alpha mRNA decay pathway can be induced by the vhs protein when present either as a virion component or as an unpackaged viral infected cell protein.

Beta mRNA stability

The stability of thymidine kinase, DNA-binding protein (ICP8), and DNA polymerase mRNAs was studied under Actinomycin D chase and that of thymidine kinase mRNA was also measured through the pulse chase protocol. Results from both techniques showed that posttranscriptional control of beta mRNA stability was regulated only by the wild type vhs gene product. Comparison of the accumulation profiles between wild type and vhs 1 infected cells suggested that the wild type vhs component plays a critical role in regulating the cytoplasmic steady state levels of thymidine kinase, ICP8, and DNA polymerase mRNAs. Kwong and Frenkel (1987) had also studied the synthesis of beta polypeptides at 5 and 7 hr post infection. The rate of beta polypeptide synthesis was measured by pulse labeling cells with ^{35}[S]-methionine. From the amounts of ^{35}[S]-methionine incorporated immediately and six hours after the addition of Actinomycin-D, this allowed the authors to conclude that the wild type vhs gene product was involved in the down regulation of beta polypeptide synthesis.

Our studies on the half-lives of beta mRNA strongly suggest that the wild-type vhs gene product modulates the functional stability of beta mRNAs by affecting their physical stability. Indeed the stability of beta mRNAs was found to be under vhs control when studied either by the Actinomycin D chase protocol or by the pulse chase protocol. The

half lives of thymidine kinase, ICP8, and DNA polymerase mRNAs fell within the range of 1.5 to 2.5 h when measured in the presence of Actinomycin-D, while thymidine kinase degraded with a half life of 1.5 hr when measured through the pulse chase protocol. The defective vhs 1 gene product on the other hand, failed to regulate beta mRNA stability and caused a significant increase in the beta mRNA half lives.

Beta/gamma and gamma mRNA stability

In wild-type infected cells, accumulation profiles of the beta/-gamma messages encoding the major capsid protein ICP5, glycoprotein B, glycoprotein H, and the gamma message encoding glycoprotein C was very dissimilar to that of the beta mRNAs encoding the thymidine kinase, ICP8, and DNA polymerase from wild-type infections. The levels of beta/gamma and gamma mRNAs did not follow the pattern of early detection, followed by peak levels, and an eventual decline. Instead, high levels of beta/gamma and gamma mRNAs persisted until late in infection. Moreover, in comparison with the profile in wild-type infected cells, the overaccumulation of the beta/gamma mRNAs in vhs 1 infected cells was not as significant as to suggest an obvious lack of mRNA stability. Published reports from Kwong and Frenkel (1987), and from Kwong et al (1988), that were based on the studies on the ^{35}[S]-methionine incorporation into beta/gamma and gamma proteins in presence of Act-D, showed no major differences in the down regulation of beta/gamma and gamma polypeptide synthesis at 10 and 16 h post infection. Kwong and coworkers (1988) reasoned that the absence of clear cut results was probably due to the fact that secondary shutoff function was expressed in vhs 1

infected cells, since the infection had been carried out for 10 to 16 h in the absence of drugs. This debate on whether the beta/gamma and gamma mRNAs were indeed resistant to the wild-type vhs induced mRNA degradation, could now be tested. Our studies showed that when Actinomycin D was added to cells beginning 6 h post infection, beta/gamma and gamma mRNAs were as susceptible as alpha and beta mRNAs to wild-type vhs mediated mRNA decay. The absence of the wild type vhs function in vhs 1 mutant infected cells led to large increases in the stability of mRNAs encoding the ICP5, glycoprotein B, glycoprotein H, and glycoprotein C mRNAs in the vhs 1 mutant infected cells.

Our studies have conclusively shown that target selectivity plays little role, if any, in the vhs mediated decay of viral mRNAs. Regardless of their class affiliation, alpha, beta, beta/gamma, and gamma mRNAs were found to be equal targets for regulation by the wild type vhs gene product.

Measurement of viral mRNA half-lives

Ours is the first study of its kind that was designed to determine the half lives of messages belonging to four different classes of the herpes simplex virus type 1. A retrospective examination of decay curves for alpha mRNAs obtained under cycloheximide reversal and inhibition of de novo transcription by Actinomycin D, allowed us to make a rough estimate of the half lives of alpha messages (Oroskar and Read, 1987). A fifty percent decay in the amounts of alpha ICP4, alpha ICP0, and alpha ICP27 mRNA had occurred between 1.5 to 2 h in wild-type infections. It was for this reason that we studied the half-lives

of all viral mRNAs in the presence of Actinomycin D, starting at 6 h after infection up to three hours after the addition of Actinomycin D. Northern blots of total cytoplasmic RNA extracted at 45 min intervals from the time of Actinomycin D addition, allowed us to make two important conclusions. First, that the vhs mediated mRNA decay was characterized by a mechanism that did not involve target mRNA selectivity. Second, that the half-lives of viral mRNAs belonging to different kinetic classes fell within the range of 1 to 2.5 h in wild type infected cells. The analysis of experiments done under the influence of drugs is however always potentially colored with any resultant artifactual conditions that may inadvertently ensue. In case where measurements were done in the presence of Actinomycin-D, for instance there is never an assurance that turnover goes on normally in the face of complete mRNA inhibition. It is for this reason therefore, if similar results can be obtained with an Act-D chase and some other method like the pulse chase, this tends to validate the results obtained from both techniques.

We therefore assayed the rate of viral mRNA turnover through the pulse chase protocol. The assay involved infecting cells with the wild type HSV-1 for upto 5.5 h in the absence of drugs, followed by a 30 min pulse of tritiated uridine. At the end of the pulse, the labeled precursor was washed off. Since a few labeled nuclear molecules can feed additional label into the cytoplasm during the course of the pulse chase, the intracellular ribonucleoside triphosphate pools were diluted with excess cold cytosine and uridine. In addition, the ribonucleotides were entrapped in UDP-glucosamine to prevent the immediate utiliza-

tion of labeled ribonucleotides for RNA synthesis (Scholtissek, 1971). After the addition of unlabeled nucleotides, total cytoplasmic RNA was extracted at approximately one hour intervals for a period of 6 hours. The RNA samples were then hybridized to DNA probes representing an alpha gene encoding ICP0, and a beta gene encoding the thymidine kinase. Measurement of the decline in labeled mRNA through the filter hybridization protocol again showed that the half-lives of representative alpha and beta messages fell with the range of 1 to 2.5 hours in wild-type infected cells. In wild-type infected cells, the average half-life of viral mRNAs from Expt 1 is 1.6 h with a standard deviation of 0.48 h. From Expt 2 , the average half-life of viral mRNAs in wild-type infections is 1.7 h with a standard deviation of 0.3 h. Since the standard deviation falls within the interval during which mRNA stability was sampled, the half-lives of all viral mRNAs do not appear to be very different from each other. Results from the pulse chase protocol also validate the viral mRNA half-lives calculated from the Actinomycin-D chase protocol and therefore reinforce our earlier conclusions.

While there may be small differences in the actual half lives of each of the 10 different viral messages, it is indeed most striking that their half lives were even found to be similar. These results support our earlier hypothesis that in wild-type and vhs 1 infected cells, the differences in the accumulation profiles of alpha and beta mRNAs on one hand and beta/gamma and gamma mRNAs on the other, cannot solely be explained on the basis of differences in mRNA stability resulting from the regulation of the wild-type and the mutant vhs protein.

Indeed the data suggest that the vhs 1 mutant gene product not only fails to regulate viral mRNA stability but that it also causes a delay and/or reduction in the transcription of beta/gamma and gamma genes.

In the context of other eukaryotic mRNAs whose half lives have been determined, globin mRNAs are among the most stable and histone mRNAs are among the least stable (t ½ is about 10 minutes). The half life of 1 to 2.5 h of HSV-1 viral mRNAs thus falls in the category of eukaryotic mRNAs with relatively short half lives.

Role of the vhs protein in the scheme of HSV-1 viral gene expression

The expression of the herpes simplex virus genes is a very tightly regulated process. To date, all studies suggest that the establishment of the HSV-1 protein synthesis cascade is not entirely due to the direct control of transcription initiation for each temporal class. In fact, both transcriptional and posttranscriptional controls together regulate the HSV RNA expression cascade which in turn correlates with the HSV protein synthesis cascade (Marsden et al, 1983, Weinheimer and McKnight, 1987, and Smith and Sandri-Goldin, 1988). Indeed, our findings on the role of vhs in the control of viral mRNA stability, have furthered our understanding of HSV gene expression in HSV infected cells.

Alpha gene expression

HSV virions contain both positive and negative effectors which regulate alpha gene expression. The virion stimulatory protein Vmw65 forms a complex with cellular factors and specifically stimulates alpha

gene transcription through cis-acting alpha regulatory sequences that are present upstream from the alpha promoter. The alpha ICP4 coding gene is negatively regulated at the level of transcription by the alpha ICP4 (Dixon and Schaffer, 1980) as well as by the beta DNA-binding protein, ICP8 (Godowski and Knipe, 1986). The autoregulatory role of ICP4 was determined through studies on the temperature sensitive ICP4 mutants. In temperature shift up experiments carried out at 4 or 8 h post infection, alpha ICP4 was found to incorporate the ^{35}[S]-methionine label even 2 hours after the temperature shift up. This suggested that new ICP4 mRNA was synthesized, which directed the synthesis of ICP4 protein. Nuclear runoff transcription assays have been carried out to determine the rate of transcription in cells infected at nonmpermissive temperature with the temperature sensitive mutant for ICP8, the DNA-binding protein. Through such studies, Godowski and Knipe (1986) showed that transcription of alpha ICP4 mRNA failed to be inhibited in cells infected with the beta ICP8 mutant. In addition, my studies strongly suggest that the vhs-mediated posttranscriptional regulation of alpha mRNAs involves the modulation of alpha mRNA stability (Oroskar and Read, 1987). It appears that cytoplasmic levels of alpha mRNAs are achieved due to the combined effects of mRNA transcription rates, mRNA processing and transport, and the vhs-mediated regulation of alpha mRNA stability.

Although both positive and negative transcriptional controls as well as alpha mRNA stabilty influence the accumulation profile of alpha mRNAs, it is possible that posttranscriptional controls may further regulate the component of alpha protein synthesis cascade. Alpha pro-

tein synthesis is generally seen between 1 and 5 to 6 h post infection. This pattern of alpha protein synthesis does not however correlate with the accumulation profile of alpha mRNAs as seen in our present studies, or by those by Harris-Hamilton and Bachenheimer (1985), or by Weinheimer and McKnight (1987). It therefore remains to be seen whether those alpha mRNAs that were detected after 6 h post infection, are indeed translatable.

Beta gene expression

The transcription of beta mRNAs is positively regulated by alpha gene products. Evidence for this comes from infections with HSV-1 carried out in the presence of cycloheximide , and with ts alpha ICP4 mutants carried out at nonpermissive temperatures (Roizman and Spear, 1981, and Watson and Clements, 1980). Secondly, transient expression assays showed that alpha ICP0 also influences beta gene expression (Everett, 1984). Finally, it is evident from our studies that the vhs mediated posttranscriptional control of beta gene expression (Kwong and Frenkel, 1987) is actually effected by vhs mediated decay of beta mRNAs. The cytoplasmic levels of beta mRNAs also appear to be the result of controls on transcription rates, mRNA processing, transport, and the vhs-medaited beta mRNA decay.

On comparing the accumulation profiles of beta mRNAs with the rates of beta protein synthesis, it appeared that the component of beta mRNA cascade closely matched the component of beta protein synthesis cascade (Harris-Hamilton and Bachenheimer, 1985, Weinheimer and McKnight, 1987). This suggests that translational controls may not be

important in the regulation of beta protein synthesis. My present studies on the accumulation profile of beta mRNAs also supports this hypothesis. However, Yager and Coen (1988) recently showed that at times when the thymidine kinase transcripts were as abundant as the transcripts for the DNA polymerase mRNAs, the polyribosomes were found to be associated more with the thymidine kinase and the ICP8 mRNAs than with the DNA polymerase mRNAs. This indicates that translational control may also play a role in the expression of the DNA polymerase gene product.

Beta/gamma and gamma gene expression

The transcription of beta/gamma and gamma genes is positively regulated by several alpha gene products. With the temperature sensitive alpha ICP4 mutant, Watson and Clements (1980) found that following a shift to the nonpermissive temperature, the mutant infected cells incorporated the ^{32}[P]-orthophosphate label only in alpha mRNAs. Furthermore, under such conditions, these cells also showed a complete lack of mRNAs corresponding to the beta, beta/gamma, and gamma class of genes. Watson and Clements (1980) therefore concluded that the wild type gene product of alpha ICP4 was continuously required for transcription of all genes expressed subsequent to the alpha class. In studies using transient expression assays, Rice and Knipe (1988) very elegantly showed that the wild type gene product of alpha ICP27 was crucial to the expression of glycoprotein B which belongs to the beta/gamma class of messages. With regard to the true gamma class of messages, the correlation between viral DNA replication and viral gene expression

is not completely clear. It appears that high levels of expression of some gamma gene promoters can be induced in cells transfected with alpha genes alone; however, DNA replication is required for the full expression of gamma genes in normal viral infection cycles (Costa et al, 1985, and Marvomara-Nazos et al 1986). To add to this controversy, transcription rate studies using nuclear runoff transcription techniques showed conflicting results on the necessity of viral DNA replication for transcription of glycoprotein C (Godowski and Knipe, 1986, and Weinheimer and McKnight, 1987). Thus it is still not clear whether a "template" effect exists during the normal infectious cycle of the virus, or whether the activation of true gamma gene promoters requires additional regulation. Such hypotheses however cannot explain the results where glycoprotein C promoter was active without DNA replication with certain HSV mutants which were temperature sensitive in the DNA replication function (Godowski and Knipe, 1985, and Arsenakis et al, 1986). Analysis of the glycoprotein C promoter has shown it to be a structurally normal RNA polymerase II promoter (Wagner, 1984).

The DNA-binding protein or the ICP8, on the other hand negatively regulates the transcription of the beta/gammma ICP5 mRNA. Through nuclear runoff transcription assays Godowski and Knipe (1986) showed that the overaccumulation of the ICP5 mRNA was actually due to the failure to negatively regulate its transcription at the nonpermissive temperatures.

In addition, my present studies indicate that the wild type vhs gene product is involved in the regulation of beta/gamma and gamma mRNA stability. However, the kinetics of mRNA accumulation in wild-type

infected cells indicate that, despite the similarities in vhs-mediated control of the half-lives of alpha, beta, beta/gamma, and gamma mRNAs, the accumulation profiles of beta/gamma, and gamma mRNAs were quite dissimilar with that seen for the alpha and beta mRNAs. My studies suggest that these differences in accumulation kinetics were primarily due to controls at the levels of transcription, processing, or mRNA transport, rather than differential mRNA stabilities. Finally, Smith and Sandri-Goldin (1988) showed that the cytoplasmic component of beta-/gamma and gamma mRNA cascade correlated well with the that of the protein synthesis cascade for the glycoprotein B, glycoprotein C, and glycoprotein D genes.

Role of the vhs function in HSV-1 virus replication

The vhs protein is known to markedly affect host mRNA stability (Schek and Bachenheimer, 1985, and Strom and Frenkel, 1987). From my studies, the wild-type vhs gene product was also shown to cause the rapid turnover of HSV-1 viral mRNAs belonging to all four kinetic classes. The possession of such a posttranscriptional control therefore enables the herpes simplex virus to regulate gene expression in a very versatile fashion.

Generally, viral replication involves a redirection of cellular resources such that viral metabolism is carried out at the expense of the host. In the case of the HSV-1, it is quite possible that the vhs function may have actually evolved for the posttranscriptional control of viral rather than cellular mRNA metabolism. The modulation of viral mRNA stabilities would thus serve to complement the control of viral

transcription. Furthermore, the nonselective nature of the vhs mediated decay of host as well as viral mRNAs appears to be an extremely efficient way of allowing the herpes simplex virus to gain control of the cellular synthetic apparatus as well as to enable viral transcriptional to be finely controlled. The HSV mRNAs are known to be structurally similar to the cellular mRNAs and probably do not differ significantly in their requirements for the protein synthetic apparatus. Also, unlike some viral mRNAs, like that of the mengovirus, the HSV mRNAs are not superior in their ability to compete for the cellular translation machinery. The cumulative effect of limiting host and viral mRNA stability through the vhs protein may therefore serve to accentuate the importance and flexibility of transcriptional controls.

At this point however, one can only speculate about the role of vhs in viral replication. It is obvious that the vhs 1 mutant and other independently derived vhs mutants are viable and not conditional lethal mutants (Read and Frenkel, 1983). Therefore, from the phenotype of vhs 1 mutant it can be said without reasonable doubt that overexpression of many of the viral genes can be tolerated, atleast in cell culture. An overexpression of earlier genes however might result in the delayed or underexpression of some of the later genes in the virus infectious cycle. In vhs 1 mutant infections therefore, the cytoplasmic appearance of glycoprotein C mRNA actually lagged for several hours behind that of glycoprotein C from wild type infected cells. Such an effect may be an outcome either of an overload of the cellular synthetic apparatus, or due to repurcussions of unregulated cascade of RNA expression of the earlier genes, or both. The vhs 1 mutation does

affect growth potential of the herpes simplex virus type 1 mutant. In single step growth curves, the size of the vhs 1 progeny was decreased by a factor of 20 when compared with that of the wild type virus (Read and Frenkel, 1983). Infact, the wild type virus because of its growth advantage rapidly outgrows the vhs 1 mutant in mixed infections (Kwong et al, 1988). Further studies may shed light on the importance of vhs functions in the infection of animal hosts.

Mechanism of vhs dependent mRNA turnover

Perhaps the most important question that arises from our studies is how and why does the HSV-1 vhs mechanism induce mRNA degradation ? The answer to why HSV-1 has developed a mechanism to degrade cellular and viral mRNAs possibly has to do with the critical role mRNA turnover rates play in determining the level of expression of at least the alpha and beta HSV-1 genes. Indeed mRNA half-lives play a very important role in the level of expression of several eukaryotic genes (Carniero and Schibler, 1984, and Raghow, 1987).

Elucidation of the biochemical mechanism of the vhs-mediated viral mRNA turnover needs to be explored on several fronts. One of the important questions that needs to be answered is why would HSV carry in its virion a protein that mediates the decay of both cellular and viral mRNAs. It is quite possible that separate pathways, all requiring the involvement of the vhs may operate in the specific decay of viral and cellular messages. Prelimnary data from my studies on the necessity of alpha polypeptides in vhs-mediated alpha mRNA decay are consistent with this hypothesis. These results should however be tested by genetic

studies that would utilize viruses with double deletions in the vhs and alpha genes, and second site mutations in the alpha genes against the vhs 1 background.

The second question that needs to be studied is which structural elements of the individual mRNAs or a class of mRNAs, are recognized by the degradative system involving the vhs protein. For instance, 5' untranslated sequences are very important in the decay of c-myc, and the beta tubulin mRNAs whereas 3' untranslated sequences containing the 67 base AU sequences are important in the turnover of c-fos (Wilson and Triesman, 1988), c-myc (Brewer and Ross, 1988), and granulocyte monocyte-colony stimilating factor, GM-CSF (Shaw and Kamen, 1986). For a start, the already existing viable mutants partially deleted in 5' or 3' portions of the ICP4, an alpha gene (DeLuca et al, 1985), or thymidine kinase, a beta gene (McKnight, 1980), should be tested for any altered stability of these modified transcripts.

Finally, the biochemical function of the vhs protein needs to be delineated. The vhs protein could either be a ribonuclease or it could function by activating a ribonuclease and its related degradative pathway. Unpublished data from Read and Frenkel (1983) and reports presented by Kwong (1988) at the 13th International Herpesvirus meeting support either of the two hypotheses. Mixed infections with the vhs 1 mutant and the wild type virus in ratios where the vhs 1 mutant virus exceeded the wild type virus by 50-100 times, showed the vhs 1 mutation to be dominant over the wild-type. In the presence of the vhs 1 mutant protein, the wild-type vhs protein was found to be ineffective. If one assumes that a mixed multimer containing both mutant and wild-type

forms of the vhs protein is inactive in inducing mRNA decay, then the above results support either hypothesis. Under these conditions, the multimeric form containing a higher prorportion of the mutant vhs 1 protein would dominate the multimeric form containing only the wild-type vhs protein, thus leading to a decrease in mRNA decay. Alternatively, the vhs protein may physically interact with cellular proteins. If this were true, the vhs 1 mutant protein might compete with the wild-type vhs protein thus causing a decrease or an absence of mRNA decay.

Another possibility is that the vhs protein functions in a manner that causes an alteration in the structure of messenger ribonucleoproteins, making them more susceptible to the cellular mRNA degradative pathway. Data from our laboratory (Krikorian and Read, unpublished data) on the structure of messenger ribonucleoproteins in wild type and mutant infected cells is consistent with such a hypothesis. From the profile of proteins cross-linked to cellular mRNAs, it was found that in comparison with mock infected controls, a 52 kilodalton protein band decreased in abundance and a 49 kilodalton protein band increased in messenger ribonucleoproteins from wild type infected cells. On the other hand, the messenger ribonucleoprotein profile from vhs 1 infected cells was found to be similar to that of the messenger ribonucleoproteins from mock infected cells. Furthermore, the changes in the profile of messenger ribonucleoproteins occurred in ^{35}[S]-methionine labeled cells, following infection with the wild type virus in the presence of Actinomycin-D. This indicates that no virion components but, cellular factors were responsible for

changes in the messenger ribonucleoprotein profile.

From the only published report on the mRNA degradation process in HSV infected cells, it appears that recognition of one or more signals in the mRNA is important. Mayman and Nishioka (1985) observed that histone H3 mRNA was more stable than other species in HSV-infected Friend erythroleukemia cells and suggested that lack of the poly(A) tail was the reason for this stability. In addition, HSV infection resulted in an increased poly(A)$^-$ RNA pool and a concomitant decrease in the abundance of poly(A)$^+$ mRNA (Nakai et al. 1982, Stringer et al. 1977). This however, would contradict the results of Brewer and Ross (1988), and Wilson and Triesman (1988), on the decay of cellular mRNAs in uninfected cells. The stability of c-fos mRNA (Wilson and Triesman, 1988) and c-myc mRNA (Brewer and Ross, 1988) was found to be dependent on the length of poly(A) tail. In both cases, the mRNA was stable as long as the poly(A) tail was intact; after which the mRNA was degraded. Thus, the mRNA structural elements recognized by the vhs degradative pathway need to be explored.

Proposed future experiments

The mechanism of vhs induced degradation of mRNA can be best studied using model in vitro mRNA degradation systems. To isolate target messages that are functionally as well as physically intact, it would be best to use in vitro translatable mRNAs prepared from a cell-free extract (Brown et al, 1983). Cells can be infected with either the wild type or the vhs 1 virus or mock infected and incubated for 6 hr after infection. At this point, cells from each group should be

processed to make cell-free translation extracts (Brown et al, 1983). This *in vitro* translation mix should be incubated at 34 °C, and the *in vitro* degradation of cellular messages determined by analyzing the total cytoplasmic RNA at the start of the incubation period and several hours thereafter.

The mechanism can be further probed by studying the effects of the *vhs* protein and mock cell lysates on target message stability in an *in vitro* mRNA degradation system. It may be technically feasible to synthesize the *vhs* protein in bacterial expression vectors using bacterial hosts. Thus, studies on the effects of *vhs* protein and fractionated mock cell lysates on the integrity of target messages would be an important contribution to the field of eukaryotic mRNA degradation.

Finally, similar to the studies on histone mRNA degradation carried out by Jeffery Ross and his colleagues (Ross et al, 1986), one could study the direction, if any, of *vhs* mediated viral mRNA decay *in vivo*. For instance, the *in vivo* degradation of a representative viral message could be followed after the addition of Actinomycin D, starting from the time when peak levels of the particular message are detectable in the cytoplasm. Total RNA extracted at frequent intervals in the first two hours could then be analyzed by probing for the integrity of its ends with both the 5' and 3' specific probes through an S1 nuclease or RNAase protection assay. The protected DNA fragment could then be analyzed by electrophoresis in a polyacrylamide sequencing gel and subsequent autoradiography. On the basis of the physical integrity and the size of each of the two probes over the two hour period, one would be able to make conclusions about the direction of viral mRNA decay.

If one were able to duplicate the appearance of *in vivo* mRNA decay products in an *in vitro* mRNA degradation system, the *in vitro* mRNA degradation system would become a useful test system in the dissection of *vhs* induced decay of cellular and viral messages.

Our studies have enabled us to analyze factors that affect the posttransriptional metabolism of viral mRNAs in HSV infected cells. We found that the *vhs* viral gene product controlled viral mRNA stability. The *vhs* gene product shows little, if any, target selectivity in mediating the degradation of *alpha*, *beta*, *beta/gamma* and *gamma* viral mRNAs. Furthermore, the *vhs* regulation of viral mRNA stability is also unique in that 10 different viral messages representing all four kinetic classes had very similar half lives in wild type infected cells. Therefore, in conjunction with transcriptional controls, the *vhs* control of viral mRNA stability may serve a very important function in determining the RNA cascade of viral gene expression.

BIBLIOGRAPHY

Anderson, K.P., R.H. Costa, L.E. Costa, L.E. Holland, and E.K. Wagner. 1980. Characterization of herpes simplex type 1 RNA present in the absence of de novo protein synthesis. J. Virol. 34:9-27.

Arsenakis, M., L.F. Tomasi, V. Speziali, B. Roizman, and G. Campadelli-Fiume. 1986. Expression and regulation of glycoprotein C gene of herpes simplex virus 1 resident in a clonal L-cell line. J. Virol. 58:367-376.

Asher, Y., M. Heller, and Y. Becker. 1969. Incorporation of lipids into herpes simplex virus particles. J.Gen. Virol. 4:65-76.

Babich, A., and J.R. Nevins. 1981. The stability of early adenovirus mRNA is controlled by the viral 72 kd DNA-binding protein. Cell. 26: 371-379.

Bachenheimer S.L., and B. Roizman. 1972. Ribonucleic acid synthesis in cells infected with herpes simplex virus. VI. Polyadenylic acid sequences in viral messenger ribonucleic acid. J.Virol. 10:875-879.

Bartkoski, M.J. Jr.,and B. Roizman. 1976. RNA synthesis in cells infected with herpes simplex virus. XIII. Differences in the methylation patterns of viral RNA during reproductive cycle. J.Virol. 20:583-588.

Bartkoski, M.J. Jr., and B. Roizman. 1978. Regulation of herpesvirus macromolecular synthesis. VII Inhibition of internal methylation of mRNA late in infection. Virology 58:146-156.

Batterson, W., D. Furlong, and B. Roizman. 1983. Molecular genetics of herpes simplex virus. VII. Further characterization of a ts mutant defective in release of viral DNA and in other stages of viral reproductive cycle. J. Virol. 45:397-407.

Batterson, W., and B. Roizman. 1983. Characterization of the herpes simplex virion-associated factor responsible for the induction of α genes. J. Virol. 46:371-377.

Belasco, J.G., G. Nilsson, A.V. Gabain, and S.N. Cohen. 1986. The stability of E. coli gene transcripts is dependent on determinants

localized to specific mRNA segments. Cell 46:245-251.

BenZe'ev, A., S.R. Farmer, and S. Penman. 1979. Mechanism of regulating tubulin synthesis in cultured mammalian cells. Cell. 17:319-325.

Berk, A.J., and P.A. Sharp. 1977. Sizing and mappping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease digested hybrids. Cell. 12:721-732.

Bird, R.C., F.A. Jacobs, G. Stein, J. Stein, and B.H. Sells. 1985. A unique subspecies of histone H4 mRNA from rat myoblasts contains poly(-A). Proc. Natl. Acad. Sci. USA 82:6760-6764.

Birnboim, H.C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nuc. Acids Res. 7:1513-1523.

Bonneau, A.M., and N. Sonenberg. 1987. Proteolysis of the p220 component of the cap-binding protein complex is not sufficient for complete inhibition of host cell protein synthesis after poliovirus infection. J. Virol. 61:986-991.

Brawerman,G. 1987. Determinants of messenger RNA stability. Cell 48:5-6.

Brewer, G., and J. Ross. 1988. Poly(A) shortening and degradation of the 3' A+U-rich sequences of human c-myc in a cell-free system. Mol. Cell. Biol. 8:1697-1708.

Brock, M.L., and D.J. Shapiro. 1983. Estrogen stabilizes vitellogenin mRNA against cytoplasmic degradation. Cell. 34:207-214.

Brown, G.D., R.W. Peluso, S.A. Moyer, and R.W. Moyer. 1983. A simple method for the preparation of extracts from animal cells which catalyze efficient in vitro protein synthesis. J. Biol. Chem. 258:14309-143-14.

Buchman, T.G., and B. Roizman. 1978a. Anatomy of bovine mammilitis virus DNA. I. Restriction endonuclease maps of four populations of molecules that differ in the relative orientation of the long and short components. J. Virol. 25:395-407.

Buchman, T.G., and B. Roizman. 1978b. Anatomy of bovine mammilitis virus DNA.II. Size and arrangement of the deoxynucleotide sequences. J. Virol. 27:239-254.

Buckmaster, E.A., V. Gompels, and A.C. Minson. 1984. Characterization and physical mapping of an HSV-1 glycoprotein of approximately $115X10^3$ molecular weight. Virology 139:408-413.

Bzik, D.J., S. Person, and G.S. Read. 1982. The active inhibition of herpes simplex virus type 1-induced cell fusion. Virology 117:504-

509.

Cai, W., B. Gu, and S. Person. 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J. Virol. 62:2-596-2604.

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

Carniero, M., and U. Schibler. 1984. Accumulation of rare and moderately abundant mRNAs in mouse L-cells is mainly post-transcriptionally regulated. J. Mol. Biol. 178:869-880.

Cassai, E.N., M. Sarmiento, and P.G. Spear. 1975. Comparison of the virion proteins specified by herpes simplex virus types 1 and 2. J. Virol. 16:1327-1331.

Challberg, M.D. 1986. A method for identifying the viral genes required for herpesvirus DNA replication. Proc. Natl. Acad. Sci. USA 83:9094-9098.

Clements, J.B., J.B. McLaughlan, and D.J. McGeoch. 1979. Orientation of herpes simplex virus type 1 immediate early mRNAs. Nuc. Acids Res. 7:77-91.

Cleveland, D.W., and J.C. Havercroft. 1983. Is apparent autoregulatory control of tubulin synthesis nontranscriptionally regulated? J. Cell Biol. 87:919-924.

Conley, A.F., D.M. Knipe, P.C. Jones, and B. Roizman. 1981. Molecular genetics of herpes simplex virus. VII. Characterization of a temperature sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of τ polypeptides. J. Virol. 37:191-206.

Costa, R.H., G.B. Devi, K.P. Anderson, B.H. Gaylord, and E.K. Wagner. 1981. Characterization of a major late herpes simplex virus type 1 mRNA. J. Virol. 38:483-496.

Costa, R., K. Draper, G.B. Devi-Rao, R. Thomson, and E.K. Wagner. 1985. Virus induced modifications of the host is required for expression of bacterial chloramphenicol acetyltransferase gene controlled by a late herpes simplex virus promoter (VP5). J. Virol. 56:19-30.

Davis,L.G., M.D. Dibner, and J.F. Battey. 1986. Basic Methods in Molecular Biology. Elsevier, New York.

Dawid, I.B., and P.K. Wellauer. 1976. A reinvestigation of the 5' to 3' polarity in the 40S ribosomal RNA precursor of Xenopus laevis. Cell 8:443-448.

DeLisle, A.J., R.A. Graves, W.F. Marzluff, and L.F. Johnson. 1983. Regulation of histone mRNA production and stability in serum-stimulated mouse 3T6 fibroblasts. Mol. Cell. Biol. 3:1920-1929.

DeLuca, N., D.J. Bzik, V.C. Bond, S. Person, and W. Snipes. 1982. Nucleotide sequences of herpes simplex virus type 1 (HSV-1) affecting virus entry, cell fusion, and production of glycoprotein gB (VP7). Virology 122:411-423.

DeLuca, N., A.M. McCarthy, and P.A. Schaffer. 1985. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. J. Virol. 56:558-570.

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

Dolynuik, M., R. Pritchett, and E. Kieff. 1976. Analysis of the polypeptides of purified enveloped Epstein-Barr virus. J. Virol. 17:935-949.

Donovan, W.P., and S.R. Kushner. 1986. Polynucleotide phosphorylase and ribonuclease II are required for cell viability and mRNA turnover in Escherichia coli K12. Proc. Natl. Acad. Sci. USA 83:120-124.

Etchison, D., S.C. Milburn, I. Edery, N. Sonenberg, and J.W.B. Hershey. 1982. Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eukaryotic initiation factor 3 and a cap-binding protein complex. J. Biol. Chem. 257:14806-14810.

Epstein, M.A., and S.J. Holt. 1963. Adenosine triphosphatase activity at the surface of mature extracellular herpes virus. Nature 198:509-510.

Everett, R.D. 1984. Transactivation of transcription by herpes simplex virus product: requirements for two HSV-1 immediate-early poltpeptides for maximum activity. EMBO J. 3:3155-3141.

Fenwick, M.L. 1984. The effects of herpesviruses on cellular macromolecular synthesis. In Comprehensive Virology vol. 19. eds. H. Fraenkel-Conrat and R.R. Wagner. pp.359-390.

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

Fenwick, M.L., S. Morse, and B. Roizman. 1979. Anatomy of herpes simplex virus DNA. XI. Apparent clustering of functions effecting inhibi-

tion of host DNA and protein synthesis. J.Virol. 29:825-827.

Fenwick, M., and B. Roizman. 1977. Regulation of herpesvirus macromolecular synthesis. VI. Synthesis and modification of viral polypeptides in enucleated cells. J. Virol. 22:720-725.

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

Fong, C.K.Y., R.B. Tenser, G.D. Hsuing, and P.A. Gross. 1973. Ultrastructural studies of the envelopement and release of guinea-pig herpes-like virus in cultured cells. Virology 52:468-477.

Fort, P., J. Rech, A.Vie, M. Piechaczyk, A. Bonnieu, P. Jeanteur and J.M. Blanchard. 1987. Regulation of c-fos gene expression in hamster fibroblasts: initiation and elongation of transcription and mRNA degradation. Nuc. Acid Res. 15:5657-5667.

Friedman, H.M., G.H. Cohen, R.J. Eisenberg, C.A. Seidel, and D.B. Cines. 1984. Glycoprotein C of HSV-1 functions as a C3b receptor on infected endothelial cells. Nature. 309:633-635.

Friedman, H.M., J.C. Glorioso, G.H. Cohen, J.C. Hastings, S.L. Harris, and R.J. Eisenberg. 1986. Binding of complement component C3b to glycoprotein C of herpes simplex virus type 1: mapping of gC-binding sites and demonstration of conserved C3b binding in low-passage clinival isolates. J. Virol. 60:470-475.

Frink, R., K, Anderson, and E. Wagner. 1981. Herpes simplex virus type 1 Hind III fragment L encodes spliced and complementary mRNA sprecies. J. Virol. 39:559-572.

Furlong, D., H. Swift, and B. Roizman. 1972. Arrangement of herpesvirus deoxyribonucleic acid in the core. J. Virol. 10:1071-1074.

Gafner, J., M. DeRobertis, and P. Phillipsen. Delta sequences in the 5' noncoding region of yeast tRNA genes. 1983. EMBO J. 2:583-591.

Gay, D.A., T.J. Yen, J.T-Y. Lau, and D.W. Cleveland. 1987. Sequences that confer β-tubulin autoregulation through modulated mRNA stability reside within exon 1 of a β-tubulin mRNA. Cell. 50:671-679.

Gibbs, J., H. Chiou, J. Hall, D. Mount, M. Retondo, S. Weller, and D. Coen. 1985. Sequences and mapping analyses of the herpes simplex virus DNA polymerase gene predict a C-terminal substrate binding domain. Proc. Natl. Acad. Sci. USA. 82:7969-7973.

Gibson, W., and B. Roizman. 1972. Proteins specified by herpes simplex virus. VIII. Characteization and composition of multiple capsid forms of subtypes 1 and 2. J. Virol. 10:1044-1052.

Godowski, P.J., and D.M. Knipe. 1983. Mutations in the major DNA-binding protein gene of herpes simplex virus type 1 result in increased levels of viral gene expression. J. Virol. 47:478-486.

Godowski, P., and D. Knipe. 1985. Identification of a herpes simplex function that represses late viral gene expression from parental viral genomes. J. Virol. 55:357-365.

Godowski, P.J., and D.M. Knipe. 1986. Transcriptional control of herpesvirus gene expression. Gene functions required for positive and negative regulation. Proc. Natl. Acad. Sci. U.S.A. 83:256-260.

Goldin, A.L., R.M. Sandri-Goldin, M.Levine, and J.C. Glorioso. 1981. Cloning of herpes simplex virus sequences representing the whole genome. J. Virol. 38:50-58.

Gompels, V., and A. Minson. 1986. The properties and and unique sequence of glycoprotein H of herpes simplex virus type 1. Virology 153:230-247.

Grafstrom, R.H., J.C. Alwine, W.L. Steinhart, C.W. Hill, and R.W. Hyman. 1975. The terminal repetition of herpes simplex virus DNA. Virology 67:144-157.

Graham, D.E., D. Medina, and G.H. Smith. 1984. Increased concentration of indigenous proviral mouse mammary tumor virus long terminal repeat-containing transcript is associated with neoplastic transformation of mammary epithelium in C3H/Sm mice. J. Virol. 49:819-827.

Greenberg, M.E., and E.B. Ziff. 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature 311:433-438.

Guarneros, G., C. Montanez, T. Hernandez, and D. Court. 1982. Posttranscriptional control of bacteriophage lambda int gene expression from a site distal to the gene. Proc. Natl. Acad. Sci. USA 79:238-242.

Guyette, W.A., R.J. Matusik, and J.M. Rosen. 1979. Prolactin-mediated transcriptional and posttranscriptional control of casein gene expression. Cell 17:1013-1023.

Harris-Hamilton, E., and S.L. Bachenheimer. 1985. Accumulation of heres simplex virus type 1 RNAs of different kinetic classes in the cytoplasm of infected cells. J. Virol. 53:144-151.

Hayward, G.S., R.J. Jacob, S.C. Wadsworth, and B. Roizman. 1975. Anatomy of herpes simplex virus DNA: Evidence for four populations of molecules that differ in the relative orientations of their long and short segmants. Proc. Natl. Acad. Sci. USA 72:4243-4247.

Heine, J.W., R.W. Honess, E. Cassai, and B. Roizman. 1974. Proteins specified by herpes simplex virus XII. The virion polypeptides of type 1 strains. J. Virol. 14:640-651.

Heintz, N., H.L. Sive, and R.G. Roeder. 1983. Regulation of human histone gene expression kinetics of accumulation and changes in the rate of synthesis and in the half lives of individual histone mRNAs during the HeLa cell cycle. Mol. Cell. Biol. 3:1920-1929.

Hill, T.M., R.K. Sinden, J.R. Sadler. 1983. Herpes simplex virus types 1 and 2 induce shutoff of host protein synthesis in Friend erythroleukemia cells. J. Virol. 45:241-250.

Holland, L.E., K.P. Anderson, J.A. Stringer, and E.K. Wagner. 1979. Isolation and localization of herpes simplex virus type 1 mRNA abundant before viral DNA synthesis. J. Virol. 31:447-462.

Holland, L., R. Sandri-Goldin, A.L. Goldin, J. Glorioso, and M. Levine. 1984. Transcriptional and genetic analyses of the herpes simplex virus type 1 genome:coordinates 0.29-0.45. J. Virol. 49:947-959.

Honess, R.W., and B. Roizman. 1973. Proteins specified by herpes simplex virus. XI. Identification and relative molar rates of synthesis of structural and nonstructural herpesvirus polypeptides in the infected cells. J. Virol. 12 :1347-1365.

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

Honess, R.W., and B. Roizman. 1975. Regulation of herpesvirus macromolecular synthesis: Sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc. Natl. Acad. Svi. USA 72:1276-1280.

Honess, R.W., and D.H. Watson. 1977. Herpes simplex virus resistance and sensitivity to phosphonoacetic acid. J. Virol. 21:584-600.

Hope, R.G., and H.S. Marsden. 1983. Processing of glycoproteins induced by herpes simplex virus type 1:sulphation and nature of the oligosaccharide linkages. J. Gen. Virol. 64:1943-1953.

Inglis, S.C. 1982. Inhibition of host protein synthesis and degradation of cellular mRNAs during infection by infleunza and herpes simplex virus. Mol. Cell. Biol. 2:1644-1648.

Inglis, M.M., and A.A. Newton. 1981. Comparison of the activities of HSV-1 and cellular mRNAs as templates for in vitro translation. Virol. 110:1-15

Johnson, D.C., and P.G. Spear. 1982. Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and egress of virions from infected cells. J. Virol. 43:1102-1112.

Kieff, E.D., S.L. Bachenheimer, and B. Roizman. 1971. Size, composition and structure of the DNA of subtypes 1 and 2 herpes simplex virus. J. Virol. 8:125-132.

Knipe, D.M., W.T. Ruyechan, B. Roizman, and I.W. Halliburton. 1978. Molecular genetics of herpes simplex virus. Demonstration of regions of obligatory and non-obligatory identity in diploid regions of the genome by sequence replacement and insertion. Proc. Natl. Acad. Sci. USA 75:3896-3900.

Kwong, A.D. 1988. 13th International Herpesvirus Workshop. Univ. of Californ9a, Irvine. Abstract # 48.

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

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

Lang, J.C., D.A. Spandidos, and N.M. Wilkie. 1984. Transcriptional regulation of herpes simplex virus immediate early gene is mediated through an enhancer-type sequence. EMBO Journal 3:389-395.

Lang, J.C., N.M. Wilkie, and D.A. Spandidos. 1983. Characterization of eukaryotic transcriptional control signals by assay of herpes simplex type 1 thymidine kinase. J. Gen. Virol. 64:2679-2696.

Lee, G.T-Y., M.F. Para, and P.G. Spear. 1982. Location of structural genes for glycoproteins gD and gE and for other polypeptides in the S component of the herpes simplex virus type 1 DNA. J. Virol. 43:41-49.

Lemaster, S., and B. Roizman. 1980. Herpes simplex virus phosphoproteins. II. Charaterization of the virion protein kinase and of the polypeptides phosphorylated in the virion. J. Virol. 35:798-811.

Linial, M., N. Gunderson, and M. Groudine. 1985. Enhanced transcription of c-myc in bursal lymphoma cells requires continuous protin synthesis. Science 230:1126-1132.

Little, S.P., J.T. Jofie, R.J. Courtney, and P.A. Schaffer. 1981. A virion-associated glycoprotein essential for infectivity of herpes simplex virus type 1. Virol. 115:149-160.

Locker, H., and N. Frenkel. 1979. Bam I, Kpn I and Sal I restriction enzyme maps of the DNAs of herpes simplex virus Justin and F: Occurrence of heterogeneities in defined regions of the viral DNA. J. Virol. 32:424-441.

Lodish, H.F., and M. Porter. 1980. Translational control of protein synthesis after infection by Vesicular Stomatitis Virus. J. Virol. 36:719-

Luscher, B., B. Stauber, R. Schindler, and D. Schumperli. 1985. Faithful cell-cycle regulation of a recombinant mouse histone H4 gene is controlled by sequences in the 3' terminal part of the gene. Proc. Natl. Acad. Sci. USA. 82:4389-4393.

Mackem, S., and B. Roizman. 1980. Regulation of herpesvirus macromolecular synthesis: transcription-initiation sites and domains of α genes. Proc. Natl. Acad. Sci. USA 76:4117-4121.

Mackem, S., and B. Roizman. 1982a. Differentiation between α-promoter regulator regions of herpes simplex virus type 1: the functional domains and sequence of a movable α-regulator . Proc. Natl. Acad. Sci. USA 79:4917-4921.

Mackem, S., and B. Roizman. 1982b. Structural features of the herpes simplex virus α gene 4, 0, and 27 promoter-regulatory sequences which confer α regulation on chimeric thymidine kinase genes. J. Virol. 44:939-949.

Maniatis, T.M., E.F. Fritsch, and J. Sambrook. 1982. In Molecular Cloning. A laboratory manual. Cold Spring Harbor Laboratory.

Manservigi, R., P.G. Spear, and A. Buchan. 1977. Cell fusion induced by herpes simplex virus is promoted and suppressed by different glycoproteins. Proc. Natl. Acad. Sci. USA 74:3913-3917.

Marsden, H.S., L. Harr, and C.M. Preston. 1983. Processing of herpes simplex virus proteins and evidence that translation of thymidine kinase mRNA is initiated at three separate AUG codons. J. Virol. 46:434-445.

Marzluff, W.F., and N.B. Pandey. 1988. Multiple regulatory steps control histone mRNA concentrations. Trends Biochem. Sci. 13:49-52.

Matthews, R.E.F. 1982. Classification and nomenclature of viruses. Fourth Report of the International Committee on Taxonony of Viruses. Intervirology 17:1-199.

Mavromara-Nazos, P., M. Ackerman, and B. Roizman. 1986. Construction and properties of a viable herpes simplex virus 1 recombinant lacking coding sequences of the α 47 gene. J. Virol. 60:807-812.

Mavromara-Nazos, P., S. Silver, J. Hubenthal-Voss, J.C. McKnight, and B. Roiuzman. 1986. Regulation of herpes simplex virus 1 gene: alpha gene sequence requirements for transient induction of indicator genes regulated by beta or late (gamma 2) promoters. Virology 149:152-164.

Mayman, B.A., and Y. Nishioka. 1985. Differential stability of host mRNAs in Friend erythroleukemia cells infected with the herpes simplex virus type 1. J. Virol. 53:1-6.

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

McGeoch, D.J., M.A. Darlymple, A.J. Davison, A. Dolan, M.C. Frame, D. McNab, L.J. Perry, J.E. Scott, and P.Taylor. 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. 69:1531-1574.

McGrath, B.M., and W.S. Stevely. 1980. The characteristics of the cell-free translation of mRNA from cells infected with the herpes virus pseudorabies virus. J. Gen. Virol. 49:323-332.

McKnight, S.L. 1980. The nucleotide sequence and transcript map of the herpes simplex virus thymidine kinase gene. Nuc. Acids Res. 8:5949-5964.

McKnight, J.L.C., P.E. Pellett, F.J. Jenkins, and B. Roizman. 1987. Characterization and nucleotide sequence of two herpes simplex virus type 1 genes whose products modulate alpha trans-inducing factor depeneedent activation of alpha genes. J. Virol. 61:992-1001.

Meijlink, F., T. Curran, A.D. Miller, and I.M. Verma. 1985. Removal of a 67 base-pair sequence in the noncoding region of proto-oncogene c-fos converts it to a transformong gene. Proc. Natl. Acad. Sci. USA 82:4987-4991.

Merrill, G., R. Harland, M. Groudine, and S.L. McKnight. 1984. Genetic and physical analysis of the chicken tk gene. Mol. Cell. Biol. 4:1769-1776.

Miller, A.D., T. Curran, and I.M. Verma. 1984. c-fos protein can induce cellular transformation: a novel mechanism of a cellular oncogene. Cell 36:51-60.

Mocarski, E.S., and B. Roizman. 1982. The structure and role of herpes simplex virus DNA termini in inversion, circularization and generation of virion DNA. Cell 31:89-97.

Morgan, C., H.M. Rose, M. Holden, and E.P. Jones. 1959. Electron-microscopic observations on the development of herpes simplex virus. J. Exp. Med. 110:643-656.

Morgan, C., H.M. Rose, and B. Mednis. 1968. Electron microscopy of herpes simplex virus. I. Entry. J. Virol. 2:507-516.

Morse, L.S., T.G. Buchman, B. Roizman, and P.A. Schaffer. 1977. Anatomy of herpes simplex virus DNA. IX. Apparent exclusion of some parental DNA rearrrangements in the generation of some intertypic (HSV-1 X HSV-2) recombinants. J. Virol. 24:231-248.

Morse, L.S., L. Pereira, B. Roizman, P.A. Schaffer, 1978. Anatomy of

HSV DNA. XI. Mapping of viral genes by analysis of polypeptides and fuctions specified by HSV-1 X HSV-2 recombinants. J. Virol. 26:389-410.

Mullner, E.W., and L.C. Kuhn. 1988. A stem-loop in the 3' untranslated region mediates iron-dependent regulation of transferrin receptor mRNA stability in the cytoplasm. Cell 53:815-825.

Nakai, H., I.H. Maxwell, and L.I. Pizer. 1982. Herpevirus infection alters the steady state levels of cellular polyadenylated RNA in polyoma virus-transformed BHK cells. J. Virol. 42:1131-1134.

Neidhart, H., C.H. Schroder, and H.C. Kaerner. 1987. Herpes simplex virus type 1 glycoprotein E is not indispensable for viral infectivity. J. Virol. 61:600-603.

Newton, A.A. 1969. Internal virology I. In Proceedings of the 1st International Congress of Virology . Melnick, J.L. ed. S. Krager, Basel. p. 253.

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

Noble, A.G., G.T-Y. Lee, R. Sprague, M.L. Parish, and P.G. Spear. 1983. Anti-gD monoclonal antibodies inhibit cell fusion induced by herpes simplex virus type 1. Virology 129:218-224.

Nuss, D.L., H. Opperman, and G. Koch. 1975. Selective blockage of initiation of host protein synthesis in RNA-virus infected cells. Proc. Natl. Acad. Sci. USA 72:1258

O'Hare, P., and C.R. Goding. 1988. Herpes simplex virus regulatory elements and the immunoglobulin octamer domain bind a common factor and are both targets for virion transactivation. Cell 52:435-445.

O'Hare, P., and G. Hayward. 1985a. Evidence for a direct role of both the 175,000 and 110,000 - molecular weight immediate-early proteins of herpes simplex virus in the trans - activation of delayed - early promoters. J. Virol. 53:751-760.

O'Hare, P., and G. Hayward. 1985b. Three trans - acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. J. Virol. 56:723-733.

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

Paek, I., and R. Axel. 1987. Glucocorticoids enhanced stability of human growth hormone mRNA. Mol. Cell. Biol. 7:1496-1507.

Para, M.F., M.L. Parrish, A.G. Noble, and P.G. Spear. 1985. Potent neutralizing activity associated with anti-glycoprotein D specificity among monoclonal antibodies selected for binding to herpes simplex virions. J. Virol. 55:483-488.

Parris, D.S., R.A.F. Dixon, and P.A. Schaffer. 1980. Physical mapping of herpes simplex virus type 1 ts mutants by marker rescue: correlation of the physical and genetic maps. Virology 100:275-287.

Pelletier, J., and N. Sonenberg. 1988. Internal initiation of translation of eukaryotic mRNA directed from poliovirus RNA. Nature(London) 334:320-325.

Pelletier, J., and N. Sonenberg. 1989. Internal binding of eukaryotic ribosomes on poliovirus RNA:Translation in HeLa cell extracts. J. Virol. 63:441-444.

Peltz, S.W., G. Brewer, G. Kobs, and J. Ross. 1987. Substrate specificity of the exonuclease activity that degrades H4 histone mRNA. J. Biol. Chem. 262:9382-9388.

Penman, S., Y. Becker, and J.E. Darnell. 1964. A cytoplasmic structure involved in the synthesis and assembly of poliovirus components. J. Mol. Biol. 8:541-555.

Pereira, L., D.V. Dondero, D. Gallo, V. Devlin, and J.D. Woodie. 1982. Serologic analysis of herpes simplex virus types 1 and 2 with monoclonal antibodies. Infect. Immun. 35:363-367.

Periera, L,, M.H. Wolff, M.L. Fenwick, and B. Roizman. 1977. Regulation of herpes simplex virus macromolecular synthesis. V. Properties of α polypeptides made in HSV-1 and HSV-2 infected cells. Virology 77:733-749.

Piechazyk, M., J.Q. Yang, J.M. Blanchard, P. Jeanteur, and K.B. Marcu. 1985. Posttranscriptional mechanisms are responsible for accumulation of truncated c-myc mRNAs in murine plasma cell tumors. Cell 42:589-597.

Pilder, S., M. Moore, J. Logan, and T. Shenk. 1986. The Adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. Mol. Cell. Biol. 6:470-476.

Pittenger, M.R., and D.W. Cleveland. 1985. Retention of autoregulatory control of tubulin synthesis in cytoplasts: demonstration of a cytoplasmic mechanism that reglates the level of tubulin expression. J. Cell. Biol. 101:1941-1952.

Post, L.E., S. Mackem, and B. Roizman. 1981. The regulation of α genes of herpes simplex virus: Expression of chimeric genes produced by fus-

ion of thymidine kinase with α gene promoters. Cell 24:555-565.

Post, L.E., and B. Roizman. 1981. A generalized technique for the deletion of specific genes in large genomes: α 22 of herpes simplex virus type 1 is not essential for growth. Cell 25:227-232.

Powell, K.L., and R.J. Courtney. 1975. Polypeptides synthesized in herpes simplex type 2-infected Hep-2 cells. Virology 66:217-228.

Preston, C.M., M.C. Frame, and M.E.M. Campbell. 1988. A complex formed between cell components and an HSV structural polypeptide binds to a viral immediate early gene regulatory DNA sequence. Cell 52:425-434.

Raghow, R. 1987. Regulation of messenger RNA turnover in eukaryotes. Trends in Biochem. Sci. 12:358-360.

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

Read, G.S., and W.C. Summers. 1982. In vitro transcription of the thymidine kinase gene of herpes simplex virus. Proc. Natl. Acad. Sci. USA 79:5215-5219.

Rice, S.A., and D.M. Knipe. 1988. Gene-specific transactivation by herpes simplex virus type 1 alpha protein ICP27. J. Virol. 62:3814-3823.

Roizman, B. 1982. The family Herpesviridae. General description, taxonomy and classification. In: The Viruses, vol. 1 Herpesviruses. ed. B. Roizman pp.1-23, Plenum press, New York.

Roizman, B., and W. Batterson. 1986. Herpesviruses and their replication. In Fundamental Virology. B.N. Fields and D.M. Knipe eds. Raven Press, New York pp. 607-636.

Roizman, B., G.S. Borman, and M. Roysta. 1965. Macromolecular synthesis in cells infected with herpes simplex virus. Nature (London) 296:1374-1375.

Roizman, B., and D. Furlong. 1974. The replication of herpesviruses. In Comprehensive Virology, eds. H. Fraenkel-Conrat and R.R. Wagner. Plenum press, New York. pp.299-403.

Roizman, B., G. Hayward, R. Jacob, S. Wadsworth, and R.W. Honess. 1974. Human herpesvirus 1: A model for molecular organization of herpesvirus virions and their DNA. Excerpta Med. Int. Congr. Ser. No. 350 2:188

Roizman, B., B. Norrild, C. Chan, and L. Pereira. 1984. Identification of a herpes simplex virus type 2 glycoprotein lacking a known type

1 counterpart. Virology 133:242-247.

Roizman, B. and P.G. Spear. 1981. Herpes simplex viruses. In DNA tumor viruses. Molecular biology of tumor viruses. John Tooze ed. pp.615-746.

Rosen, H., G. DiSegni, and R. Kaempfer. 1982. Translation control by messenger RNA competition for eukaryotic initiation factor 2. J. Biol. Chem. 257:946-952.

Ross, J. and G. Kobs. 1986. H4 histone messenger RNA decay in cell-free extracts initiates at or near the 3' terminus and proceeds 3' to 5'. J. Mol. Biol. 188:579-593.

Ross, J., G. Kobs, G. Brewer, and S.W. Peltz. 1987. Properties of the exonuclease activity that degrades H4 histone mRNA. J. Biol. Chem. 262:9374-9381.

Ross, J., S.W. Peltz, G. Kobs, and G. Brewer. 1986. Histone mRNA degradation in vivo: the first detectable step occurs at or near the 3' terminus. Mol. Cell. Biol. 6:4362-4371.

Ruyechan, W.T., S.L. Morse, D.M. Knipe, and B. Roizman. 1979. Molecular genetics of herpes simplex virus type II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. J. Virol. 29:677-697.

Sacks, W.R., C.C. Greene, D.P. Aschman, and P.A. Schaffer. 1985. Herpes simplex virus ICP27 is an essential regulatory protein. J. Virol. 55:796-805.

Sacks, W.R., and P.A. Schaffer. 1987. Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. J. Virol. 61:829-839.

Sarmiento, M., M. Haffey, and P.G. Spear. 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7(β_2) in virion infectivity. J. Virol. 29:1149-1158.

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

Schmeissner, U., K. McKinney, M. Rosenberg, and D. Court. 1984. Removal of a terminator structure by RNA processing regulates int gene expression. J. Mol. Biol. 176:39-53.

Schumperli, D.. 1986. Cell-cycle regulation of histone gene expression. Cell 45:471-472.

Scholtissek, C. 1971. Deletion of an unstable RNA in chick fibroblasts after reduction of the UTP pool by glucosamine. Eur. J. Biochem. 24:358-365.

Schwartz, J., and B. Roizman. 1969. Similarities and differences in the development of laboratory strains of herpes simplex viruses in HEp-2 cells: Electron microscopy. J. Virol. 4:879-889.

Sharp, J.A., M.J. Wagner, and W.C. Summers. 1983. Transcription of herpes simplex virus genes in vivo:Overlap of a late promoter with the 3' end of the early thymidine kinase gene. J. Virol. 45:10-17.

Shaw, G., and R. Kamen. 1986. A conserved AU sequence from the 3'untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46:659-667.

Sheiness, D., L. Puckett, and J.E. Darnell. 1975. Possible relationship of poly(A) shortening to mRNA turnover. Proc. Natl. Acad. Sci. USA 72:1077-1081.

Sheldrick, P., and N. Berthelot. 1975. Inverted repetitions in the chromosome of herpes simplex virus. Cold Spring Harbor Symposium Quant. Biol. 39:667-678.

Silver, S., and B. Roizman. 1985. Gamma-2 thymidine kinase chimeras are identically transcribed but regulated as gamma-2 genes in herpes simplex virus genomes and as beta genes in cell genomes. Mol. Cell. Biol. 5:518-528.

Silverstein, S., R. Millette, P. Jones, and B. Roizman. 1976. RNA synthesis in cells infected with herpes simplex virus. XII. Sequence complexity and properties of RNA differing in the extent of adenylation. J. Virol. 18:977-991.

Sittman, D.B., R.A. Graves, and W.F. Marzluff. 1983. Histone mRNAs are regulated at the level of transcription and mRNA degradation. Proc. Natl. Acad. Sci. USA 80:1849-1853.

Smiley, J.R., C. Smibert, and R.D. Everett. 1987. Expression of a cellular gene cloned in herpes simplex virus: Rabbit beta-globin is regulated as an early viral gene in infected fibroblasts. J. Virol. 61:2368-2377.

Smith, I.L., and R.M. Sandri-Goldin. 1988. Evidence that transcriptional control is the major mechanism of regulation of glycoprotein D in herpes simplex virus type 1-infected cells. J. Virol. 62:1474-1477.

Spear, P.G. 1976. Membrane proteins specified by herpes simplex virus I. Identification of four glycoprotein precursors and their products in type I infected cells. J.Virol. 17:991-1008.

Spear, P.G. 1984. Glycoproteins specified by herpes simplex viruses. In: The herpesviruses, Vol. 3, B. Roizman, ed. Plenum press, New York.

Spear, P.G., and B. Roizman. 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J. Virol. 9:431-439.

Stanssens, P., E. Remaut, and W. Fiers. 1986. Inefficient translation initiation causes preature transcription termination in the lac Z gene. Cell 44:711-718.

Stow, N.D., J.H. Subak-Sharpe, and N.M. Wilkie. 1978. Physical mapping of temperature-sensitive mutations of herpes simplex virus type 1 by intertypic marker rescue. Virology 90:1-11.

Stringer, J.R., L.E. Holland, R.I. Swanstrom, K. Pivo, and E.K. Wagner. 1977. Quantitation of herpes simplex virus type 1 RNA in infected HeLa cells. J. Virol. 21:889-901.

Strnad, B.C., and L. Aurelian. 1976. Proteins of herpes virus type 2 I. Virion, nonvirion, and antigenic polypeptides in infected cells. Virology 69:438-452.

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

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

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

Thompson, D.B. P.B. Challoner, P.E. Neiman, and M. Groudine. 1986. Expression of c-myb proto-oncogene during cellular proliferation. Nature(London) 319:374-3880.

Treisman, R. 1985. Transcript accumulation of c-fos RNA following serum stimulation requires a conserved 5' element and c-fos 3' sequences. Cell 42:889-902.

Tognon, M., E. Cassai, A. Rotola, and B. Roizman. 1983. The heterogeneous regions in herpes simplex virus 1 DNA. Microbiologica 6:191-198.

Wadsworth, S., R.J. Jacob, and B. Roizman. 1975. Anatomy of herpes simplex virus DNA. II. Size, composition, and arrangement of inverted terminal repetitions. J. Virol. 15:1487-1497.

Wagner, E.K. 1984. Individual HSV transcripts: characterization of specific genes. pp. 45-104. In Herpesviruses vol. 3, ed. B. Roizman, Plenum press, New York.

Wagner, E.K., and B. Roizman. 1969. Ribonucleic acid synthesis in

cells infected with HSV. I. The patterns of RNA synthesis in productively infected cells. J. Virol. 4:36-46.

Wagner, M.M., and W.C. Summers. 1978. Structure of the joint region and the termini of the DNA of herpes simplex virus type 1. J. Virol. 27:374-387.

Watson, R.J., and J.B. Clements. 1978. Characterization of transcription-deficient temperature-sensitive mutants of herpes simplex virus type 1. Virology 91:364-379.

Watson, R.J., and J.B. Clements. 1980. A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis. Nature 285:329-330.

Watson, R.J., C.M. Preston, and J.B. Clements. 1979. Separation and characterization of herpes simplex virus type 1 immediate-early mRNAs. J. Virol. 31:42-52.

Watson, R.J., M. Sullivan, and G.F. Vande Woude. 1981. Structure of two spliced herpes simplex virus type 1 immediate-early mRNAs which map at the junctions of the unique and reiterated regions of the virus DNA S components. J. VIrol. 37:431-444.

Weber, P.C., M.D. Challberg, N.J. Nelson, M. Levine, and J.C. Glorioso. 1988. Inversion events in the HSV-1 genome are directly mediated by the viral DNA replication machinery and lack sequence specificity. Cell 54:369-381.

Weber, P.C., M. Levine. and J.C. Glorioso. 1987. Rapid identification of nonessential genes of herpes simplex virus type 1 by Tn5 mutagenesis. Science 236:576-579.

Weinheimer, S.P., and S.L. McKnight. 1987. Transcriptional and posttranscriptional controls establish the cascade of herpes simplex virus protein synthesis. J. Mol. Biol. 195:819-833.

Weller, S.K., K.J. Lee, D.J. Sabourin, and P.A. Schaffer. 1983. Genetic analysis of temperature sensitive mutants which define the gene for the major herpes simplex virus type 1 DNA-binding protein. J. Virol. 45:354-366.

Westmoreland, D., and J.F. Watkins. 1974. The IgG receptors induced by herpes simplex virus:studies using radioiodinated IgG. J. Gen. Virol. 24:167-178.

Wildy, P., and D.H. Watson. 1963. Electron microscopic studies on the architecture of animal viruses. Cold Spring Harbor Symposium Quant. Biol. 27:25-47.

Wilson, M.C., and J.E. Darnell Jr. 1981. Control of messenger RNA concentration by differential cytoplasmic half-life. Adenovirus messenger

RNAs from transcription units 1A and 1B. J. Mol. Biol. 148:231-251.

Wilson, T., and R. Treisman. 1988. Removal of poly(A) and consequent degradation of c-fos mRNA facilitated by 3' AU-rich sequences. Nature 336:396-399.

Wong, H.C., and S. Chang. 1986. Identification of a positive retroregulator that stabilizes mRNAs in bacteria. Proc. Natl. Acad. Sci. USA 83:3233-3237.

Woodland, H.R., and F.H. Wilt. 1980. The functional stability of sea urchin histone mRNA injected into oocytes of Xenopus laevis. Dev. Biol. 75:199-213.

Wu, C.A., N.J. Nelson, D.J. McGeoch, and M.D. Challberg. 1988. Identification of herpes simplex virus type 1 genes required for origin dependent DNA synthesis. J. Virol. 62:435-443.

Yager, D.R., and D.M. Coen. 1988. Analysis of the transcript of the herpes simplex virus DNA polymerase gene provides evidence that polymerase expression is inefficient at the level of translation. J. Virol. 62:2007-2015.

Yen, T.J., P.S. Machlin, and D.W. Cleveland. 1988. Autoregulated instability of β-tubulin mRNAs by recognition of the nascent amino terminus of β-tubulin. Nature 334:580-585.

Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains:nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103-119.

Zhang, Y.-F., G.B. Devi-Rao, M. Rice, R.M. Sandri-Goldin, and E.K. Wagner. 1987. The effect of elevated levels of herpes simplex alpha gene products on the expression of model early and late genes in vivo. Virology 157:99-106.

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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 requirements for the degree of Doctor of Philosophy.

4/17/89
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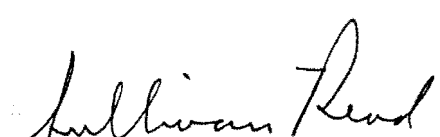
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