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LOYOLA UNIVERSITY CHICAGO

STUDIES OF mRNA DEGRADATION MEDIATED BY THE VIRION HOST SHUTOFF FUNCTION OF HERPES SIMPLEX VIRUS TYPE 1

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INTRODUCTION

Documentation of human herpesvirus infections extends back to the ancient Greeks. Indeed, the word "herpes" derives from a Greek word meaning "to creep or crawl", a reference to the way skin lesions spread in infected individuals (19,225). However, modern scholarship in the study of herpes simplex virus did not begin until the late 1960s. It was in 1968 when Nahmias and Dowdle first demonstrated the existence of two antigenic strains of the virus, and furthermore that herpes simplex type 1 (HSV-1) was associated with infections above the belt (nongenital infection) while herpes simplex type 2 (HSV-2) was associated with infection below the belt (genital herpes) (135). The following 20-odd years has led to many dramatic developments in the field, but numerous questions about the biology of herpes simplex virus remain.

The two strains of herpes simplex virus belong to the family Herpesviridae (123). Members of this family share several features. All have a large, linear double-stranded DNA genome, wrapped around a fibrillar spool, enclosed in the core of the virion. The core is surrounded by an icosahedral nucleocapsid, consisting of 162 capsomeres. The nucleocapsid is enclosed in an amorphous material called the tegument. The entire structure is then housed in an envelope covered with viral glycoprotein spikes.

Herpesviruses are ubiquitous in nature. Upon examination, most vertebrate species can be infected by at least one herpesvirus. The range extends from humans and other primates, through horses, cattle, pigs, chickens, and fish. These viruses fall into one of three subfamilies (58). Members of the subfamily Alphaherpesvirinae have a variable host range, reproduce relatively quickly, spread rapidly in culture, destroy infected cells efficiently, and can establish latent infections in nerve ganglia. This subfamily contains two genera, Simplexvirus, which includes HSV-1 and HSV-2, and Varicellavirus, which includes varicella-zoster virus (the causative agent of chicken pox and shingles) and pseudorabies virus (which infects pigs).

Members of the second subfamily, the Betaherpesvirinae, are characterized by a more restricted host range than the Alphaherpesvirinae, a long reproductive cycle, and slow growth in culture. Infected cells often become enlarged (cytomegalia). These viruses can maintain latent infections in secretory glands, lymphoreticular cells, kidneys, and other tissues. The primary genus in this subfamily is Cytomegalovirus, with both human and murine representatives.

The subfamily Gammaherpesvirinae contains members whose experimental host range is very limited. <u>In vitro</u>, these viruses all can replicate in lymphoblastoid cells, with specificity for either T or B cells. Latent infection is typically in these lymphoid tissues. The principal genus is the Lymphocryptovirus, the archetype being Epstein-Barr virus, the causative agent of infectious mononucleosis. The Gammaherpesvirinae also contain the recently identified human herpesvirus 6.

Despite this recent discovery, as well as descriptions of nearly 100 other herpesviruses, the majority of the extant information comes from studies involving HSV-1 and HSV-2. Much is known on the biology, morphology, and genetics of these viruses exists, some of which will be reviewed below.

Viral Replication

Sequence analysis indicates that HSV-1 contains 74 open reading frames encoding 72 polypeptides (two of the open reading frames are reiterated; 126). Of that number, at least 33 are designated as virion polypeptides (77). Eight of these virion proteins are glycosylated and are found on the surface of the virion. These glycoproteins are involved mainly in the attachment to and penetration of the host cell. Glycoprotein B (gB) and gC are primarily responsible for attachment to receptor molecules on the surface of the host cells (110,227). The recently reported gH is required early in infection, although it may be involved in penetration rather than attachment (28).

Penetration of the host cell is a multistep event involving several of the glycoproteins. Penetration is thought to occur not by phagocytosis but rather by fusion of the viral envelope with the plasma membrane (131). Two virion glycoproteins, gB and gD, are implicated in this fusion process. A temperature sensitive mutation in gB leads to a virus that can attach but not penetrate host cells (121). Likewise, viruses carrying a deletion of gB can only attach to host cells (30). Deletion of gD leads to the same phenotype (93). In cell culture, gC, gE, gG, gI, and gJ are all dispensable, for either entry or egress of viral particles (223). The other three glycoproteins, gB, gD, and gH are essential.

Once the virus penetrates the plasma membrane, the intact nucleocapsid is transported to the nuclear pores. The viral DNA is then released into the nucleoplasm. Infection with wild type virus leads to accumulation of empty nucleocapsids at the nuclear pores, and parental viral DNA accumulates in the nucleus. This event requires a viral function, as the temperature sensitive mutant HSV-1(HFEM)<u>ts</u>B7 accumulates at nuclear pores and only releases its DNA upon downshift to the permissive temperature (11).

The HSV genome has a structure unique to the Herpesviridae (Figure 1). The genome is approximately 150 kilobase pairs in size, with a 68% G+C content (HSV-1), or 69% G+C content (HSV-2) (13,100). The genome consists of two covalently linked segments, L (long) and S (short). Each segment contains unique sequences bracketed by inverted repeats

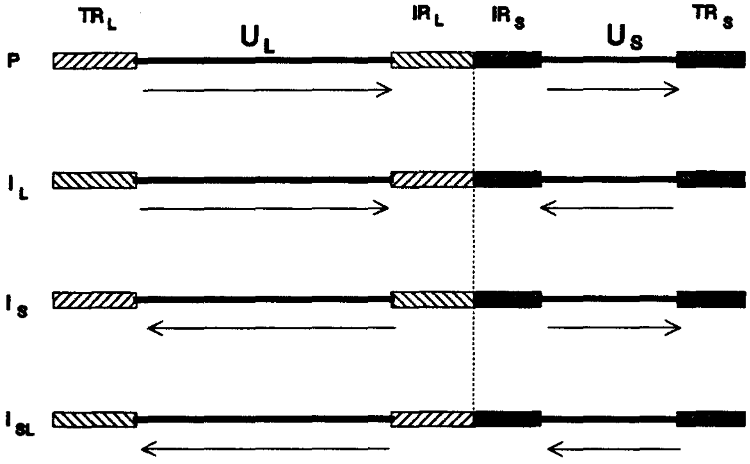


Figure 1. Schematic Diagram of the Herpes Simplex Type 1 Genome. The four possible isomeric forms of the wild type genome are diagrammed here: P is the prototypical isomer, I_S is inverted in the S segment, I_L is inverted in the L segment, and I_{SL} is inverted in both the S and L segments. The arrows refer to the orientation of the segments relative to the P isomer. Refer to the text for other definitions.

(194). The repeats on the L segment are designated ab and b′a′, whereas the repeats of the S component are called ca and a′c′ (219). The genome can be represented schematically thus:

$$a_La_nb - U_L - b'a'_mc' - U_S - ca_S$$

where a_L and a_S are terminal a sequences as defined below, a_n and a_m are terminal a sequences directly repeated zero or more times (n) or present in one to many copies (m), U_L is the large unique component, and U_S is the small unique component (116,165).

The a sequence has a highly conserved structure, but contains an inconstant number of repeat elements. The repeat elements of the HSV-1 strain F have been defined, where the a sequence consists of a 20 base pair direct repeat called DR1, a 65 base pair unique sequence designated U_b, a 12 base pair direct repeat (DR2) present in 19-23 copies per a sequence, a 37 base pair direct repeat (DR4) present in two to three copies, a 58 base pair unique sequence (U_c), and finally another copy of DR1 (128,129). The number of copies of DR2 and DR4 determine the size of the a sequence for a given strain. The a sequence can be schematically represented as:

$$DR1 - U_b - DR2_n - DR4_m - U_c - DR1$$

where n and m are the number of copies of DR2 and DR4 respectively.

The most unique feature of this genome is that the L and S components can invert relative to one another. These inversions yield four distinct isomers, designated as P (prototype), I_L (inversion of the L component), I_S (inversion of the S component), and I_{SL} (inversion of both the S and L components) (45,76). Wild type isolates can carry any one of these four isomers with a 25% probability. However, genomes "frozen" in a particular isomer by deletion of the internal inverted repeats generate viable progeny (92,157). Therefore, the reason for this isomerization is unclear.

Regardless of the isomer involved, once the genome is liberated into the nucleoplasm, viral gene expression begins. Viral genes are expressed in one of three broad kinetic classes, designated α (immediate-early), β (early), and γ (late). These three classes are "coordinately controlled in a cascade fashion" (82), such that α gene products regulate the expression of β genes, whose products subsequently control the expression of γ genes (82). The regulation of α gene transcription is effected by a component of the incoming virion, called α-trans-induction factor (αTIF) or Vmw 65 (12, 31). A cis-acting site has been identified in the 5′ noncoding region of all five α genes (105). A host nuclear protein binds to this site (65,141); αTIF binds to the ensuing nuclear protein-viral DNA complex, or binds the nuclear protein and this protein-protein complex then binds the cis-acting site (158,212). This interaction presumably allows αTIF to transactivate α gene transcription.

Notwithstanding the precise mechanism of trans-activation, the α genes can be operationally defined as those capable of being expressed in the absence of any other viral gene expression (153). There are five such genes, whose products are designated as infected cell polypeptides (ICP) 0, 4, 22, 27, and 47. The synthesis of these proteins can be detected as early as one hour post infection (p. i.), peaks around two to four hours p. i., and declines thereafter (83). All α proteins, with the possible exception of ICP47, are thought to have regulatory functions. ICP4 is the best characterized α protein, and it is essential throughout infection for efficient transcription of all classes of viral genes (47). One interesting set of <u>ts</u> mutants in ICP4 overproduces α proteins but fails to induce the expression of any other class of viral genes (46,222).

The α protein ICP27 is also essential. Deletion mutants exhibit reduced synthesis of viral DNA and a virtual absence of late gene expression (124). Analysis of viral mutants as well as results from transient expression assays indicate that ICP27 has either positive or negative regulatory effects, depending upon the gene examined (51,163).

ICP0, another of the α proteins, has been examined primarily through the use of transient expression systems. These studies suggest that ICP0 can trans-activate co-transfected viral genes, either alone or in concert with ICP4 (52, 64,142). Deletion of the gene for ICP0 does not greatly reduce the growth of mutant viruses in tissue culture (181, 203). These mutants produce significant amounts of viral DNA and exhibit only a small reduction in the synthesis of late proteins compared to wild type.

ICP22 deletion mutants make viral DNA but do not express late genes effectively, although this appears to depend upon the host cell used for infection (191). As a group, then, the α proteins play a major role in all subsequent viral gene expression, although ICP4 and ICP27 appear to be the only essential α proteins.

Expression of β genes requires the presence of functional α proteins, and β polypeptide synthesis peaks at five to seven hours p. i. (83). The β genes are divided into two kinetically distinct classes, namely β_1 and β_2. The β_1 class appears very early in infection, and its members have been mistaken for α proteins (36). In fact, β_1 genes differ from α genes by the requirement of the former for functional ICP4 for their synthesis (81,82). Representative β_1 genes include the one coding for the large subunit of the viral ribonucleotide reductase (ICP6) and the gene for the major DNA-binding protein (ICP8). The latter is an essential gene, required for the synthesis of viral DNA (40, 66) and control of later viral gene expression (67,68). The β_2 class includes the genes for the viral thymidine kinase and DNA polymerase. Most of these genes are involved in the synthesis of viral DNA, an event that heralds the onset of γ gene expression.

The late, or γ, genes have been divided into two groups,

γ_1 and γ_2, depending on the stringency of their requirement for DNA synthesis (41,80). Prototypical γ_1 genes include the genes for glycoprotein B and H, and the major capsid protein gene (ICP5). The expression of these genes is somewhat affected by DNA synthesis inhibitors, probably as a result of gene dosage effects. As the number of genome copies decreases, so too does the expression of the γ_1 genes. In contrast, the expression of γ_2 genes, such as the glycoprotein C gene, is totally repressed in the presence of DNA synthesis inhibitors (82,83). As a group, the γ gene products become structural components of the progeny virions, which appear as early as 12 hours p. i.

Viral DNA Synthesis

The synthesis of viral DNA can be detected as early as three hours after the start of infection, and proceeds at least until 15 hours p. i. (166,167). Of the starting (parental) DNA, only a small fraction is actually replicated (89). Although within the virus the viral DNA is linear, in the infected cell, free ends of viral DNA cannot be detected. The DNA is either circular, or in head-to-tail concatamers (88). The evidence so far implies that herpes simplex virus replicates by a rolling-circle mechanism (15,88), although such a structure has not yet been isolated.

The origins of replication of HSV DNA have been isolated and well defined. Origins were first defined by examining defective interfering viral genomes (59,198). The current definition is functional. That is, an origin of replication is defined as those sequences required to allow a fragment of HSV DNA transfected into permissive cells to be amplified. This transfected fragment requires viral functions provided in trans, either by co-transfection with a viral genome or individual viral genes, or by infection with helper virus. Using this functional definition, HSV-1 has three origins of DNA replication. Two of these map to the repeat sequences of the S component. Ori_S1 is found between the promoters for the α genes encoding ICP4 and ICP22, whereas ori_S2 is situated between the ICP4 and ICP47 gene promoters (43,130, 202, 218). The third origin, ori_L, is in the middle of the L component, between the promoters for the major DNA-binding protein (ICP8) and the DNA polymerase, both β genes (117, 199). This third origin, ori_L, consists of an A+T-rich, 144 base pair perfect palindrome (118,224). The S component origins are shorter than ori_L. They both contain an A+T-rich palindrome related to that of ori_L, but the S component palindrome is smaller and not perfectly symmetrical (202).

Once the origins of viral DNA replication were described and defined, experimentation turned to defining the viral functions required in trans for that replication. Chalberg (34), in an elegant series of experiments, transfected a plasmid containing an origin of replication along with various viral genes and looked for amplification of the plasmid.

Seven essential genes were identified: a viral DNA polymerase (UL 30), a single-strand DNA binding protein (ICP8; UL 29), an origin binding protein (UL 52), a double-strand DNA binding protein (UL 42), and three additional protein which act as a complex with both primase and helicase functions (UL 5, UL 8, and UL 9). Presumably these viral proteins act in concert with cellular proteins to perform the essential task of replicating the viral DNA. There are undoubtedly other genes, both viral and cellular, that are needed for further processing and packaging of the newly replicated viral DNA.

As mentioned above, the replicated viral DNA lacks free ends (88). This DNA must be processed and packaged into empty capsids. This involves amplification of a sequences and cleavage of the DNA into genome length. Isomerization of the DNA occurs throughout these events. Cleavage and packaging are considered to occur simultaneously (44,109). The full capsids then are enveloped with modified nuclear membranes (49,138) and are secreted via the Golgi apparatus in a manner similar to that of secreted cellular proteins (94). These events terminate the viral replication cycle.

Effects of HSV Infection on the Host Cell

One of the earliest structural changes of the host cell is the enlargement and fragmentation of the nucleolus. At the same time, host chromosomes are marginated, with the nucleus ultimately becoming distorted and multilobed (164, 168). The appearance of cellular membranes is also greatly altered at late times after infection. Both the nuclear and cytoplasmic membranes display thickened patches along their length; ultimately, these patches fold upon themselves, appearing as double membranes (131,138).

The best studied effect of HSV infection is that on host macromolecular synthesis. There is a rapid and generalized cessation of a variety of host metabolic activities. Host DNA synthesis ceases (169), host protein synthesis rapidly declines (160,167,209) and host ribosomal RNA synthesis is curtailed (221). The most extensive information exists on the ability of the virus to shutoff host protein synthesis.

This shutoff involves a virion structural component, and does not require any viral gene expression (56,139). Therefore, this shutoff can occur in physically or chemically enucleated cells, in cells treated with transcription inhibitors (dactinomycin) or translation inhibitors (cycloheximide), or in cells infected with UV-inactivated virus (55,56, 107,160,184). Host shutoff is characterized by disaggregation of host polyribosomes (139), and destabilization and degradation of host messenger RNA (53,139,184).

The unravelling of the mechanism of this shutoff began with the isolation of viruses with mutations in this function, called vhs (virion host shutoff) mutants (160). These mutants failed to induce rapid shutoff of host polypeptide synthesis, but unexpectedly also overproduced α polypeptides.

Indeed, the entire temporal pattern of viral gene expression was prolonged in these mutants. Still, these mutants inhibited host protein synthesis late in a productive infection, although not as completely as wild type virus. This late shutoff was termed secondary shutoff by Read and Frenkel (160), and apparently is unrelated to the vhs gene, since a vhs deletion mutant still displays secondary shutoff (Read and Knight, unpublished data).

Further studies on one particular mutant, designated vhs 1, revealed that the functional stability of α mRNAs was increased in cells infected with this virus (143). In a later experimental tour de force, Oroskar and Read (144) surveyed representative mRNAs from all kinetic classes to show that the half-lives of all messages examined were increased substantially in cells infected with vhs 1. This indicates that the vhs function is non-selective, and can affect the half-life of any mRNA present in the cell.

Frenkel and colleagues (107,206) have also studied the consequences of infection with vhs 1. In the absence of viral polypeptide synthesis, cells infected with vhs 1 showed no decay of several host mRNAs including β-actin, α-tubulin, or heat shock protein 70. In contrast, cells infected with wild type virus under the same conditions rapidly degraded these messages. Likewise, α mRNAs were more stable in vhs 1 infected cells, compared to wild type infected cells. While mRNA half-lives were not obtained, these results again indicate that the vhs function does not discriminate mRNAs.

A hint as to the genetic locus of the vhs gene came from early studies by Fenwick et al. (54). Here, they document that HSV-2 causes a more rapid host shutoff than HSV-1. Therefore, by analyzing the shutoff characteristics of HSV-1 x HSV-2 intertypic recombinants, they were able to map this function to between map units 0.52 and 0.59 on the HSV-2 genome. The isolation of vhs 1, an HSV-1 mutant (160) allowed the use of marker rescue to map the mutation. In marker rescue, intact mutant virus DNA is co-transfected with known fragments of the wild type genome into permissive cells. The resulting progeny virus are then screened for wild type shutoff activity. Kwong et al. (108) also exploited the fact that vhs 1 grows somewhat slower in culture than its wild type counterpart. This allowed the enrichment of recombinants before screening. The end result was that a 265 base pair fragment spanning from 0.604 to 0.606 on the wild type HSV-1 genome rescued the mutation in vhs 1. Examination of the viral genome sequence in this region implicates an open reading frame designated UL41 that encodes a protein with an apparent molecular weight of 58,000 daltons and a predicted molecular weight of 54,914 daltons (126). A 1.6 kb intronless mRNA is derived from that region, and it is regulated as a $\gamma 1$ gene (60).

Inhibition of Host Translation in Other Viral Systems

Although infection of a host cell by a virus frequently results in gross alterations in the translation pattern in the infected cell, the method used by HSV, namely the active degradation of host mRNA, is seldom found in other viruses. Indeed, the function is not essential to the growth of HSV in culture (160). Kozak (102) proposes several mechanisms whereby a virus can usurp the translational machinery of an infected cell. The first, and conceptually most straightforward, is competition of viral and cellular mRNAs for the same, unaltered cellular translational machinery.

This competition can occur in several ways. The virus may simply overwhelm the translational capacity of the host with so many copies of viral mRNAs that host mRNA becomes the minority species in the cell. Or, the viral mRNA may be more efficiently translated than host message. The competing host mRNA can also be eliminated by degradation. Any of these modes may also be involved in the switch from early to late viral protein synthesis that occurs in many viruses. In effect, the late viral mRNAs are competing with early messages for translation.

The second method for preferential translation of viral mRNAs is to alter the normal translational apparatus by inactivating a component necessary for translation of host mRNA but not required for viral mRNA translation. The third method also involves altering the translational apparatus, in this case by production of a viral inhibitor of host mRNA translation. This method is complicated by having to invoke mechanisms whereby the viral mRNAs bypass this inhibition. The final method involves the production of a viral factor that preferentially fosters the translation of viral mRNAs.

The available evidence supports the idea that the vast majority of viruses use the first mechanism, that of competition, described above to preferentially translate their mRNAs. For instance, vesicular stomatitis virus (VSV) mRNA is as efficient as host mRNA for translation, but the sheer number of viral transcripts favors the production of VSV proteins (119). Vaccinia virus, a member of the poxvirus family, induces the active degradation of host cell mRNA (162). By eliminating the competition, vaccinia virus mRNA is preferentially translated. Influenza virus also degrades host mRNA (85). This is coupled with a high efficiency of _in vitro_ translation of influenza mRNAs, making these mRNAs very competitive in the host cell.

As described above, herpes simplex virus degrades not only host cell mRNA but also viral mRNA of the various kinetic classes (143,144,184). The initial degradation of host mRNA apparently allows for efficient translation of viral α mRNAs, while the degradation of early viral mRNAs allows late mRNAs to be translated. This nonspecific _vhs_-induced mRNA degradation coupled with specific transcriptional control thus regulates the levels of mRNA in cells infected with wild type HSV. The virus does not require this degradation activity in culture (160), but it does provide the virus with a growth advantage (108).

Poliovirus offers the only definitive example of the second method for preferential translation of viral mRNAs, as described above. This method, in which a cellular function necessary for the translation of host mRNA is altered by the infecting virus, is accomplished in poliovirus by the cleavage of a 220 kilodalton (kda) protein designated p220 (50). This protein is thought to be a part of a cellular initiation factor that binds to the 5′ cap structure found on most eukaryotic mRNAs. Poliovirus mRNA lacks this 5′ cap (57), and does not require the cap binding initiation factor. Pelletier and Sonenberg (149,150) recently showed that initiation of translation of poliovirus mRNA occurs by ribosomal binding to internal 5′ noncoding regions, obviating the need for a cap binding initiation factor.

A difficult to classify mechanism is present in cells infected with adenovirus. Late in infection, viral transcripts are preferentially translated even though host message is present in undegraded form (154). Translation proceeds in these cells because an interferon-induced inactivation of eukaryotic initiation factor eIF2α is blocked by a virus associated RNA called VA RNA I (187,195). VA RNA I apparently binds a 68 kda cellular protein kinase, thereby blocking its autophosphorylation. The phosphorylated form of the protein kinase normally phosphorylates eIF2α in response to the liberation of interferon, which leads to the inactivation of this necessary initiation factor. What this means is that the generation of interferon, a cellular response to adenovirus infection, leads to the shutoff of translation. A viral response, namely the production of VA RNA I, restores translation by preventing this interferon-mediated shutoff (98). What remains to be defined is how the active form of eIF2α interacts specifically with adenovirus transcripts to allow their preferential translation, while host cell transcripts are still apparently under the influence of the inactivated eIF2α. Kozak suggests that this may again be a consequence of effective competition of the viral transcripts for a limited amount of active translational machinery in the cell, that is, that VA RNA I is capable of blocking the inactivation of only a fraction of the total pool of eIF2α which is then available to translate the more efficient or more abundant viral mRNA. Obviously, much more work remains to be done to resolve this issue.

No definitive examples of the other mechanisms for effecting viral translation in infected cells have yet been described. Therefore, the examples cited above suggest that viruses have a limited arsenal of weapons with which to attack the host cell translational machinery. Most likely, the infecting virus overwhelms the cellular macromolecular synthetic capacity to produce progeny virus. Occasionally, the virus may have an active component to effect this takeover, such as the degradation of cellular mRNA. This makes the study of these active mechanisms most interesting, to help understand viral mechanisms of infection as well as cellular responses to that infection.

Stability of mRNA in Eukaryotic Cells

Gene expression in eukaryotic cells can be regulated at many levels. This regulation can occur at several stages in the maturation and translation of the mRNA. Events such as the post-transcriptional processing of the nascent transcript, transport of the mature transcript to the cytoplasm, or the ability of that transcript to participate in translation, can all be regulated (137). The most straightforward way to regulate the expression of a gene is to either produce functional mRNA from that gene, or not (i. e., transcriptional regulation). Obviously, there are many subtleties involved in transcriptional regulation, but the end result is still the same: either a transcript is produced (at some level), or its production ceases. By and large, prokaryotic gene expression is controlled at the level of transcription, owing to the extremely short half-lives of most bacterial mRNAs (101).

Transcriptional regulation may not be as effective in down-regulating gene expression in eukaryotic cells, especially when the gene encodes a long-lived mRNA as are found in these cells (173). Such long messenger half-lives mean that terminating transcription does not halt the expression of a gene. The mRNA for that gene will perhaps exist through an entire cell cycle, far too long for effective control of gene expression. Hence, most control occurs at the post-transcriptional level. This broad category covers many mechanisms that regulate the production of the polypeptide endproduct of the gene. One such mechanism is to prevent the transcripts from interacting with ribosomes, a process termed translational control. Another way of controlling gene expression post-transcriptionally is to degrade the transcript before it has a chance to interact with the translational machinery. This type of control, by regulating the degradation of mRNA, will be the focus of this section.

Several hormones have been shown to act as external stimulants that regulate the degradation of specific mRNAs. Estrogen, for example, greatly increases the half-life of vitellogenin mRNA (26). Prolactin has the same effect on casein message, increasing its stability some 20-fold (75). Human growth hormone message is stabilized in response to glucocorticoid stimulation (145). This list is expected to grow, as cellular responses to hormone stimulation continue to be studied. How hormones regulate mRNA stability is as yet unknown.

The stability of mRNA can also be regulated by cis-acting sequences that have been found in several transcripts.

Transferrin receptor mRNA, for instance, responds negatively to increases in intracellular concentrations of iron (132). A sequence that can form a stem-loop structure in the 3′ untranslated region of that message has been implicated in this control. Apparently, increases in iron concentrations reduce the affinity of an mRNA binding protein for this 3′ stem-loop sequence, and this in turn decreases the stability of transferrin receptor mRNA (133,177). Evidently, the mRNA binding protein protects the stem-loop from attack by endogenous ribonucleases. Histone mRNA also has a 3′ sequence that can form a stem-loop structure, whose presence is required to couple the decline in histone mRNA levels to the decline in DNA synthesis (113). This stem-loop structure appears to be the target of nucleolytic attack both in vitro (174, see below) and in vivo (176).

The 5′ untranslated region of mRNA has been implicated in affecting the stability of mRNA encoding the proto-oncogene c-myc (155,159). A large stem-loop structure is present in the first exon, which is often translocated in transformed cells carrying an activated c-myc (183). The loss of this cis-acting structure may affect the binding of some regulatory protein, leading to a change in the stability of the c-myc message.

The mRNA structure most often implicated in the control of message stability is the 3′ end, and its associated poly(A) tail, a feature present on most eukaryotic messages

(22). The poly(A) tail does not exist free in the cell, but rather is complexed with a ribonucleoprotein known as the poly(A) binding protein (see below). This poly(A)-poly(A) binding protein complex is thought to be a major determinant of mRNA stability, as studied both in vivo and in vitro.

Some early work on the role of the poly(A) tail involved injecting globin mRNA with poly(A) tracts of various lengths into Xenopus oocytes (140). Globin mRNA with poly(A) tracts of 32 residues could direct translation as efficiently as mRNA with 150 residues. Messages with 16 or less adenylate residues in their poly(A) tails were ten-fold less efficient, at a level comparable to deadenylated mRNA. The deadenylated mRNA molecules were rapidly degraded in the Xenopus oocytes, while mRNA molecules with normal poly(A) tails were stable.

This work suggests that some minimum length of poly(A) tail is required for a given message to remain functionally active. Likewise, the poly(A) binding protein requires a minimum length of about 27 residues for efficient binding. This minimum length requirement may explain why globin mRNA is rapidly degraded if the poly(A) tail is less than 16 residues long, and may explain data concerning the poly(A) tail of metallothionein mRNA. When newly synthesized, this mRNA has a poly(A) tail of about 200 residues; over the next ten hours, the length of this tail decreases to about 30 residues (127). Subsequently, the steady-state level of this mRNA declines. The simplest explanation for these data is that mRNA molecules with 30 or fewer residues lack a poly(A) binding protein, and are rapidly degraded, leading to a decline in the steady-state level. In fact, two separate reports, one on c-<u>myc</u> and the other on c-<u>fos</u>, indicate that the first step in the degradation of these two mRNA species is the nucleolytic attack of the poly(A) tail, followed by rapid degradation of the body of the mRNA in a 3′ to 5′ direction (24, 226).

A conserved 3′ AU-rich sequence has been identified in these and other short-lived messages. This sequence has been found in the 3′ untranslated regions of the granulocyte monocyte-colony stimulating factor (GM-CSF;193), the proto-oncogene c-<u>myc</u> (96), and the proto-oncogene c-<u>fos</u> (226). Several investigators have suggested a link between alteration of the stabilities of these messages and subsequent cellular oncogenic transformation (112,114,148,188). Indeed, the removal of a 3′ noncoding region containing this AU-rich sequence from the c-<u>fos</u> proto-oncogene makes the resulting transcript much more stable (226). Conversely, the addition of this sequence to the 3′ end of the normally stable β-globin mRNA makes the chimeric message relatively unstable (211).

Two facts, therefore, emerge. One is that, in the case of c-<u>myc</u> and c-<u>fos</u>, the degradation of these messages is preceded by the rapid removal of their poly(A) tails. The second fact is that both of these messages contain a conserved AU-rich sequence in their 3′ untranslated regions.

One proposed model to account for these facts involves postulating that the affinity of the poly(A) binding protein for the poly(A) tail is influenced by the AU-rich region, which might expose the poly(A) tail to attack by endogenous nucleases (18).

Additional work *in vitro* supports this hypothesis. Ross and colleagues have developed and exploited an *in vitro* system derived from the polysomes of K562 cells, a human erythroleukemia cell line (174). Initial studies with this system involved analysis of histone mRNA, a class of message that normally lacks a poly(A) tail, which is tightly regulated with respect to the cell cycle (189). Histone message is barely detectable during G_1 phase, accumulates during S phase, and is rapidly degraded at the end of S phase. Histone mRNA, when microinjected into *Xenopus* oocytes, is degraded rapidly; the addition of a poly(A) tail increases its stability (84). Likewise, the addition of a poly(A) tail to histone mRNA stabilizes it 10 to 20-fold *in vitro* (151). The degradation of normal nonpolyadenylated histone mRNA commences at a 3′ stem-loop sequence, and proceeds from 3′ to 5′ (174). The addition of a poly(A) tail may protect the 3′ stem-loop from this degradation, leading to this increase in stability.

The degradation of c-*myc* mRNA was examined in this same *in vitro* system. This species of mRNA has a poly(A) tail, unlike normal histone mRNA, but it is rapidly degraded (see above). Incubation of this mRNA _in_ _vitro_ showed that the poly(A) tail was removed prior to nuclease attack on the 3′ untranslated region of c-_myc_ mRNA (24). The poly(A) tail of β-globin mRNA, a long-lived message, was unaffected under the same conditions.

A paradox emerges from these _in_ _vitro_ data. On the one hand, the addition of a poly(A) tail to an otherwise unstable histone mRNA stabilizes it; likewise, the stable β-globin mRNA remains so _in_ _vitro_. On the other hand, a normally polyadenylated mRNA like c-_myc_ is highly unstable in this _in_ _vitro_ system, mimicking its _in_ _vivo_ instability. It would seem that a poly(A) tail would behave in a like manner with respect to all messages, since there are no sequence differences that would influence the specificity of poly(A)-specific nucleases. Therefore, the answer must lie elsewhere. One possible influencing factor is the poly(A) binding protein (PABP).

The biochemistry and genetics of the PABP are discussed extensively in the next section. This discussion will focus on the role of the PABP in stabilizing mRNA. Early data demonstrated that a protein-free poly(A) tail is much more sensitive to snake venom exonuclease than a PABP-bound poly(A) tail (16). Ross and coworkers examined the role of the PABP on the long half-life of β-globin mRNA _in_ _vitro_ (17). In the first series of experiments, _in_ _vitro_ synthesized β-globin mRNA was incubated in the cell-free system along with a ribosomal salt wash (this provides PABP and nucleases). When competitor poly(A) was present in the reaction mix, the rate of decay of the β-globin target was seven-fold greater than when the competitor was absent. The addition of poly(G), poly(U), or poly(C) had no such effect on the rate of β-globin mRNA degradation. It is conceivable that the competitor poly(A) binds the free PABP, leaving the poly(A) tails of the β-globin mRNA accessible to nucleolytic attack. Therefore, the presence or absence of PABP on the poly(A) tails of β-globin mRNA may determine the stability of the message.

This hypothesis was examined by looking at the stability of the β-globin transcripts in a cell-free extract whose ribosomal salt wash was depleted of free PABP by prior passage of the salt washes through a poly(A)-Sepharose column. The rate of β-globin mRNA degradation was seven-fold higher when treated ribosomal salt wash was added to the cell-free system. This degradation is characterized by removal of the poly(A) tail prior to sequential 3′ to 5′ digestion of the body of the β-globin mRNA. Addition of purified PABP to this system increased the stability of this mRNA, while addition of purified PABP had no effect on the decay rate of histone mRNA or on deadenylated β-globin mRNA.

Hence, the PABP-poly(A) complex appears to regulate message half-life, at least <u>in vitro</u>. This partially satisfies the hypothesis concerning the 3′ AU-rich sequence found in many short-lived messages, as discussed earlier. This hypothesis involves an alteration in the affinity of the PABP for the poly(A) tail of these transcripts by the AU-rich sequence. This AU-rich sequence may bind a regulatory protein that decreases the affinity of the PABP for the poly(A) tail. The migration of the PABP off these poly(A) tails may allow endogenous nucleases to attack the poly(A) tail, initiating the progressive 3′ to 5′ degradation of these messages (18). What remains is the examination of the affinity of the PABP on the poly(A) tails of various messages, with and without the 3′ AU-rich sequence.

The study of the degradation of tubulin mRNA suggests that the stimulus for degradation may not reside in the RNA sequence itself. Tubulin is the predominant subunit of eukaryotic microtubules (37). Tubulin mRNA has long thought to be autoregulated; drugs that increase the levels of free tubulin in the cell (e. g. colchicine and nocodazole) decrease synthesis of new tubulin, while drugs that decrease the amounts of free tubulin (e. g. vinblastine and taxol) increase tubulin synthesis (14,32,38). The direct microinjection of purified tubulin into cells has the same effect as drugs that increase the concentration of free tubulin, that is, to rapidly inhibit tubulin synthesis (39).

This inhibition occurs at the level of mRNA stability rather than at the transcriptional level. Enucleated cells react to the drugs mentioned above in the same way as intact cells (156). Furthermore, alterations in the synthesis of tubulin in response to the various drugs were matched with alterations in the levels of tubulin mRNA (33). Lastly, a hybrid gene construct, with the tubulin promoter being replaced with a metallothionein promoter, was still autoregulated (63). These data all suggest that the autoregulation of tubulin synthesis is specified by directed regulation of the stability of tubulin mRNA.

Transfection experiments using a hybrid tubulin/thymidine kinase gene showed that the first 13 nucleotides of the coding region of tubulin mRNA were necessary and sufficient for this autoregulated message instability (228). Transcripts derived from a variety of in vitro mutagenized tubulin genes showed that mutations (either point or frameshift) that changed the amino acid sequence of the first four amino acids abolished autoregulation of these transcripts; changes in the third nucleotide of each codon were tolerated if they resulted in conservative replacements of the amino acids (229). Likewise, relocation of these 13 nucleotide to an internal position of the transcript abrogated the autoregulated control.

The novel suggestion is that the autoregulated degradation centers on the first four amino acids of the nascent polypeptide rather than on a nucleotide sequence of the transcript. A model has been proposed to explain these observations (228). As levels of free tubulin increase in the cell, the autoregulatory protein (presumably the free tubulin) binds to these first four amino acids of the nascent polypeptide chain. This protein-nascent protein interaction somehow triggers the degradation of the presently translating tubulin mRNA. This could either be by activating a specific nuclease, or by causing the ribosomes to pause, thereby exposing transcripts to attack by endogenous nucleases. In either case, this hypothesis is different from the others described above in that the target of the ribonucleases appears to be actively translating mRNA. The mechanism of ribonuclease-mediated mRNA degradation may, however, be very similar in all of the systems described.

Ribonucleoprotein Particles: a Review of the Literature

In all known cells information passes from deoxyribonucleic acid (DNA) to protein via a ribonucleic acid (RNA) intermediate (4). In eukaryotic cells, the primary RNA transcript is known as heterogeneous nuclear RNA (hnRNA), and, as its name implies, it is found in the nucleus. The hnRNA is modified in several ways before it is termed mRNA. Most hnRNA contains intervening sequences (introns) that are subsequently spliced out. The hnRNA has a cap structure added to its 5′ end and a polyadenosine [poly (A)] tail added to its 3′ end. The end product of these reactions is mRNA, and it is found in the cytoplasm (137). Neither type of RNA exists naked in the cell. Rather, the RNA is associated with protein in a complex structure called a ribonucleoprotein particle (RNP). This discussion will focus on the RNPs derived from hnRNA and mRNA, called hnRNP and mRNP (reviewed in 48,122). The existence of these RNA-protein structures has been widely debated, but recent experiments eliminated any doubt that such structures are found in vivo.

The earliest evidence for the existence of mRNPs came from Spirin and coworkers in 1964 (reviewed in 200). They established the criteria used for mRNP identification that lasted for the following decade. Essentially, the technique involved pulse-labeling cells with radioactive RNA precursors, then passing the cytoplasm over a sucrose gradient to isolate the labeled material. This material is then fixed with formaldehyde and analyzed by isopycnic centrifugation in a CsCl gradient. The fixed material was found to have a buoyant density between 1.35 g/cm^3 and 1.5 g/cm^3. By comparison, free RNA has a buoyant density of about 1.9 g/cm^3 and ribosomes band at 1.55 g/cm^3. Spirin suggested that the fixed material was a complex of protein and RNA (201).

Other workers (78,95,125) at the time believed these RNA-protein complexes to be what are today called initiation complexes, mRNA in association with the 40S ribosomal subunit. Their error was in analyzing only a portion of the sucrose gradient; Spirin and coworkers analyzed both subribosomal and postpolysomal fractions for mRNP structures (201). Henshaw and Loebenstein confirmed this work, but raised the "possibility that the complexes of polydisperse RNA with protein are an artifact of homogenization" (79).

This caveat led to the development of a variety of isolation protocols designed to control for such artifacts. Blobel (20) isolated mRNPs from dissociated rabbit reticulocyte ribosomes in the presence of 500 mM KCl; presumably, the high salt would discourage any nonspecific interactions and lead to the identification of mRNA-specific proteins. He demonstrated the existence of two proteins, one with an apparent molecular weight of 52 kda and the other, 78 kda. Greenberg (70) isolated unfixed mRNPs from mouse L cells on cesium sulfate density gradients, arguing that this isolation protocol would serve to remove all but the most tightly bound proteins. This procedure yielded a more complex protein profile, but did show the presence of a 76 kda protein he felt corresponded to the 78 kda protein demonstrated by Blobel. Other workers, using isotonic isolation procedures, also showed complex protein profiles, but all showed proteins of about 78 kda and 50 kda (8,62,207).

Blobel (21) is credited with the next breakthrough in the field. He demonstrated that the 78 kda protein isolated by his high salt protocol was associated with the poly(A) tail of polysomal RNA. In these experiments, L cells were incubated with either [^{3}H]adenosine or [^{3}H]uridine, and mRNPs were isolated from dissociated polysomes. After selective digestion with pancreatic ribonuclease (RNase) and RNase T1, which leaves poly(A) tails intact, the digestion products were separated on sucrose gradients. The 78 kda protein was shown to be associated with an 11S [^{3}H]adenosine peak, whereas [^{3}H]uridine was evenly distributed throughout the gradient. Base composition analysis showed approximately 80% AMP in the peak associated with the 78 kda protein. Kwan and Brawerman (106) repeated the same experiment with mouse sarcoma 180 ascites cells and obtained similar results.

More definitive demonstrations followed upon the development of a new technique using oligo(dT)-cellulose to isolate mRNA that contains a poly(A) tail (5). This allowed investigators to separate polyadenylated mRNPs, as well as the poly(A) tail itself, free from contaminating fragments. Lindberg and Sundquist (115) were the first to develop a technique for isolating mRNPs on oligo(dT)-cellulose. The eluted material was shown to be mRNP by the buoyant density criterion established by Spirin ten years earlier (201). The protein composition of this material was examined by labeling KB cells with [^{35}S]methionine, isolating the mRNPs on oligo-(dT)-cellulose, and resolving the proteins on polyacrylamide gels. KB cells were shown to contain four mRNP proteins of apparent molecular weights of 125 kda, 78 kda, 68 kda, and 56 kda. The isolation was done in the presence of moderate salt concentrations, suggesting the RNA-protein interactions were specific.

Other investigators used this new procedure to isolate mRNPs from various cell types, all reporting a variety of protein components (86, 91,207). Again, the presence of the poly(A) binding protein of about 72 kda and a protein of about 50 kda were consistently present. No other pattern of proteins emerged from this work. This suggested that most of these proteins were artifacts of the isolation procedure used, and were not true mRNP proteins associated with mRNA _in vivo_. An observation by Smith (197) in 1969 suggested a way around this quandary. He showed that DNA could be efficiently crosslinked to amino acids with ultraviolet (UV) light. Indeed, the interaction was such that only molecules in close association with the nucleic acid would be covalently attached. Greenberg (71) adapted this finding to be used in the isolation of mRNPs by exposing polysomes to UV light and isolating the resulting covalent complexes under more stringent conditions.

Setyono and Greenberg (192) identified six major protein components of polysomal mRNPs UV crosslinked in this manner. The mRNP material, isolated on oligo(dT)-cellulose, contained proteins of 98 kda, 78 kda, 75 kda, 68 kda, 62 kda, and 52 kda. That the 78 kda protein associated with the poly(A) tail was confirmed by digesting the UV crosslinked mRNPs with RNases A and T1 in high salt, and chromatographing the digestion products through oligo(dT)-cellulose. This procedure should leave the poly(A) tail and its crosslinked proteins bound to the column, while other RNA fragments and proteins flow through. Upon elution of the poly(A) tail with a low salt buffer, the remaining RNA was digested with micrococcal nuclease (a nonspecific nuclease) and the proteins analyzed on a polyacrylamide gel. Prior labeling of the proteins with [^{35}S]methionine allowed autoradiography of the gel; a single protein of 78 kda was detected.

The UV crosslinking procedure Greenberg and colleagues used was still open to artifacts, since the crosslinking took place after lysis of the cells. To eliminate the risk of nonspecific protein interactions occurring during the isolation procedure, Wagenmakers et al. (220) developed the technique of irradiating intact living cells with UV light, then isolating the mRNP complexes in the presence of 2-mercaptoethanol, sodium dodecylsulfate, and at elevated temperatures (90°C) to discourage any nonspecific protein binding. After isolating the mRNPs on oligo(dT)-cellulose, the RNA moiety of the mRNP was digested with a mixture of RNase A and micrococcal nuclease and the [^{35}S]methionine-labeled proteins were resolved on polyacrylamide gels. Prominent bands were found at 73 kda, 69 kda, and 52 kda; several minor bands of varying intensity were also noted. Unirradiated controls showed no protein bands when isolated under identical conditions.

Adam et al. (1), using a similar protocol, identified a larger set of proteins crosslinked to the mRNA of HeLa cells. They showed polypeptides of 135 kda, 93 kda, 72 kda, 68 kda, 53 kda, 50 kda, 43 kda, and 36 kda. The major proteins banded at 72 kda, 68 kda, 53 kda, 50 kda, and 36 kda. The 72 kda protein was shown to be the poly(A) binding protein.

Very little is known about the function of these proteins, although the locations on the mRNA of several proteins is known. The 72 kda protein is known to bind to the poly(A) tail (1,21), while several others bind to the 5′ cap structure (72). The 50 kda major mRNP protein has been reported to associate with both the 5′ cap structure and the body of the mRNA, but not with the poly(A) tail (72). Still, there is some speculation about the possible functions for these mRNP proteins. First, these proteins may serve to protect the mRNA from degradation by endogenous nucleases. Hence, the functional stability of the mRNA may be regulated by these proteins. Secondly, these proteins may participate in translation of the mRNA into protein, or conversely may serve to prevent the translation of certain messages. That is, these proteins may serve in translational control of eukaryotic gene expression. Lastly, these proteins may serve a transport role, moving the mRNA from the nucleus into the cytoplasm and ultimately to the ribosomal subunits for translation (23). Clearly, these functions are not mutually exclusive, and may not even be separable. The function of at least certain proteins in hnRNP particles is in splicing (35).

As mentioned earlier, the poly(A) binding protein is the best characterized of all the known mRNP proteins. Several functions have been suggested for this protein. Schwartz and Darnell (190) suggested that the protein in HeLa cells is involved in transport from the nucleus to the cytoplasm, but this observation has not been confirmed. Van Venrooij et al. (216) suggested that "free" mRNA (i. e., that mRNA not associated with the polysome fraction) lacked the poly(A) binding protein, although polysomal mRNA carried this protein. This implies that the poly(A) binding protein is involved in translational control. Vincent et al. (217) showed that a variety of protein differences were found between free and polysomal globin mRNPs, including the lack of the poly(A) binding protein on free mRNPs. It was also shown that free mRNPs could not be translated in vitro, again strongly implicating the poly(A) binding protein in translational control.

Jacobsen and Favreau (90) showed that the addition of exogenous poly(A) tracts to in vitro translation extracts would, in a dose-dependent manner, inhibit the efficiency of translation of polyadenylated mRNA. This inhibition could be overcome by translating mRNPs instead of naked mRNAs, suggesting that a protein component of the mRNPs was the rate-limiting factor. A group at the University of Stuttgart, extending the observation of van Venrooij et al. (216), demonstrated that free globin mRNA lacked the poly(A) binding protein, but the protein could be found in mRNPs isolated from 48S initiation complexes as well as in polysomes (186). This group also extended the findings of Jacobsen and Favreau

(90), showing that homopolyribonucleotides other than poly(A) did not effect the efficiency of translation. Lastly, this group showed that in vitro translation extracts depleted of RNA binding proteins would not translate naked mRNA but would translate exogenous mRNPs. The naked mRNA could be translated if certain RNA binding proteins were added back (185).

Munroe and Jacobson (134) examined a set of in vitro synthesized transcripts which differ only with respect to the lengths of their poly(A) tails. These transcripts were analyzed in in vitro translation reactions for their efficiency of translation. Nonpolyadenylated mRNA was unable to be efficiently translated, even though it was no more unstable than identical polyadenylated transcripts. The authors discovered that this translational defect stemmed from a reduced capacity of nonpolyadenylated mRNA to form 80S initiation complexes. The authors suggest that the presence of a poly(A) tail somehow influences events at the 5′ end, enhancing the binding of an initiation factor or ribosomal subunit there. Jackson and Standart (87) speculate that this 3′ influence over 5′ events, which they term "cross-talk", may be the result of alternative secondary structures propagating at the 3′ end, or may involve direct interaction between the 3′ and 5′ ends. These events are probably mediated by proteins such as the poly(A) binding protein and other mRNP proteins.

Indeed, several investigators have demonstrated that naked mRNA, when added to an in vitro translation extract, forms a structure indistinguishable from polysomal mRNPs isolated _in vivo_ (61,73). Butcher and Arnstein (29) demonstrated that radioiodinated mRNPs could engage in efficient translation, and that the labeled proteins always remained associated with 80S ribosomes and polysomes _in vitro_. All this evidence together strongly suggests that one or more protein components of mRNPs are involved in translation, and that the 3′ poly(A) tail and its associated protein may be an important factor in enhancing the translation of mRNA.

Another apparent function of the poly(A) binding protein is in the formation of a periodic structure of the poly(A) tail. The repeating structure of the poly(A) tail can be seen by mild digestion of poly(A) tails from mRNPs with nonspecific nucleases. The resulting RNA fragments range in size from about 15 to 150 adenosine residues, with a periodicity of about 25 bases (6,99). This structural property of the poly(A) binding protein was used to purify a 75 kda protein from rat liver (7). All indications suggest this 75 kda protein is identical to the poly(A) binding protein found tightly associated with mRNPs. These data suggest that the poly(A) binding protein induces a periodic structure in the poly(A) tail, in a manner similar to the periodic structure induced by histones in eukaryotic DNA (4).

Rose et al. (170) reported that poly(A) polymerase and the poly(A) binding protein share related antigens. Polyclonal rabbit antibody was raised against poly(A) polymerase isolated from rat hepatoma nuclei. Poly(A) binding protein isolated from HeLa cell polysomal mRNPs could compete for this antibody in a competitive radioimmunoassay to the same level as purified poly(A) polymerase, suggesting both molecules share similar, if not identical, epitopes. That these are not the same proteins is evidenced by the different molecular weights of the proteins (60 kda for poly(A) polymerase and 72 kda for HeLa poly(A) binding protein) and the different cellular locations (nucleus for poly(A) polymerase and cytoplasm for poly(A) binding protein).

More recent reports indicate that there may be several forms of poly(A) polymerase in HeLa cells. Ryner et al. (178) detected two forms of the enzyme in nuclear fractions and one form of the enzyme in cytoplasmic fractions. Each enzyme activity, when mixed with appropriate nuclear fractions containing necessary cofactors, could catalyze the cleavage and polyadenylation of target pre-mRNA containing the canonical AAUAAA sequence (230). The three forms of poly(A) polymerase were also shown to be similar in size and immunochemical cross-reactivity, suggesting that these three enzyme activities are closely related or identical. Whether any of these multiple forms of poly(A) polymerase is identical to the poly(A) binding protein of hnRNPs, as proposed by Dreyfuss (48), remains to be ascertained.

Recently, the gene for the poly(A) binding protein of the yeast Saccharomyces cerevisiae has been isolated and sequenced (3,179). The protein structure consists of two principal regions. The N-terminal region consists of four homologous domains, each of which contains a short sequence conserved among several other RNA binding proteins. The C-terminal region is rich in proline, glutamine, and alanine. To date, its function is unknown, although it may interact with other proteins to modulate the binding of the poly(A) binding protein to the poly(A) tail. Genetic analysis of the poly(A) binding protein gene showed that it is an essential gene in Saccharomyces (180). However, a yeast strain carrying a single N-terminal domain is viable. This suggests that the poly(A) binding function of the protein is critical to the cell.

Another approach to examining the possible functions of mRNP proteins is by using viral systems to study both host and viral mRNP particles. The first study of mRNP structure in virus-infected cells was by Lindberg and Sundquist (115). They reported that KB cells infected with adenovirus type 2 contained mRNPs that carried a novel 110 kda protein along with the other proteins found in uninfected cells. This finding was confirmed by another group (210,214); it must be noted that all groups used conventional mRNP isolation procedures relying on isolation on sucrose gradients. When van Venrooij et al. (215) repeated this experiment using UV crosslinking, no such protein was found. They reported that the protein composition in infected cells was identical to that of uninfected cells. In every report, mRNPs were isolated at late times after infection. Hence, the lack of any difference as reported by van Venrooij et al. could not be explained by a difference in time of isolation. Instead, other explanations must be forwarded. For example, the protein may not be efficiently crosslinked by UV light. Or the protein may interact with mRNP particles through protein-protein interactions instead of directly with the mRNA. This latter possibility is likely because the labeling protocol used [^{3}H]nucleosides to label the mRNA directly, in a label transfer protocol. After UV irradiation and RNase digestion, some of the labeled nucleosides remain covalently associated with the proteins to allow for detection by autoradiography.

Adam and Dreyfuss (2) attempted to resolve this issue by using UV crosslinking with [^{35}S]methionine labeling of proteins, [^{3}H]ribonucleoside labeling of RNA, and immunoblotting using antiserum against the adenovirus 110 kda protein. All analyses indicate that, at late times after infection, the 110 kda protein was associated with both host and viral mRNAs. These mRNAs were also associated with the normal complement of host cell RNP proteins, indicating that adenovirus infection does not lead to a dissociation of host mRNPs. Adam and Dreyfuss speculate that the contradictory results obtained by van Venrooij et al. stem from an inefficient infection of the host cells by the latter group. Adam and Dreyfuss cite results derived from abortive infection of CV-1 cells, where the 110 kda protein was not crosslinked to mRNA, even though the protein was abundantly present in the infected cells.

The data concerning cells infected with vesicular stomatitis virus (VSV) is much more consistent when conventional versus UV crosslinking data is compared. VSV mRNA is transcribed in the cytoplasm by a virally encoded RNA polymerase that is resistant to the effects of dactinomycin. As such, virus-specific message can easily be followed by infecting cells in the presence of 5 μg/ml dactinomycin (1). Grubman and Shafritz (74), using this protocol to label viral mRNP proteins with [^{35}S]methionine, found that a 52 kda protein with a tryptic digestion pattern identical to the major virion nucleocapsid (N) protein was associated with isolated viral mRNPs.

Cohen's group (171,172) confirmed this finding, but suggested that viral mRNA was associated exclusively with N protein. They isolated polysomal mRNPs from VSV infected cells, identified the mRNA components as virus-specific by in vitro translation, and identified the protein component as N protein by metabolic labeling with [^{35}S]methionine. Most significant was their finding that viral mRNP particles could not be translated efficiently in vitro. This suggests that the mRNA is sequestered in vivo such that, although associated with polysomes, translation does not occur (171). Expansion of this work suggested that formation of the ternary complex was blocked in vitro by VSV mRNP or purified nucleocapsids, both of which contain N protein, and that this block could be overcome by the addition of excess eukaryotic initiation factor eIF-2 (172). This result shows that VSV mRNP blocks translation by blocking the first step in initiation of translation.

Dreyfuss' group (1) repeated the isolation of VSV mRNP using UV crosslinking. By label transfer using [^{3}H]nucleotide they showed that both host and VSV mRNA was associated with the same set of proteins. Both mRNPs also carried the 72 kda poly(A) binding protein of HeLa cells on their poly(A) tails. To test if N protein was there but could not be resolved on the polyacrylamide gels, polyclonal antiserum was raised against purified VSV virions and used in Western blots of mRNP proteins. This experiment showed that the N protein was indeed there, but could not be seen because it co-migrates with the 50 kda protein of uninfected HeLa cell mRNPs. In the case of VSV mRNPs, both conventional and UV cross-linking protocols led to the same conclusion.

The last report of a virus system used to examine mRNP proteins is one using herpes simplex virus. Bartkoski (10) analyzed the mRNP proteins of polysomal mRNAs in HEp-2 (human epithelial) cells infected with HSV type 1 (HSV-1). The protocol used involved the pelleting of polysomes in sucrose gradients, then labeling the mRNP proteins by radioiodination using lactoperoxidase. The polysomes were then dissociated with EDTA and the mRNPs were isolated on oligo(dT)-cellulose columns. Upon polyacrylamide gel analysis, uninfected cells were shown to contain proteins of 74 kda and 52 kda, corresponding to the molecular weights of known mRNP proteins, including the 74 kda poly(A) binding protein. At three hours post-infection, the mRNPS contained reduced levels of these two proteins but carried a new protein of 47 kda. By six hours post-infection, the 74 kda putative poly(A) binding protein was missing altogether, the 52 kda protein was markedly reduced, and the 47 kda protein was abundant.

These data are quite interesting and suggest that a radical change in the composition of mRNPs in HSV-1 infected cells takes place. However, the typical reservations remain about these data. First, the protein associations may be artifactual owing to the lysis protocol used. For instance, the number of mRNP proteins in uninfected cells reported here appears to be a subset of the total number of mRNP proteins reported by UV crosslinking experiments (48). Secondly, the radioiodination protocol may have led to inefficient labeling of the protein components of the mRNPs. Metabolic labeling is though to better label proteins, and may allow the distinction of cellular versus viral proteins in the mRNPs. Thirdly, only polysomal mRNPs were examined. The nature of free mRNPS in infected cells was not considered. By examining total cytoplasmic mRNPs, a broader picture of the events during infection could be obtained. Lastly, mutants such as vhs 1 were not studied side by side with wild type virus, making the relevance of these earlier data uncertain.

Proposed Studies in This Dissertation

The focus of the experiments in this dissertation is to begin to elucidate the mechanism of regulation of mRNA stability mediated by the vhs function of herpes simplex virus. Initial experiments involve the use of marker rescue to begin to map the mutation in vhs 1. These studies involve the use of a cloned fragment of the wild type genome co-transfected with the intact vhs 1 genome. If the appropriate recombination events occur such that the defect in the vhs 1 genome is replaced with wild type sequences, then the resulting progeny virus should display wild type vhs activity. By using smaller and smaller wild type fragments, the defect in the vhs 1 genome could be mapped.

Another set of experiments addresses the possible role of the proteins associated with mRNA in cells infected with herpes simplex virus. As discussed above, one role for these mRNP proteins is in the regulation of mRNA stability. By using a UV crosslinking protocol to deduce a definitive picture of the proteins associated with mRNA in cells infected with both wild type and vhs 1, it may be possible to determine if the vhs activity influences the structure of mRNPs.

Another major avenue of experimentation centers on the use of a cell-free system to study the rudiments of vhs-mediated mRNA decay. The development of such a system would allow for a more detailed biochemical study of the vhs function, as well as opening up the possibility of a study of the broader question of mRNA stability in eukaryotic cells. The experiments described here involve the initial analysis of an in vitro mRNA degradation system mediated by the vhs function, and these experiments provide the basis for a discussion of a model for vhs-mediated mRNA degradation.

MATERIALS AND METHODS

Cells and Virus

HeLa S3 and Vero cells were purchased from the American Type Culture Collection (ATCC) and grown in Eagle's minimum essential medium (MEM; GIBCO) supplemented with antibiotics and 10% (vol/vol) calf serum. Stocks of wild type HSV-1, strain KOS, and the KOS-derived mutant vhs 1 were prepared from and titred on Vero cell monolayers as previously described (143). In all experiments virus was allowed to adsorb onto cell monolayers for one hour in MEM containing 5% calf serum. The inocula were then aspirated and the cells overlaid with fresh MEM plus 5% calf serum. Mock infected cells were treated in the same way, except that the cells were exposed to an uninfected Vero cell lysate prepared in the same way as the virus stocks.

Plasmids

The plasmid pSG124 (kindly provided by M. Levine) contains the EcoR1 fragment A (0.49 to 0.63 map units) of wild type HSV-1, strain KOS, inserted at the EcoR1 site of pBR325 (69). The plasmid pHcGAP contains a 1.2 kb cDNA insert encoding a portion of human glyceraldehyde-3-phosphate dehydrogenase (GAPD), and was obtained from the ATCC. The plasmid pHSV106, which contains a 3.4 kb BamH1 fragment encoding the HSV-1 thymidine kinase (TK), was purchased from Bethesda Research Laboratories. The plasmid pXlr11 contains a 4.6 kb fragment of *Xenopus laevis* rDNA inserted into the EcoR1 site of colicin E1 (42), and was kindly provided by J. Doering. The plasmids pSG124 and pXlr11 were maintained in *Escherichia coli* strain HB101, while pHcGAP and pHSV106 were maintained in strain DH5α (Bethesda Research Laboratories). Plasmid DNA was prepared by ethidium bromide-CsCl density gradient centrifugation as described previously (120).

Transfection Procedures

Two sets of transfections were carried out according to the protocol of Stow et al. (204). One set consisted of the intact *vhs* 1 DNA alone; the other set was a co-transfection of the intact viral DNA and pSG124 DNA digested with EcoR1. In either case, the procedure involved the following. First, approximately 4 x 10^6 Vero cells were seeded into a $25cm^2$ flask containing 5 ml of MEM plus 5% calf serum at about 6 h prior to transfection to ensure a subconfluent monolayer of cells. To prepare the DNA for transfection, appropriate quantities of *vhs* 1 DNA with or without pSG124 DNA were resuspended in 1.0 ml of Hank's buffered saline (HBS: per liter, 8.0 g NaCl, 0.37 g KCl, 0.125 g $Na_2HPO_4 \bullet H_2O$, 1.0 g dextrose, 5.0 g N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.05]). The final concentration of DNA was brought up to 20 μg/ml using salmon sperm carrier DNA. To this DNA mixture was added 75 μl of 2 M $CaCl_2$ to form a fine calcium phosphate precipitate. The precipitate was allowed to form for one hour.

After formation of the precipitate, the medium was poured off the cells in the flasks, and 1.0 ml of precipitate was added and allowed to incubate at 34°C for 45 min. After 45 min., the cells were overlaid with 5 ml fresh MEM plus 5% calf serum and allowed to incubate a further 3 h 45 min. At this point (4 h after the start of transfection) the old medium was poured off and the cells were shocked with 25% dimethyl sulfoxide (DMSO) in HBS for 4 min. After the DMSO was removed, the cells were washed twice with 5 ml fresh MEM plus 5% calf serum, then the same was placed on the cells and incubation continued for about 5 more days, until cytopathic effect was observed.

The viruses tested in this report were generated by using 5μg intact vhs 1 DNA, 2.5 μg pSG124 digested with EcoR1, and 13.5 μg carrier DNA. Progeny viruses from the transfections were recovered by one cycle of freeze-thawing the flasks followed by sonication in a bath sonicator. The progeny were titrated on Vero cells in 24-well trays, and single virus plaques were obtained by plaque-purification in 96-well trays.

Recombinant Screening

Small stocks of plaque-purified viruses were grown on Vero cells in 24-well trays at 34°C until cytopathic effects were extensive. These trays were then freeze-thawed three times, and the resulting lysates were used to assay vhs function (160). This assay was performed on Vero cells grown in 24-well trays. The old medium was aspirated from a well of cells, then the cells were infected with 400 μl of the prepared viral lysate in the presence of a final concentration of 5 μg/ml dactinomycin (Calbiochem). At the end of a 5 h incubation, the infected cells were labeled with [^{35}S]methionine (DuPont-NEN) for 1 h. The amount of radioactive counts incorporated into trichloroacetic acid-precipitable material was used as a measure of ongoing host translation. Wild type viral lysates and mock infected cell lysates were examined in the same way.

Ultraviolet Light Irradiation and mRNP Preparation

HeLa cells were grown to subconfluent densities and radiolabeled for the intervals described in the text by exposing cells to MEM containing one-tenth the normal amount of methionine, 20 μCi/ml [^{35}S]methionine (DuPont-NEN), and 5% calf serum.

Ultraviolet light irradiation and mRNP isolation were performed essentially as described by Adam et al. (1). After labeling, cell monolayers were washed twice with ice-cold phosphate buffered saline (PBS), then overlaid with the same buffer. The cells were then exposed for three min to a 30 watt germicidal lamp (Sylvania G30T8) placed 4.5 cm from the monolayer surface. This provides a UV dose of approximately 6.5×10^3 ergs/mm^2, as measured by a model UVX Digital Radiometer (Ultra-Violet Products, San Gabriel, CA). After irradiation, the cells were allowed to swell in ice-cold RSB (10 mM Tris HCl, pH 7.4; 10 mM NaCl; 1.5 mM $MgCl_2$) containing 0.5% aprotinin (Sigma), 1 μg/ml leupeptin (Sigma), 1 μg/ml pepstatin (Sigma), and 10 mM vanadyl ribonucleoside complex (VRC; Bethesda Research Laboratories). The cells were lysed by the addition of Triton X-100 (Bio-Rad) to 0.5%, Tween 40 (Sigma) to 1%, and sodium deoxycholate (Calbiochem) to 0.5%. The cells were then homogenized by four passes through a 25-gauge needle. The nuclei were pelleted by low speed centrifugation, and the supernatant was considered the cytoplasmic fraction.

This fraction was adjusted to 0.5% SDS, 1% 2-mercaptoethanol, and 10 mM ethylenediaminetetraacetic acid (EDTA), heated to 65°C for five min, then chilled on ice. LiCl was added to 0.5 M, and the cytoplasmic material was mixed for 15 min with 0.5 ml of oligo(dT)-cellulose (type 3; Collaborative Research) preequilibrated with binding buffer (10 mM Tris HCl, pH 7.4; 0.5 M LiCl; 1 mM EDTA; 0.5% SDS). After mixing, the resin plus cytoplasm was poured into a column, and the flowthrough was reapplied to the resin twice. The column was then extensively washed with at least 10 column volumes of binding buffer, then poly(A)$^+$ mRNPs were eluted with 2.5 ml of elution buffer (10 mM Tris HCL, pH 7.4; 1 mM EDTA; 0.5% SDS). The volume of the eluate was reduced with 2-butanol and the mRNPs were precipitated at -20°C overnight by the addition of one-tenth volume 3M NaOAc and three volumes of ethanol.

The precipitate was recovered by a 15 min spin in a microcentrifuge, dried under vacuum for 15 min, then re-suspended in a small volume of RNase buffer (10 mM Tris HCl, pH 7.4; 1 mM $CaCl_2$), and digested at 37°C for 60 min with 25 μg/ml RNase A (Sigma) and 400 U/ml micrococcal nuclease (Pharmacia). This reaction also contained 0.5% aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin to prevent proteolysis. After digestion, the proteins were precipitated by storage at -20°C for at least two hours by adding LiCl to 0.5M and three volumes of ethanol.

Protein Gel Electrophoresis

The proteins were recovered by a 15 min spin in a microcentrifuge, dried for 15 min, and were resuspended in SDS sample buffer (125 mM Tris HCl, pH 6.8; 1% SDS; 5% 2-mercaptoethanol; 10% glycerol; bromophenol blue). Samples were boiled for three min, and the proteins were resolved by SDS polyacrylamide gel electrophoresis. The running gel contained 12% acrylamide, 0.3% DATD, and 0.1% SDS in 0.375 M

Tris HCl, pH 9.1. The stacking gel was 4% acrylamide, 0.44% BIS, and 0.1% SDS in 0.125 M Tris HCl, pH 6.8. Following electrophoresis, the gels were fixed in 10% acetic acid-30% methanol and treated with Entensify (DuPont-NEN) as directed by the manufacturer. Dried gels were exposed to Kodak X-AR 5 film for fluorography.

Cell-free Extracts

Cytoplasmic extracts for studying *in vitro* mRNA degradation were prepared from infected or mock infected HeLa cells using a modification of the procedure described by Brown and colleagues for the preparation of *in vitro* translation extracts (27). At the indicated times post-infection, cells were washed twice with ice-cold wash solution (0.15 M sucrose, 33 mM NH_4Cl, 7 mM KCl, 4.5 mM $Mg(OAc)_2$, and 30 mM HEPES, pH 7.4), then permeabilized by the addition of 300 μg/ml lysolecithin (L-α-lysophosphitidyl choline; Sigma) in wash buffer to the cells for 60 s. The lysolecithin solution was removed, and the cells from one 100 mm dish were then scraped into 200 μl of standard reaction mix (0.1 M HEPES, pH 7.4, 0.2 M NH_4Cl, 20 mM Mg $(OAc)_2$, 7 mM KCl, 1 mM dithiothreitol, 1 mM ATP (dipotassium salt), 1 mM GTP (sodium salt), 40 μM of each of the 20 amino acids, 0.1 mM S-adenosylmethionine, 1 mM spermidine, 10 mM creatine phosphate (dipotassium salt), 40 units/ml creatine kinase, and 100 units/ml placental ribonuclease inhibitor (RNasin; Promega), and disrupted by 10 passages through a 25 gauge needle. The nuclei were removed by a low-speed spin and the cytoplasmic supernatant was kept on ice until ready to use.

The extracts were incubated at 30°C for the times indicated. A zero time point was taken just before the extracts were placed at the incubation temperature. Same volume aliquots were removed from each sample, and placed in an equal volume of urea buffer (7 M urea, 10 mM Tris HCl, pH 7.9, 0.35 M NaCl, 10 mM EDTA, and 1% SDS) then extracted twice with phenol:chloroform and twice with chloroform, as previously described. RNA was precipitated at -20°C overnight with 3 vol 95% EtOH.

Polysome Preparation

Polysomes were prepared using a modification of the procedure of Ross et al. (175). Cells were washed and treated with lysolecithin as before. After removal of the lysolecithin, the cells from one 100 mm dish were scraped into 200 μl of buffer A (1 mM potassium acetate, 1.5 mM magnesium acetate, 2 mM dithiothreitol, 10 mM Tris HCl (pH 7.6)). The cells were lysed by 10 passages through a 25-gauge needle, and nuclei were spun out by a low speed spin. The supernatant derived from two 100 mm Petri dishes of cells was layered onto a 1 ml cushion of 30% (w/v) sucrose in buffer A. Polysomes were pelleted by spinning in the TLS 55 rotor (Beckman) at 25,000 rpm for 1 h. The supernatant was discarded and the pellet gently washed twice with buffer A. The pellet was then resuspended in standard reaction buffer. Undissolved chunks were broken up by homogenizing the pellet through a 25-gauge needle. The polysomes were incubated at 30°C as described above.

The preparation of supernatant material for the "mix-and-match" analysis of various polysomal supernatant and pellet combinations was essentially as described above. After pelleting polysomes as described above, both the pellet and entire supernatant were retained. Care was taken to measure the volume of the polysomal supernatant so that the components of the standard reaction mix were present in the correct proportions. Additions, if necessary, were done using concentrated stock solutions. The final sucrose concentration in these supernatants was approximately 10%, owing to the retained sucrose cushion. The appropriate supernatant fraction was then mixed with the appropriate polysomal pellet, and the pellet was resuspended by homogenization through a 25-gauge needle, as indicated above. Incubations were carried out at 30°C as above.

Extract Pretreatment

To test the sensitivity of the extracts to brief heat treatment, standard *in vitro* degradation reactions were heated at 90°C for 10 min, cooled to 4°C, and then analyzed for *in vitro* decay of mRNAs according to the standard protocol. To test the sensitivity of the extracts to pretreatment with protease, standard extracts were supplemented with proteinase K (Sigma; Molecular Biology Grade) to a concentration of 1 mg/ml, and incubated at 30°C for 30 min before analysis for in vitro mRNA degradation according to the standard protocol.

To test the effect of pretreating the extracts with micrococcal nuclease, standard in vitro degradation reactions were supplemented with micrococcal nuclease (Pharmacia) to 1000 U/ml and $CaCl_2$ to 1 mM, and then incubated at 30°C for 10 min. Ethyleneglycol-bis(β-aminoethyl ether) N,N′-tetraacetic acid (EGTA) was then added to 2 mM and the extracts were chilled on ice for 10 min. Deproteinized total cytoplasmic RNA from an equivalent number of uninfected HeLa cells was added to each reaction mixture, which was then incubated at 30°C and analyzed according to the standard protocol.

Preparation of Soluble Virion Extracts

Virion extracts of both wild type HSV-1 and vhs 1 were performed essentially as described by Preston et al. (158). Virus was prepared from infected Vero cells as described above, and the viral stocks were clarified by brief centrifugation in a table top centrifuge. The virus was then pelleted by centrifugation at 25,000 x g for two h, and the pellet was resuspended in 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, and NP-40 to a final concentration of 0.5%. The virus was incubated at 4°C for one h. Soluble supernatant was prepared by centrifugation of the viral extract at 50,000 x g for one h, and the supernatant was stored at -70°C until used.

RNA Electrophoresis, Northern Blotting, and Hybridization

RNA was separated by agarose gel electrophoresis as previously described (161). The RNA was recovered from ethanol by a 15 min spin at 16,000 x g at 4°C. The dried pellet was dissolved in a small volume of diethyl pyrocarbonate (DEPC) treated H_2O. Aliquots of the RNA were incubated in 1 M deionized glyoxal, 10 mM NaH_2PO_4, pH 7.0, and 50% DMSO for 60 min at 50°C. The RNA was size fractionated on a 1% agarose gel in 10 mM NaH_2PO_4, pH 7.0, at 115 V for three hours with constant buffer recirculation.

The fractionated RNA was capillary blotted onto Nytran nylon filters (Schleicher and Schuell) as directed by the manufacturer. The blotting buffer used was 10X SSPE (1X SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4 (pH 7.7), 1 mM EDTA). The dried filters were prehybridized for 1-2 h at 42°C with constant agitation, and hybridized for 12-20 h under the same conditions, as directed. Prehybridization buffer consists of 50% deionized formamide, 5X Denhardt's solution (1X Denhardt's contains 0.02% Ficoll, 0.02% polyvinylpyrollidone, and 0.02% BSA), 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, and 5X SSPE. Hybridization buffer is identical to prehybridization buffer except the former buffer contains 2.5X Denhardt's solution instead of 5X Denhardt's solution as in the latter. The nick-translated probes were denatured by boiling for 10 minutes, then chilled on ice briefly before being added to the hybridization buffer. Viral TK was probed with nick-translated pHSV106, whereas cellular GAPD was detected with nick translated pHcGAP. Nick translated pXlr11 was used to detect 28S rRNA. Nick translation kits were purchased from Bethesda Research Laboratories and used as directed by the manufacturer.

Blot washing was done as previously described (143), with two 45 min washes with 2X SSPE and 0.4% SDS at room temp, followed by two 15 min washes with 0.1X SSPE at 60°C. Moist filters were exposed to Kodak X-AR5 film with intensifying screens.

Quantitation of mRNA Levels

To quantitate the levels of host and viral mRNAs and of 28S rRNA, autoradiograms from the Northern blots were scanned using a Gilford Response UV-VIS Spectrophotometer fitted with an autoradiogram holder for gel scanning.

RESULTS

The experiments described in this section concern attempts to elucidate the mechanism by which the vhs function degrades host and viral mRNA. Initial experiments involved efforts at mapping the mutation in vhs 1, the best characterized of the vhs mutants originally described by Read and Frenkel (160). From a mechanistic point of view, it is important to map the mutation in vhs 1 in order to show whether the vhs function acts alone or requires ancillary viral functions which may also have been mutated in this virus. By rescuing the mutation in vhs 1 with a sufficiently small cloned fragment from the wild type genome, it is possible to demonstrate that the vhs function is required for shutoff of host and viral polypeptide synthesis. These analyses do not exclude other ancillary viral or cellular factors required for this shutoff, but the recognition of one such factor, the vhs function, provides an extremely useful starting point for the examination of the shutoff process.

The second set of experiments involved the analysis of the protein components of infected cell mRNPs. One possible mechanism of vhs function is the alteration of cellular mRNP structure, making the mRNA moiety more susceptible to attack by ribonucleases. Experiments by Bartkoski (10) first described changes in the protein components of mRNP in HSV-1 infected cells. A major conclusion of that work was that the levels of poly(A) binding protein associated with mRNA isolated from wild type infected cells was decreased relative to the amounts detected in uninfected cells. However, as described above, the method used was open to artifacts, and there were no relevant mutants available for similar analyses. The experiments described in this section employed a UV-crosslinking procedure first described by Greenberg (71) and modified by Dreyfuss (1), a procedure more resistant to artifactual RNA-protein interactions. Also, the mRNPs examined in these experiments derived from cells infected by both wild type and vhs 1 viruses. This allowed a determination of the effects of the vhs function on the proteins associated with mRNA in infected cells.

The final series of experiments concerns the development of an in vitro mRNA degradation system to study the vhs function. The in vitro system consisted of cytoplasmic extracts from HeLa cells infected with either wild type HSV-1 or the mutant vhs 1. The preparation of these extracts was a modification of a procedure for an in vitro translation system first described by Brown et al. (27). The development of the in vitro degradation system described here should eventually allow a more detailed biochemical analysis of the vhs function to be undertaken, and may lead to a better understanding of the process of mRNA degradation in mammalian cells.

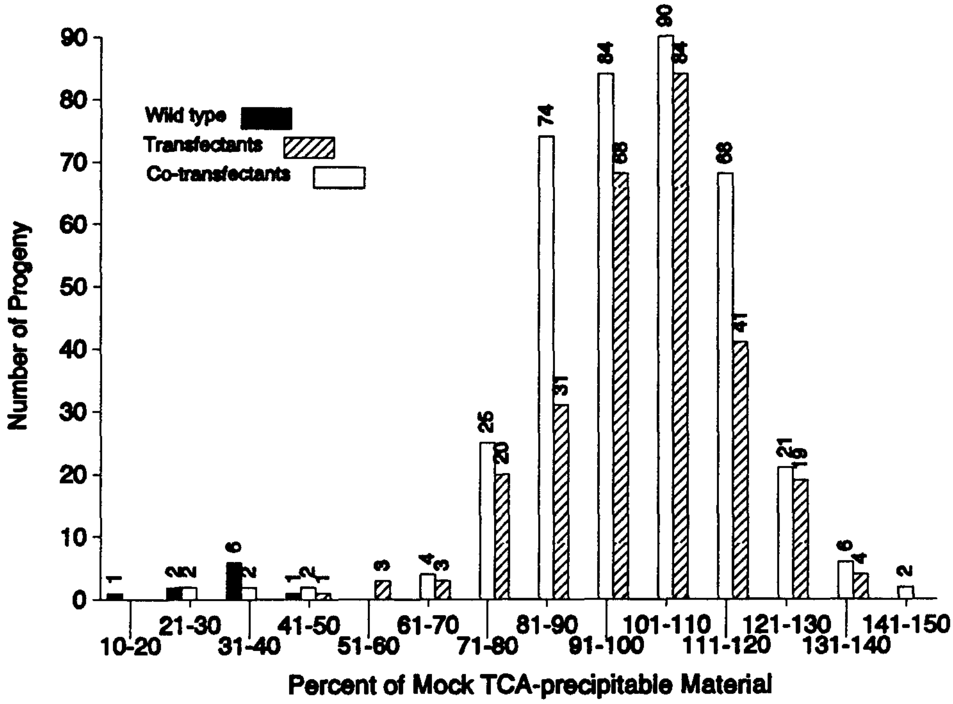
Marker Rescue of the Vhs 1 Mutation

To map the vhs mutation, Vero cells grown in 25 cm^2 flasks were transfected according to the protocol of Stow et al. (204). The transfection mixes contained 5 μg intact vhs 1 DNA, 2.5 μg plasmid pSG124, containing the EcoR1 fragment A from wild type HSV-1 strain KOS, and 12.5 μg salmon sperm carrier DNA. Control transfections contained 5 μg intact vhs 1 DNA plus 15 μg carrier DNA. Progeny viruses were screened to determine the fraction exhibiting wild type vhs activity. This screening involved picking individual progeny virus plaques and expanding them into small stocks by infecting Vero cells in 24-well trays. One half of the virus from each well was used to infect a fresh well of cells in the presence of 5 μg dactinomycin per ml. These cultures were labeled with [^{35}S]methionine from five to six h post-infection, and the amount of radioactivity incorporated into trichloroacetic acid (TCA) precipitable material was used as a measure of ongoing host translation (160). Host translation in mock infected cells as well as cells infected with wild type virus was also measured in the same way. This provided a base line value for wild type host shutoff, in order to compare the degree of shutoff of the progeny viruses derived from the transfections.

Measurement of radioactivity incorporated into TCA precipitable material provides the easiest method for screening large numbers of virus for vhs activity. However, it would be impractical to screen statistically significant numbers of progeny from independent transfections involving each of the EcoR1 fragments of the viral DNA. Fortunately, earlier data suggested a region of the genome on which to focus. Many strains of HSV-2 induce a more rapid shutoff than HSV-1 strains. By examining HSV-1 x HSV-2 intertypic recombinants, Fenwick and coworkers were able to correlate the rapid HSV-2 shutoff with the presence within the recombinant of a portion of the HSV-2 genome from between 0.52 and 0.59 map units (54).

The experiments described below therefore used the plasmid pSG124, which contains the EcoR1 fragment A (from 0.49 to 0.63 map units) of wild type HSV-1 inserted into the EcoR1 site of pBR325 (69). Co-transfections consisted of a mixture of pSG124, intact vhs 1 viral DNA, and carrier; control transfections consisted of the vhs 1 DNA and carrier. The results of screening 380 progeny from the experimental transfection and 274 progeny from the control transfection are tabulated to show the number of virus for which the level of incorporation into TCA-precipitable material was an indicated percentage of the mock infected level (Figure 2). The progeny from the transfection involving vhs 1 DNA plus carrier formed a distribution centered at 100%. In contrast,

Figure 2. Host Shutoff Activity of Progeny from Transfections. The number of progeny from each type of transfection at a particular percent of the mock transfection is shown as a vertical bar, with the total number of progeny indicated above the bar. The figure legend indicates which vertical bar corresponds to which type of transfection. The experiment was performed as described in the text.


Number of Progeny
0
10
20
30
40
50
60
70
80
90
Wild type ■
Transfectants ▨
Co-transfectants □
1
2
2
6
2
1
2
1
3
4
3
25
20
74
31
84
68
90
84
68
41
21
19
6
4
2
10-20
21-30
31-40
41-50
51-60
61-70
71-80
81-90
91-100
101-110
111-120
121-130
131-140
141-150
Percent of Mock TCA-precipitable Material

the control transfection proved to be mutants upon retesting (Table 1), indicating that none of the 274 control progeny were revertants. In contrast six of the 10 progeny from the experimental transfection proved to be wild type for vhs activity. Since the control experiment yielded no revertant progeny, these six progeny apparently represent authentic recombinant viruses, signifying a frequency of marker rescue of 6/380, or 1.6×10^{-2}.

These six recombinant progeny were next screened to see whether they were mutant or wild type with respect to the shutoff of α polypeptide synthesis. This involves the actual resolution of polypeptides on polyacrylamide gels, and subsequent identification of viral α polypeptides. Vero cells were infected with the recombinant progeny, along with wild type and vhs 1 controls. The infections were carried out for five h in the presence of 50 μg cycloheximide per ml. Infection in the presence of cycloheximide allows viral α mRNAs to be transcribed, but inhibits the synthesis of any polypeptides. After five h, the cycloheximide containing medium was removed and replaced with medium containing 5 μg dactinomycin per ml. This inhibits further transcription, but allows any existing mRNAs to be translated. The cultures were labeled with [^{35}S]methionine at various times to assay the rates of ongoing α translation.

In accord with previous results (143,160), α polypeptide synthesis continued for significantly longer in vhs 1 in-

Table 1

Rescreening of Progeny from Transfections

	Virus	Primary Screen	Rescreen
Vhs 1 plus WT EcoR1 A	60	0.49	0.49, 0.50, 0.63, 0.47
	86	0.20	0.33, 0.39, 0.40, 0.27
	93	0.35	0.26, 0.40, 0.39, 0.30
	108	0.30	0.20, 0.19, 0.33, 0.26
	215	0.20	0.19, 0.21, 0.20, 0.19
	291	0.50	0.24, 0.25, 0.33, 0.24
	37	0.68	1.21, 1.37, 1.26, 1.13
	115	0.69	1.06, 1.07, 0.97, 0.96
	332	0.61	1.21, 0.88, 1.09, 0.89
	340	0.68	1.09, 1.03, 0.98, 1.01
Vhs 1	404	0.63	1.03, 1.08, 0.91, 0.85
	428	0.59	0.97, 1.03, 0.87, 0.87
	429	0.59	1.16, 1.02, 1.04, 0.93
	452	0.69	1.01, 1.16, 1.01, 0.92
	461	0.50	0.85, 0.93, 1.04, 0.95
	C5	0.50	0.93, 1.11, 1.05, 0.86
	C25	0.62	1.07, 1.07, 0.89, 1.01
WT control			0.39, 0.46, 0.49, 0.46

fected cells than in cells infected with wild type virus (Figure 3). In wild type infected cells, synthesis of ICPs 4 and 0 could barely be detected at three h post-reversal of the cycloheximide block (lane 2), while the synthesis of these polypeptides was undetectable by seven h post-reversal (lane 4). Synthesis of ICP 27 was greatly reduced by seven h post-reversal. This all occurs against a background of cellular polypeptide synthesis which was also greatly reduced by seven h post-reversal (lane 4). In contrast, the three α polypeptides marked in Figure 3 continued being synthesized in abundant quantities at seven h post-reversal in cells infected with the mutant <u>vhs</u> 1 (lane 8). All six recombinants were clearly wild type for α shutoff, that is, the synthesis of ICP 4, 0, and 27 were all significantly reduced by seven h post-reversal (Figure 4), just as in the wild type control. The background synthesis of host polypeptides was also greatly reduced in these recombinants by seven h post-reversal. These results indicate that the mutation(s) affecting the host and α shutoff activities of <u>vhs</u> 1 map between 0.49 and 0.63 map units in the viral genome. Since all six recombinants demonstrated wild type levels of both host and α shutoff, this is a strong indication that both events are controlled by the same or closely linked genes. These data, taken together, strongly suggests that the <u>vhs</u> function is intimately involved in the shutoff of both host and viral polypeptide synthesis, either alone or in conjunc-

Figure 3. Synthesis of α Polypeptides by Wild Type and Vhs 1 Virus. Cells were infected with 50 pfu per cell of wild type virus (lanes 1 through 4) or vhs 1 (lanes 5 through 8), all in the presence of 50 μg cycloheximide per ml. At 5 h post-infection, the cycloheximide-containing medium was removed and replaced with medium containing 5 μg dactinomycin per ml. The cells were pulse labeled for 1 h intervals beginning at 0.5 h (lanes 1 and 5), 3 h (lanes 2 and 6), 5 h (lanes 3 and 7) or 7 h (lanes 4 and 8) after removal of the cycloheximide. The labeled polypeptides were analyzed by electrophoresis on 9.25% polyacrylamide gels as previously described (160). The α polypeptides ICP 4, 0, and 27 are labeled to the left of lane 1 and are marked by dots to the left of lane 5.

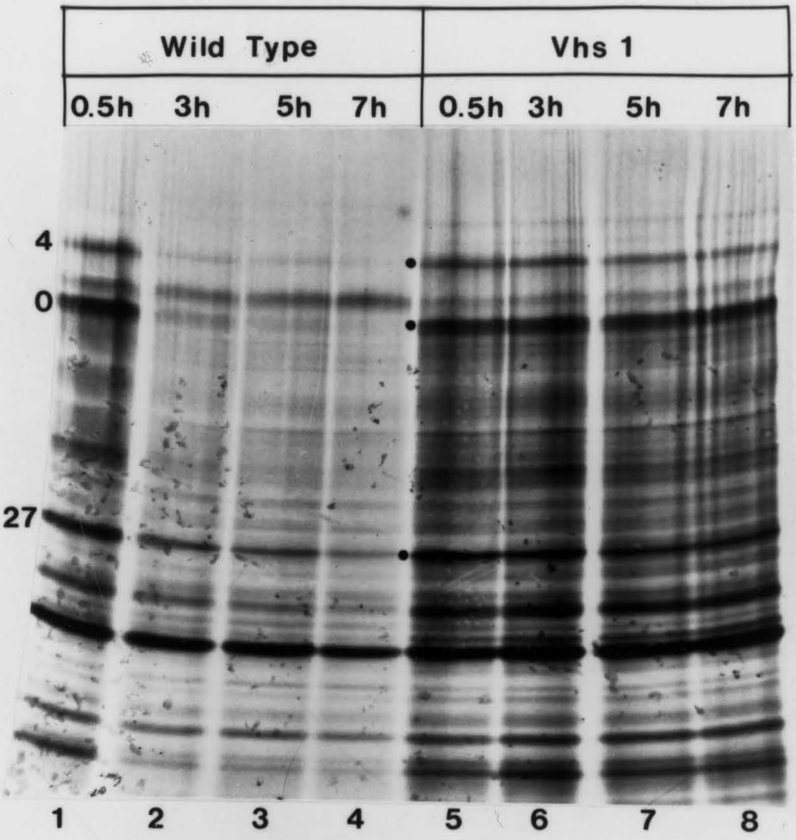
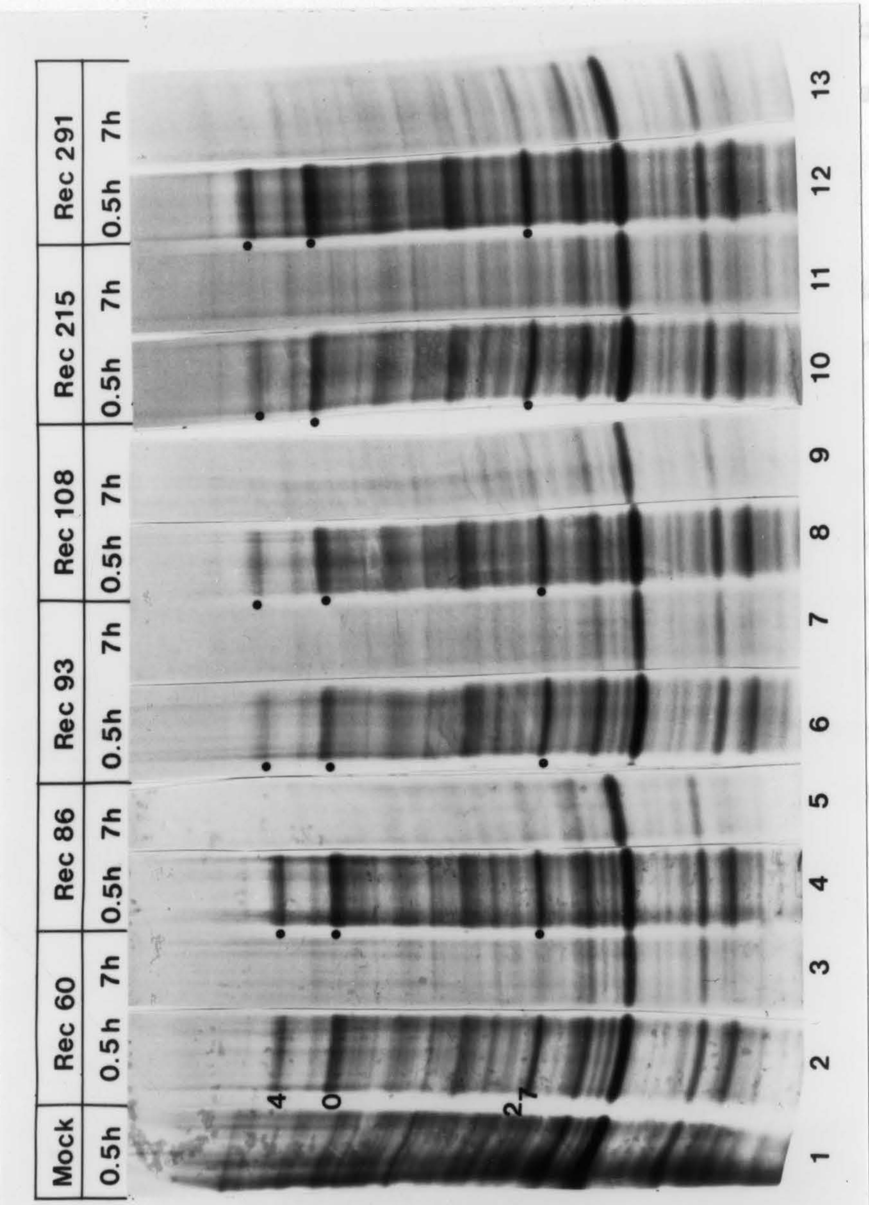

Wild Type
Vhs 1
0.5h
3h
5h
7h
0.5h
3h
5h
7h
4
0
27
1
2
3
4
5
6
7
8

Figure 4. The Synthesis of α Polypeptides by Recombinant Virus. Cells were mock infected (lane 1) or infected with 50 pfu per cell of the indicated recombinant virus, all in the presence of 50 μg cycloheximide per ml. The cycloheximide-containing medium was removed at 5 h post-infection and replaced with medium containing 5 μg dactinomycin per ml. The cells were pulse labeled for 1 h intervals beginning at 0.5 h (lanes 1, 2, 4, 6, 8, and 10), or 7 h (lanes 3, 5, 7, 9, 11, and 13) after removal of the cycloheximide. Labeled polypeptides were analyzed as described for Figure 3. The α polypeptides ICP 4, 0, and 27 are labeled to the left of lane 2 and are marked by dots to the left of lanes 4, 6, 8, 10, and 12.


Mock
Rec 60
Rec 86
Rec 93
Rec 108
Rec 215
Rec 291
0.5h
0.5h
7h
0.5h
7h
0.5h
7h
0.5h
7h
0.5h
7h
0.5h
7h
4
0
27
1
2
3
4
5
6
7
8
9
10
11
12
13

tion with other viral and/or cellular functions. Subsequent data from another laboratory demonstrated that both the host shutoff and α shutoff phenotype of vhs 1 could be rescued by a 256 bp fragment, and that this fragment mapped to a single open reading frame, named UL 41. This is in complete agreement with the data presented here.

Proteins Associated with mRNA in Infected Cells

One possible function for the protein component of mRNP is that of regulating mRNA stability (48). It is therefore possible that one effect of the vhs function is to alter the proteins associated with mRNA in infected cells, to thereby increase the susceptibility of the mRNA to endogenous ribonucleases. To test this hypothesis, cells were mock infected or infected with wild type or vhs 1 virus and the intact cells were exposed to ultraviolet (UV) light (1). This method covalently crosslinks only those proteins intimately associated with mRNA (197). After cells were lysed with detergent, the cytoplasmic material was passed over an oligo-(dT)-column (5), and messenger ribonucleoprotein particles were recovered. Metabolic labeling of the proteins using [^{35}S]methionine allowed the protein components of mRNPs to be visualized by digesting the mRNA with nucleases and separating the proteins by SDS-polyacrylamide gel electrophoresis followed by fluorography (1).

The first order of business was to demonstrate that the

UV crosslinking procedure would yield results in agreement with other researchers' work. HeLa cells were labeled with [^{35}S]methionine for four h, then mock infected for three h in the absence of label. The cells were then UV-irradiated and mRNPs were prepared as described in Chapter 2. At least seven protein bands could be resolved in mRNPs from mock infected cells (Figure 5A, lane 1). Prominent bands were seen at 125, 73, and 52 kda, with minor bands at 49, 39, 34, and 32 kda. The 73 kda protein has been shown to be the cytoplasmic poly(A) binding protein (1, 21).

The isolation of these proteins as part of poly(A)$^+$ mRNPs was dependent upon irradiation of the cells prior to lysis (compare Figure 5B, lanes 1 and 2). This indicates that the proteins were intimately associated with mRNA within the intact cell and that the association was not due to artifactual interactions with the mRNA during cell fractionation.

The proteins associated with mRNA in cells infected with wild type HSV-1 was then examined. The protein profiles of mRNPs from mock and wild type virus infected cells were very similar (Figure 5A, lanes 1 and 2). All of the major components of mRNPs from mock infected cells were also components of mRNPs from wild type infected cells. Nevertheless, several quantitative changes were consistently seen in mRNPs from cells infected with wild type virus. The major host polypeptide of 52 kda was decreased in abundance, while the minor

Figure 5. Messenger RNPs from Wild Type and Mock Infected Cells. A. HeLa cells were prelabeled for 4 h with [^{35}S]-methionine and then mock infected (lane 1) or infected with 20 pfu per cell of wild type HSV-1 (lane 2). The cells were incubated in the absence of label from 0 to 3 h post-infection, at which time mRNPs were prepared and analyzed. The molecular weights (in kilodaltons) of prominent proteins are shown to the left of lane 1. The 52 kda polypeptide whose abundance is decreased by wild type virus infection is marked by an open circle to the right of lane 1, while the 49 kda protein that is increased in abundance is labeled by a closed circle. B. Parallel cultures were labeled as in part A and infected with 20 pfu per cell of wild type HSV-1. At 3 h post-infection the cells were either irradiated with UV light (lane 1) or not irradiated (lane 2). The cells were then lysed and mRNP samples prepared according to the remainder of the normal protocol described in the Materials and Methods.

host band of 49 kda was increased, both relative to the 73 kda PABP. There was no apparent decrease in the levels of PABP in cells infected with wild type HSV 1 as described by
Bartkoski in
10).

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viral polypep
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infection mi
cells were mock
infected or i
either wild type
or vhs 1 and
s zero to three h
post infectio
amount of labeled
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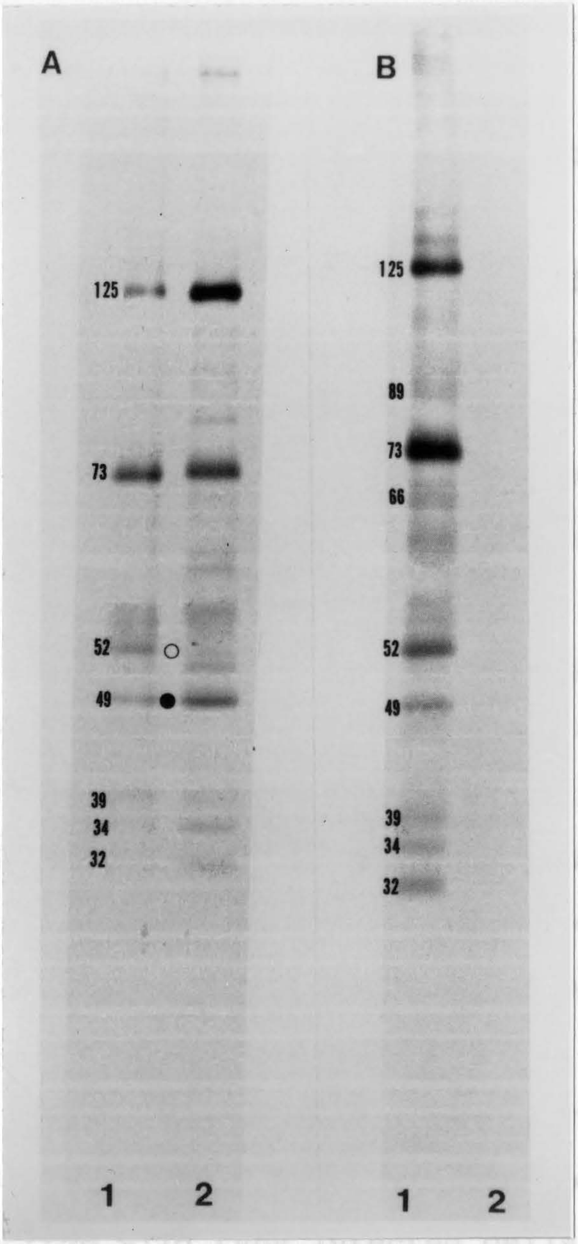
ell mRNP compared
to mock or v
rved (Figure 6A).
This decrease
protein synthesis
caused by inf
hs 1 virus (160).

To compa
s to compare the
protein compo
d type, and vhs 1
infected cel
sity exposures of
the three lar
sed (Figure 6B).
Once again, s
bserved for mRNPs
from mock an
The decrease in
abundance of
rease in the 49
kda protein
were observed for
mRNP proteins from wild type infected cells, but were not as obvious in these gel exposures as in Figure 5A. In addition, a minor 47 kda host polypeptide was decreased in abundance while a new minor band of 46 kda was observed (Figure 6B,

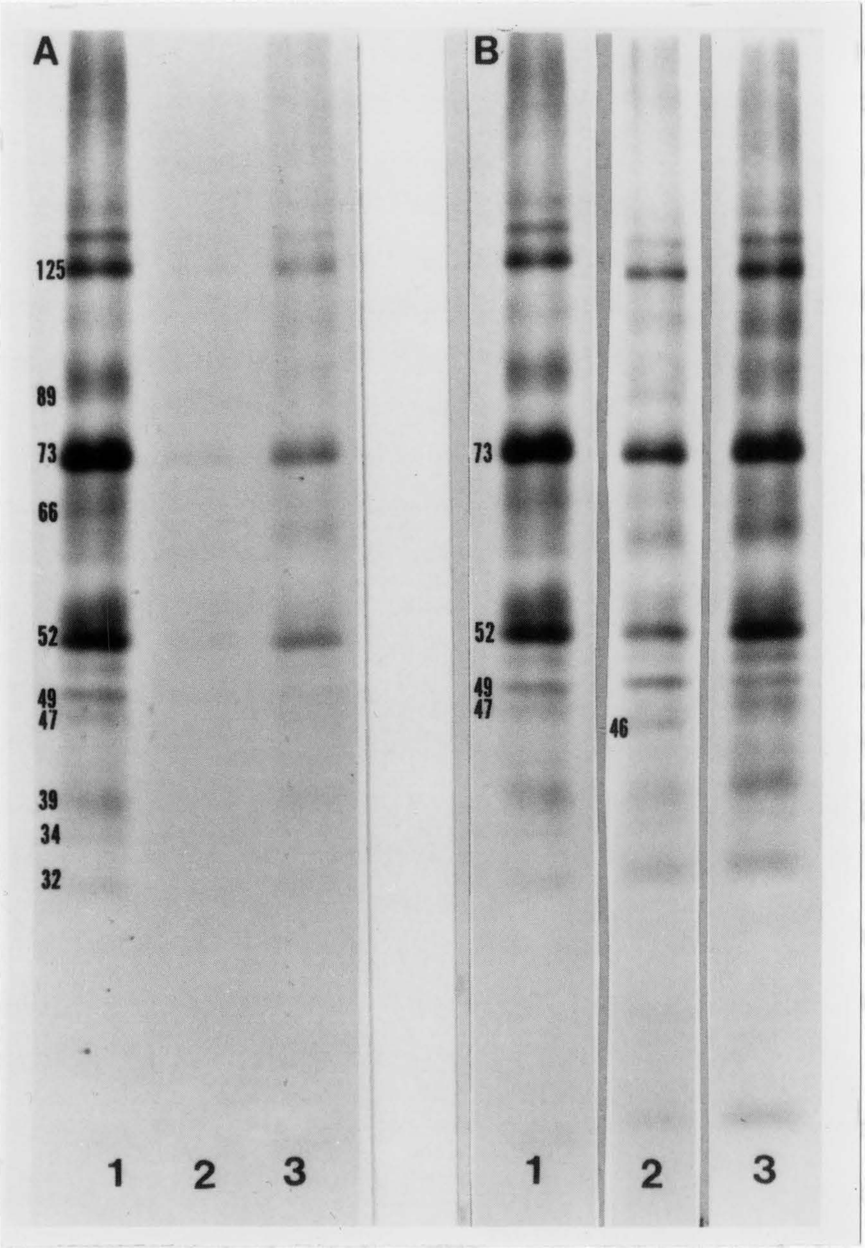


host band of 49 kda was increased, both relative to the 73 kda PABP. There was no apparent decrease in the levels of PABP in cells infected with wild type HSV 1 as described by Bartkoski in previously published results (10).

To examine the possibility that de novo synthesized viral polypeptides or cellular proteins synthesized following infection might be mRNP components, HeLa cells were mock infected or infected with 20 pfu per cell of either wild type or vhs 1 and labeled with [^{35}S]methionine from zero to three h post infection. A striking decrease in the amount of labeled protein recovered from wild type infected cell mRNP compared to mock or vhs 1 infected cells was observed (Figure 6A). This decrease reflects the shutoff of host protein synthesis caused by infection with wild type but not vhs 1 virus (160).

To compensate for this difference so as to compare the protein composition of mRNPs from mock, wild type, and vhs 1 infected cells, approximately equal intensity exposures of the three lanes from Figure 6A were juxtaposed (Figure 6B). Once again, similar protein profiles were observed for mRNPs from mock and wild type infected cells. The decrease in abundance of the 52 kda polypeptide and increase in the 49 kda protein relative to the 73 kda PABP were observed for mRNP proteins from wild type infected cells, but were not as obvious in these gel exposures as in Figure 5A. In addition, a minor 47 kda host polypeptide was decreased in abundance while a new minor band of 46 kda was observed (Figure 6B,

Figure 6. Messenger RNPs Synthesized Following Wild Type, _Vhs_ 1, or Mock Infection. A. HeLa cells were mock infected (lane 1) or infected with 20 pfu per cell of wild type virus (lane 2) or _vhs_ 1 (lane 3). The cells were labeled with [^{35}S]methionine from 0 to 3 h post-infection, and mRNPs were prepared and analyzed at 3 h. Material from approximately 3 x 10^7 cells were loaded onto each lane of the gel. B. Multiple exposures were obtained for the gel shown in part A, and exposures resulting in similar intensities for the mock (lane 1), wild type (lane 2), and _vhs_ 1 (lane 3) infections were juxtaposed. The molecular weights (in kilodaltons) of prominent proteins are shown to the left of lane 1 in both A and B.


A
125
89
73
66
52
49
47
39
34
32
1
2
3
B
73
52
49
47
46
1
2
3

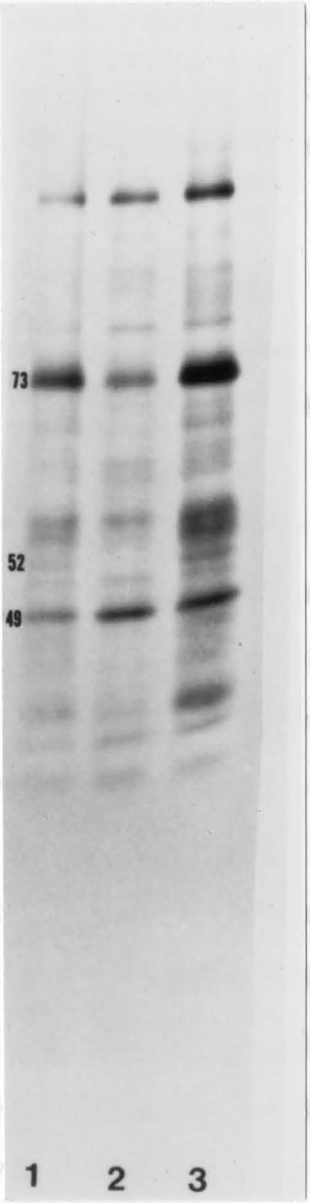
lane 2). Significantly, the profile of mRNP proteins from cells infected with vhs 1 was similar to the protein pattern of mock infected cells, and did not show the changes characteristic of wild type virus infection (Figure 6B, lane 3). Furthermore, no de novo synthesized viral proteins were detected in mRNPs from either wild type or vhs 1 infected cells.

To test the apparent correlation between changes in mRNP composition and vhs activity, experiments were performed to determine whether the changes induced by wild type virus still occurred following infection under conditions that prevent viral gene expression, a hallmark of vhs induced host shutoff. Cells were prelabeled with [^{35}S]methionine for four h, then mock infected or infected with 50 pfu per cell of either wild type or vhs 1 in the presence of 5 μg dactinomycin per ml. Wild type virus induced the same changes in the presence of dactinomycin (Figure 7, lane 2) as in a productive infection. Cells infected with vhs 1 in the presence of dactinomycin showed the same pattern of proteins as mock infected cells (Figure 7, lanes 1 and 3).

Taken together, these experiments suggest that infection of HeLa cells with wild type virus leads to a change in the proteins associated with mRNA, as determined by UV crosslinking. Since these changes were detected in the presence of dactinomycin, this implicates a component of the incoming virion. Likewise, the absence of any change in the protein

Figure 7. Changes in mRNP Structure Occur in Wild Type Infections in the Absence of Viral Gene Expression. HeLa cells were prelabeled with [^{35}S]methionine for 4 h and then mock infected (lane 1) or infected with 50 pfu per cell of wild type virus (lane 2) or vhs 1 (lane 3), all in the presence of 5 μg dactinomycin per ml. Messenger RNPs were prepared at 3 h post-infection. The molecular weights (in kilodaltons) of prominent proteins are shown to the left of lane 1.

profile of mRNPs isolated from cells infected with vhs 1, a mutant defective in the virion-associated host shutoff of translation, further implicates the vhs function as perhaps involved in the alte proteins (103). No reproducible changes in t y(A) binding protein were ever detected, a res with previously published data (10).



An In Vitro Degrad Study the Vhs Function

The HSV virion vhs) protein is known to induce rapid degrad and viral mRNAs in the cytoplasm (107,143,1 . In an effort to develop an in vitro mR system to study the vhs function, the rates ion in in vitro translation extracts prepa nfected HeLa cells, from wild type HSV-1 infe and HeLa cells infected with the mutant vhs a defective vhs polypeptide (160), were co lls were chosen because they are readily i V and because numerous studies have proven rticularly suitable cell line for the prepar ro translation extracts. Although in vivo s that vhs induced mRNA degradation does not require efficient ongoing translation of the mRNA (184,206), in vitro translation extracts were prepared initially because it was reasoned that these would be the best initial approximation of a functional cytoplasm.

profile of mRNPs isolated from cells infected with vhs 1, a mutant defective in the virion-associated host shutoff of translation, further implicates the vhs function as perhaps involved in the alteration of mRNP proteins (103). No reproducible changes in the amount of poly(A) binding protein were ever detected, a result in conflict with previously published data (10).

An In Vitro Degradation System to Study the Vhs Function

The HSV virion host shutoff (vhs) protein is known to induce rapid degradation of host and viral mRNAs in the cytoplasm (107,143,144,160, 184,206). In an effort to develop an *in vitro* mRNA degradation system to study the vhs function, the rates of mRNA degradation in *in vitro* translation extracts prepared from mock infected HeLa cells, from wild type HSV-1 infected HeLa cells, and HeLa cells infected with the mutant vhs 1, which encodes a defective vhs polypeptide (160), were compared. HeLa cells were chosen because they are readily infected with HSV and because numerous studies have proven them to be a particularly suitable cell line for the preparation of *in vitro* translation extracts. Although *in vivo* studies indicate that vhs induced mRNA degradation does not require efficient ongoing translation of the mRNA (184,206), *in vitro* translation extracts were prepared initially because it was reasoned that these would be the best initial approximation of a functional cytoplasm.

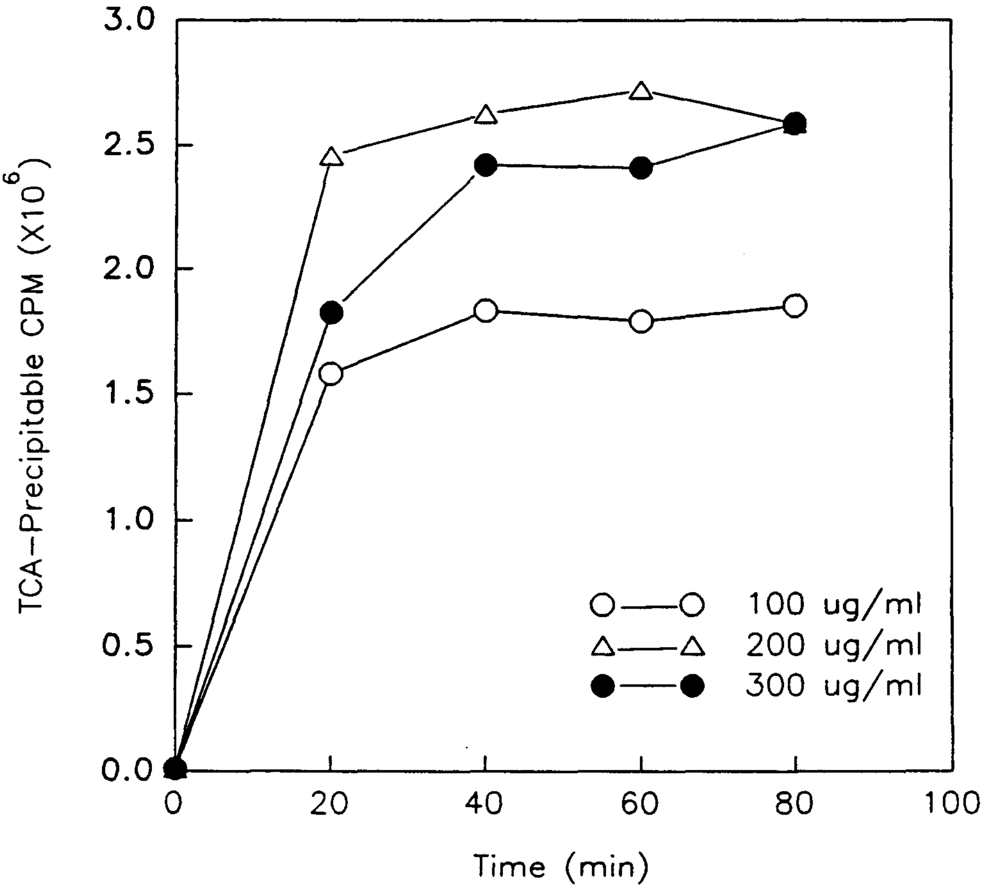
Extracts were prepared using a procedure shown by Brown and coworkers to be suitable for the preparation of highly active *in vitro* translation extracts from a variety of cultured cells (27). This procedure involves brief exposure of the cell monolayers to lysolecithin in order to permeabilize the cells, followed by harvesting the cells directly into buffer containing the components required for *in vitro* translation, disruption of the cells by repeated passage through a 25-gauge needle, and removal of the nuclei by low speed centrifugation. This protocol was chosen because it is simple and rapid, making it possible to prepare and analyze the activity of extracts on the same day. This allowed all the experiments to be performed using freshly prepared extracts, and eliminated the need to freeze any part of the cell-free system, an obvious advantage in view of the possible detrimental effects that freezing and thawing might have upon the activities of as yet uncharacterized factors.

To optimize the concentration of lysolecithin needed to permeabilize HeLa cells and to verify that this procedure yielded active *in vitro* translation extracts, standard reaction mixtures were prepared using a variety of lysolecithin concentrations in the permeabilization buffer. Unlabeled methionine was omitted from the reaction buffer and [^{35}S]methionine was added to a concentration of 75 µCi per ml. At various times after the start of incubation at 30°C, aliquots were withdrawn, and the amount of radioactivity incorporated into TCA-precipitable material was determined as previously described (160). Exposure of the HeLa cells to 300 μg lysolecithin per ml was found to be optimal for in vitro translation. As can be seen in Figure 8, for extracts prepared with this concentration of lysolecithin, efficient in vitro translation continued for approximately 40 min, after which the amount of incorporated label leveled to a plateau.

In Vitro Degradation of Host mRNAs

At this point, experiments were begun to study the in vitro degradation of host mRNAs. The vhs function was originally identified on the basis of its ability to induce rapid degradation of host mRNAs and the concomitant shutoff of host polypeptide synthesis (see above). To determine whether HeLa cell lysates would be suitable for in vitro studies of the vhs function, standard translation extracts were prepared from mock infected cells and from cells five h after infection with 20 pfu per cell of either wild type HSV-1 or the mutant vhs 1. The extracts were incubated at 30°C, aliquots were withdrawn at various times, extracted with phenol and chloroform, and the decay of specific cellular mRNAs analyzed by Northern blotting. To control for the total amount of RNA loaded onto each lane of the gel, the blots were also probed for 28S ribosomal RNA, and the amount of mRNA was normalized to the amount of 28S rRNA prior to plotting the mRNA decay curve.

Figure 8. *In Vitro* Translation by HeLa Cell Extracts and Determination of Appropriate Lysolecithin Concentration. *In vitro* mRNA degradation extracts were prepared from mock infected HeLa cells using three different concentrations of lysolecithin. Each extract was then examined for its efficiency of translation. Each extract was prepared in standard reaction buffer containing 2.5 mM $Mg(OAc)_2$, lacking unlabeled methionine, and supplemented with $[^{35}S]$methionine to 75 μCi per ml. The reaction mixture was incubated at 30°C. At various times samples were withdrawn, and the amount of radioactivity incorporated into trichloroacetic acid-precipitable material was determined as described previously (160).


3.0
2.5
2.0
1.5
1.0
0.5
0.0
TCA–Precipitable CPM (X10^6)
0
20
40
60
80
100
Time (min)
○——○ 100 ug/ml
△——△ 200 ug/ml
●——● 300 ug/ml

In these initial experiments the decay of endogenous cellular mRNAs was studied, instead of the decay of exogenously added mRNAs. This was because the structure of messenger ribonucleoprotein particles (mRNPs) reconstituted on exogenous mRNAs might differ from that of endogenous mRNPs. This mRNP structure could easily affect mRNA stability, and, as shown above, HSV infection has been shown to induce changes in mRNP structure that correlate with a wild type virion host shutoff function (103). Focussing upon the decay of endogenous mRNAs should, therefore, remove one potential variable from the experiments. In addition, the results involving the decay of endogenous mRNAs should provide a base line for later attempts to study the degradation of exogenous message.

The in vitro decay of the cellular mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPD) is shown in Figures 9 and 10. GAPD mRNA was chosen for study because it has a long in vivo half-life in uninfected cells (213), but is sensitive to vhs induced degradation upon infection with wild type HSV-1 (184). Thus, any vhs induced reduction in message stability should be more easily detected for this mRNA than for a message that is inherently unstable. GAPD mRNA was relatively stable for at least four h in extracts prepared from mock infected cells (Figure 9, lanes 1-5; Figure 10). In contrast, this message decayed rapidly in extracts from cells infected with wild type HSV-1, so that little detect-

Figure 9. In Vitro Degradation of Host mRNAs. Standard *in vitro* mRNA degradation extracts were prepared from HeLa cells 5 h after mock infection (lanes 1 through 5), or infection with 20 pfu of wild type HSV-1 (lanes 6 through 9) or *vhs* 1 (lanes 10 through 14) per cell. Samples were withdrawn from the reactions at 0 h (lanes 1, 6, and 10), 1 h (lanes 2, 7, and 11), 2 h (lanes 3 and 13), 3 h (lanes 4, 8, and 13), or 4 h (lanes 5, 9, and 14). The samples were extracted twice with phenol-chloroform and twice with chloroform, and the RNAs were precipitated from ethanol. Samples of total cytoplasmic RNA were denatured with glyoxal, electrophoresed through 1% agarose gels, and transferred to Nytran membranes by capillary blotting as described in the Materials and Methods. The membranes were then probed to detect GAPD mRNA and 28S rRNA as described in the Materials and Methods.

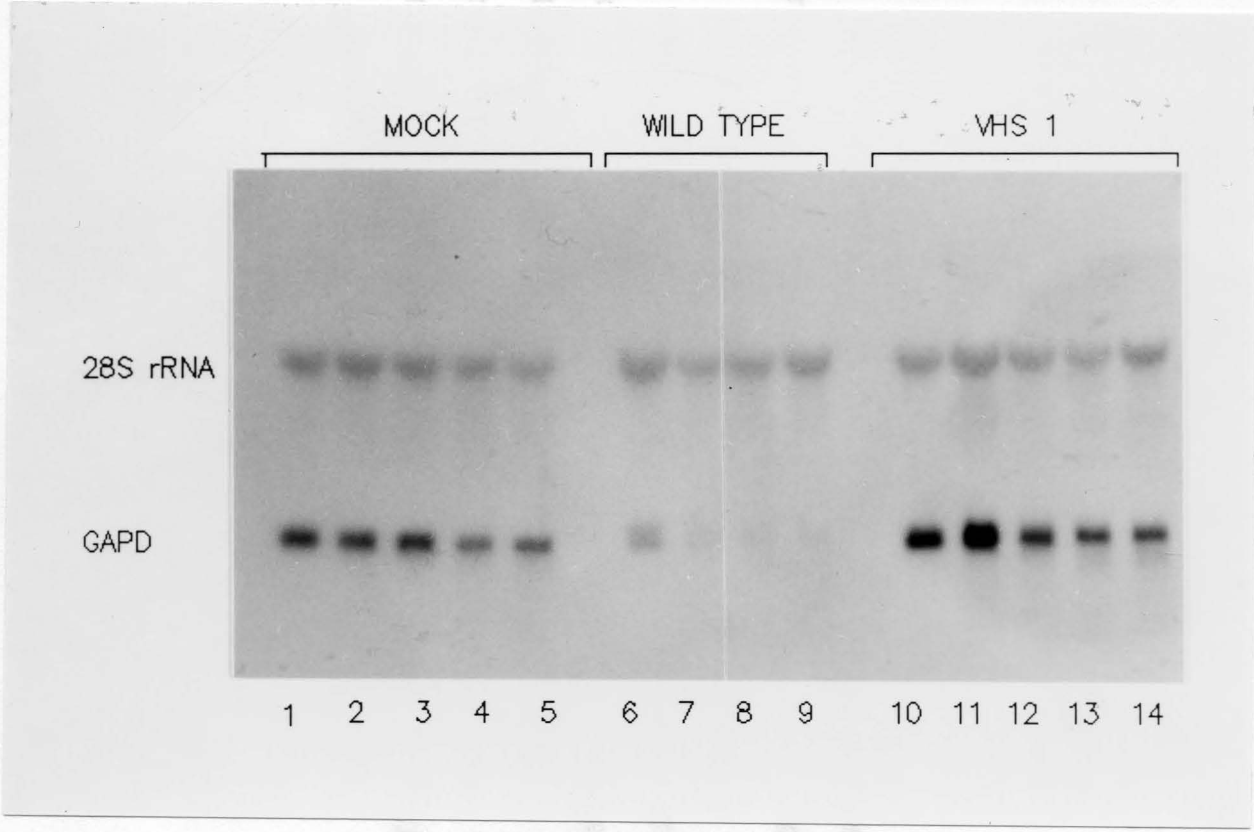
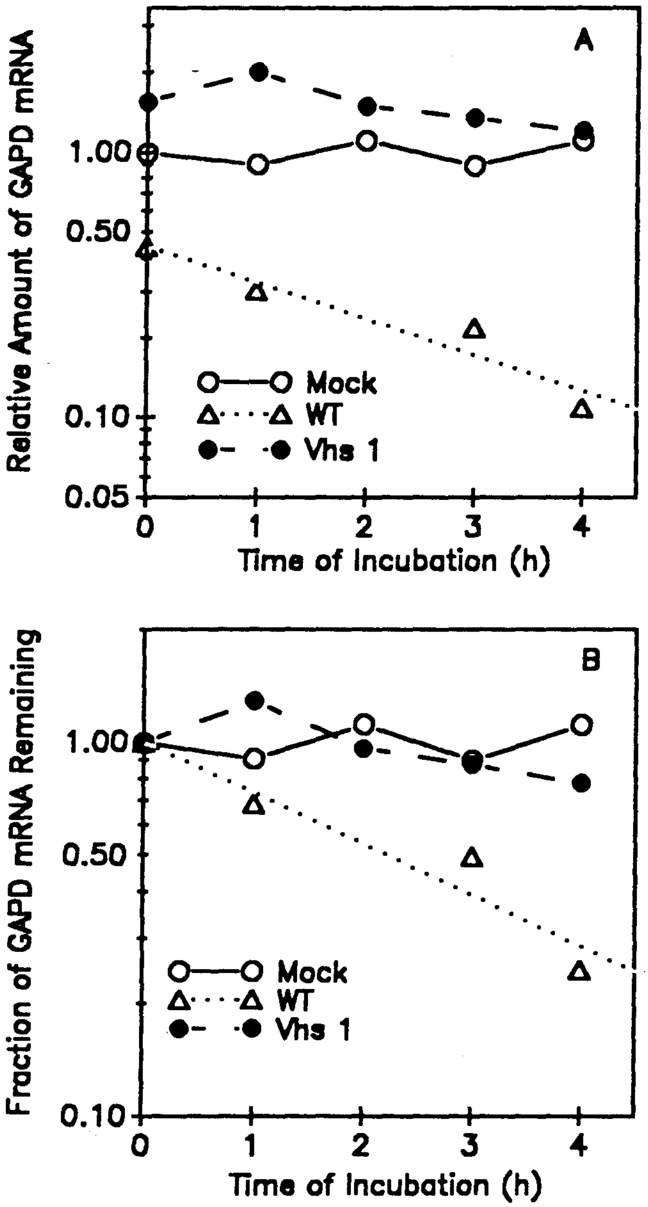

MOCK
WILD TYPE
VHS 1
28S rRNA
GAPD
1
2
3
4
5
6
7
8
9
10
11
12
13
14

Figure 10. Quantitation of <u>In Vitro</u> Decay of Host mRNAs. The autoradiogram shown in Figure 9 was scanned, and the amount of GAPD mRNA in each lane was normalized to the amount of 28S rRNA. In panel A, the relative amounts of GAPD mRNA in mock infected, wild type virus infected, and <u>vhs</u> 1 infected cell extracts are plotted in arbitrary units. In panel B the amount of GAPD mRNA present in a sample from any of the three types of extracts is expressed as a fraction of the amount present at 0 h in that kind of extract.


A
Relative Amount of GAPD mRNA
1.00
0.50
0.10
0.05
0
1
2
3
4
Time of Incubation (h)
Mock
WT
Vhs 1
B
Fraction of GAPD mRNA Remaining
1.00
0.50
0.10
0
1
2
3
4
Time of Incubation (h)
Mock
WT
Vhs 1

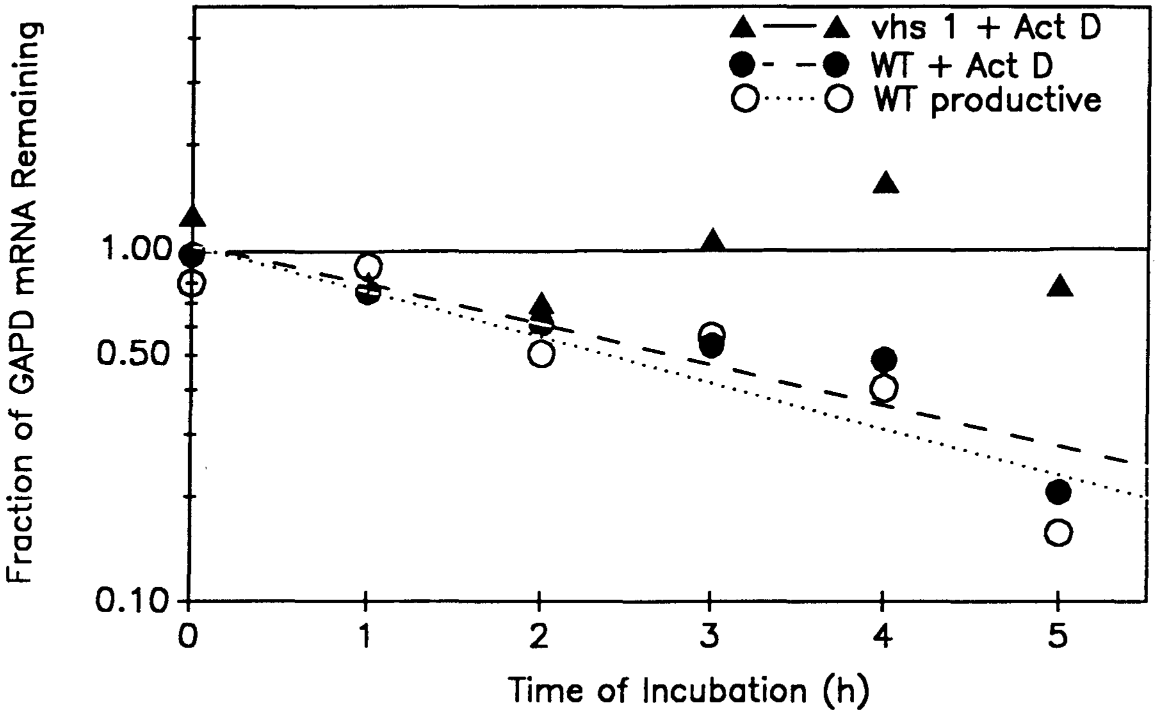
able message remained by one h after the start of incubation (Figure 9, lanes 6-9; Figure 10). That considerable vhs induced degradation had occurred in vivo in wild type cells prior to the preparation of extracts is indicated by the fact that the intensity of the band formed by GAPD mRNA at zero h of incubation for wild type extracts was reduced considerably relative to that observed at zero h for mock of vhs 1 infections (compare Figure 9, lanes 1, 6, and 10 with Figure 10). In contrast to the case for wild type infected cell extracts, in extracts prepared from cells infected with vhs 1, GAPD mRNA was as stable as in extracts from mock infected cells (Figure 9, lanes 10-14; Figure 10). In vitro decay was specific for mRNA, as evidenced by the fact that 28S rRNA was equally stable in mock, wild type, and vhs 1 infected cell extracts. All told, the rank order of in vitro decay rates of GAPD mRNA was the same as that observed in mock, wild type, and vhs 1 infections in vivo.

The vhs protein is a structural component of virions, and is, therefore, able to induce degradation of host mRNAs in the absence of prior de novo viral gene expression (see above). Thus, virion host shutoff is induced by UV-inactivated virus, as well as following infection of cells in the presence of dactinomycin to block viral transcription. To determine whether the accelerated degradation of GAPD mRNA observed in extracts from cells infected with wild type virus was induced by a virion component or required de novo viral gene expression, in vitro degradation extracts were prepared from cells five h after a productive wild type virus infection, or five h after infection with 50 pfu per cell of wild type virus or vhs 1 in the presence of 5 μg dactinomycin per ml. As can be seen in Figure 11, degradation of the GAPD mRNA was equally rapid in extracts from cells infected with wild type virus in the presence or absence of dactinomycin. In contrast, GAPD mRNA was stable for at least five h of incubation in extracts from vhs 1 infected cells. Thus, the accelerated degradation of host mRNAs that was observed in extracts from cells infected with wild type HSV-1 was not dependent on de novo viral gene expression, and was, therefore, induced by a component of the infecting virions (104).

In Vitro Degradation of Viral mRNAs

Although vhs mutants were originally isolated on the basis of their defects in the degradation of cellular mRNAs and the shutoff of host protein synthesis, recent studies indicate that the vhs protein plays a central role in determining the half-lives of both viral and cellular mRNAs in the infected cell (107,143,144). Measurements of the half-lives of ten different viral mRNAs in cells infected with wild type virus or the mutant vhs 1 revealed several things. First, in wild type infections the half-lives of all ten messages, representing all kinetic classes of viral mRNAs, were very similar. Second, the mutation in vhs 1 caused a dramatic

Figure 11. In Vitro Degradation in Extracts from Cells Infected in the Presence of Dactinomycin. Standard in vitro degradation extracts were prepared from cells 5 h after infection with 50 pfu of wild type virus or vhs 1 per cell in the presence of 5 µg of dactinomycin per ml or 5 h after infection with 50 pfu of wild type virus per cell in the absence of any drugs. The extracts were incubated at 30°C for the indicated times, at which point samples were withdrawn and total RNAs were extracted and analyzed for GAPD mRNA and 28S rRNA by Northern blotting as described in the legend to Figure 9. Autoradiograms were scanned with a Hoeffer model GS300 scanning densitometer, and the amount of GAPD mRNA was normalized to the amount of 28S rRNA. The amount of GAPD mRNA remaining at various times was plotted as a fraction of the amount present at 0 h.


▲—▲ vhs 1 + Act D
●- -● WT + Act D
○·····○ WT productive
Fraction of GAPD mRNA Remaining
1.00
0.50
0.10
0
1
2
3
4
5
Time of Incubation (h)

increase in the stabilities of all ten messages (144). Thus, the vhs protein induces the largely nonselective degradation of both viral and cellular mRNAs in vivo.

To determine whether degradation of viral mRNA was also accelerated in the in vitro message degradation system, in vitro degradation extracts were prepared from cells five h after infection with wild type virus or vhs 1, and analyzed for the degradation of the mRNA encoding the viral thymidine kinase (TK). As can be seen in Figures 12 and 13, TK mRNA was degraded rapidly in in vitro extracts from cells infected with wild type HSV-1, but was relatively stable for at least four h in extracts from cells infected with vhs 1. Therefore, once again the in vitro results paralleled those observed in vivo.

Characterization of the In Vitro mRNA Degradation System

In all respects examined to this point, in vitro degradation of host and viral mRNAs observed in HeLa cell extracts were comparable to vhs induced mRNA degradation observed in vivo. This system, therefore, was presumed to be an adequate in vitro model system to study vhs mediated mRNA degradation. The next set of experiments were designed to characterize some of the biochemical requirements of the in vitro mRNA degradation system.

A preliminary experiment was undertaken to determine the effect of the placental ribonuclease inhibitor RNasin upon

Figure 12. <u>In</u> <u>Vitro</u> Degradation of Viral mRNAs. Standard <u>in vitro</u> mRNA degradation extracts containing 5 mM Mg^{2+} were prepared from cells 5 h after infection with 20 pfu of wild type HSV-1 (lanes 1 through 6) or <u>vhs</u> 1 (lanes 7 through 13) per cell. The extracts were incubated at 30°C for the indicated times, at which point samples were withdrawn and total RNAs were prepared at 0 h (lanes 1 and 7), 15 min (lanes 2 and 8), 30 min (lanes 3 and 9), 1 h (lanes 4 and 10), 2 h (lanes 5 and 11), 3 h (lanes 6 and 12), and 4 h (lane 13) after the start of incubation. The amount of viral thymidine kinase (TK) mRNA was then analyzed by Northern blotting as described in Figure 9.

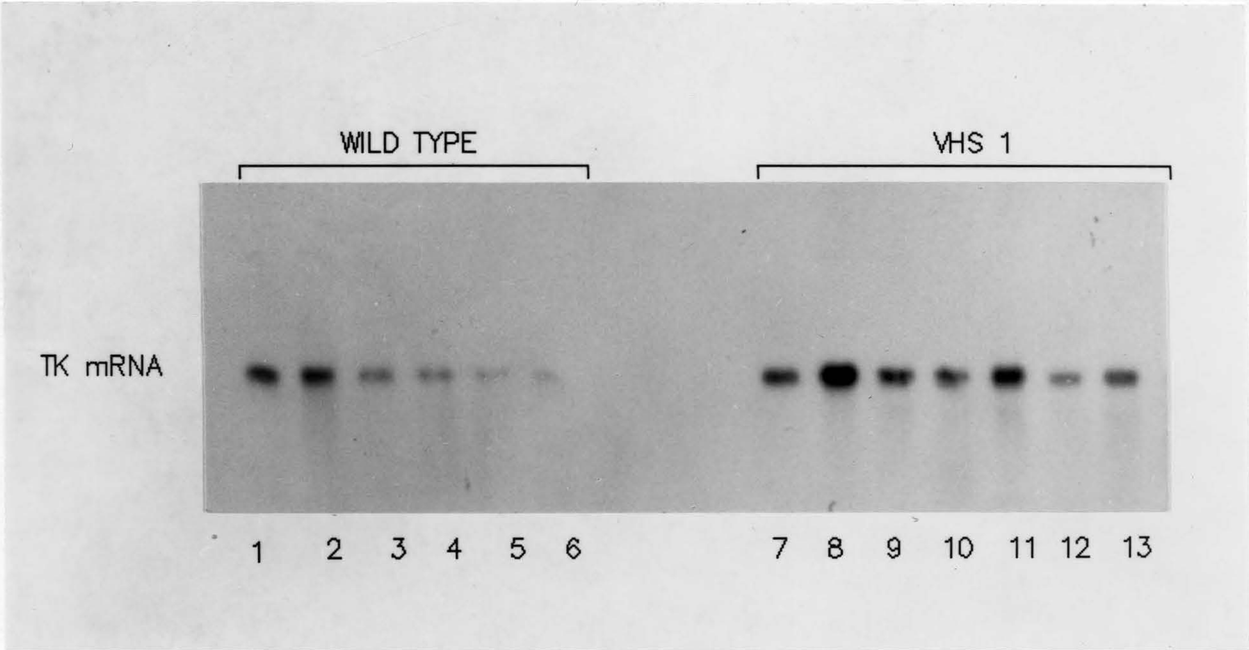
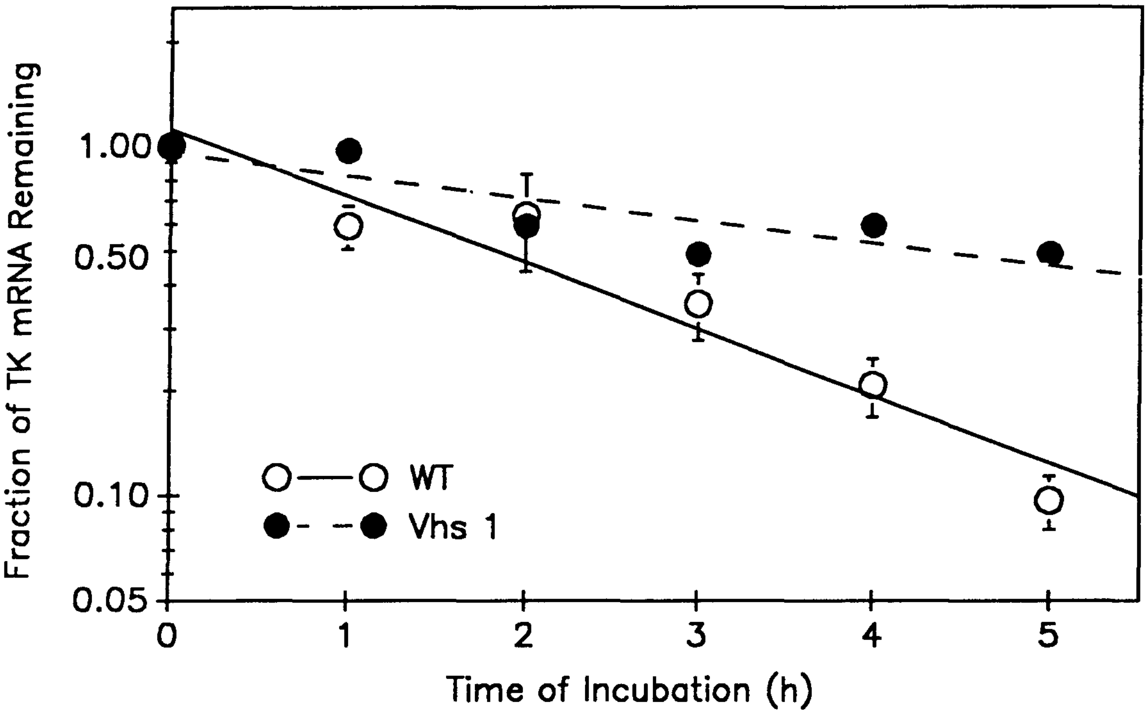

WILD TYPE
VHS 1
TK mRNA
1
2
3
4
5
6
7
8
9
10
11
12
13

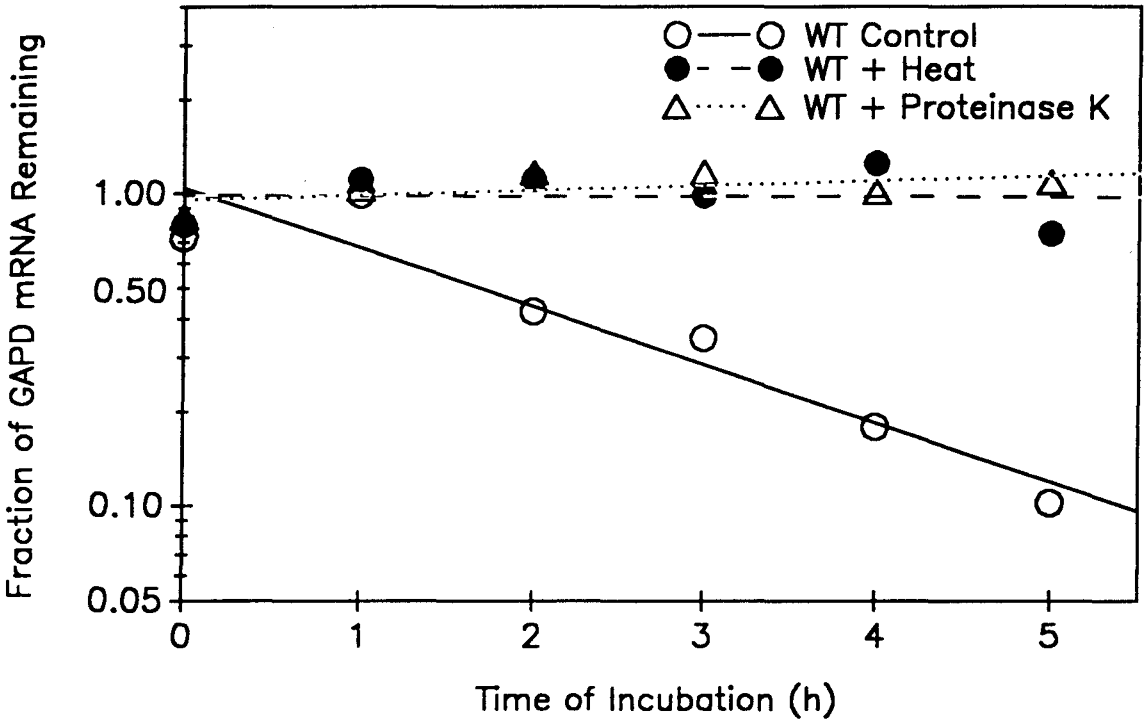
Figure 13. Quantitation of In Vitro Degradation of Viral mRNAs. Standard in vitro mRNA degradation extracts containing 20 mM Mg^{2+} were prepared from cells 5 h after infection with 20 pfu of wild type HSV-1 or vhs 1 per cell. The extracts were incubated at 30°C for the indicated times, at which point samples were withdrawn and total RNAs were extracted and analyzed for TK mRNA and 28S rRNA by Northern blotting as described in Figure 9. Autoradiograms were scanned with a Hoeffer model GS300 scanning densitometer, and the amount of TK mRNA remaining at various times was plotted as a fraction of the amount present at 0 h. Error bars indicate the standard errors of the means determined from replicate experiments.


Fraction of TK mRNA Remaining
1.00
0.50
0.10
0.05
0
1
2
3
4
5
Time of Incubation (h)
WT
Vhs 1

vhs induced mRNA decay. Parallel degradation extracts were prepared from HeLa cells five h after infection with 20 pfu per cell of either wild type or vhs 1. RNasin was included at a concentration of 100 units per ml in half of the reaction mixtures, while it was omitted from the other half. Regardless of its presence or absence, mRNAs were very stable in extracts derived from cells infected with vhs 1, while they decayed rapidly in extracts from cells infected with wild type virus (data not shown). Because the addition of RNasin did not inhibit vhs induced degradation and its inclusion might inhibit spurious nucleases, RNasin was included as a component in the standard in vitro reaction buffer and was present in all of the other experiments described in this section.

The next set of experiments was undertaken to ascertain whether the in vitro vhs activity could be inactivated by pretreating the extracts with heat or proteinase K. Three parallel in vitro degradation extracts were prepared from HeLa cells five h after infection with 20 pfu per cell of wild type HSV-1. One extract was heated to 90°C for 10 min, then chilled on ice. A second extract was supplemented with proteinase K and digested at 30°C for 30 min, while the third extract was left untreated. All three extracts were then analyzed for in vitro vhs activity. As can be seen in Figure 14, pretreatment of the extracts by either heating or proteinase K digestion completely abolished vhs mediated in

Figure 14. Effect of Heat and Proteinase K Pretreatment upon *Vhs*-induced *In Vitro* Degradation. Standard *in vitro* mRNA degradation extracts were prepared from cells 5 h after infection with 20 pfu of wild type HSV-1 per cell. Extracts were pretreated either by the addition of proteinase K and digestion for 30 min or by heating to 90°C for 10 min, after which the extracts were returned to 4°C. Control un-pretreated extracts were left at 4°C until the start of incubation. The extracts were incubated at 30°C for the indicated times. Samples were withdrawn and total RNAs were extracted and analyzed for GAPD mRNA and 28S rRNA by Northern blotting, and the amount of GAPD mRNA in each sample was normalized to the amount of 28S rRNA.



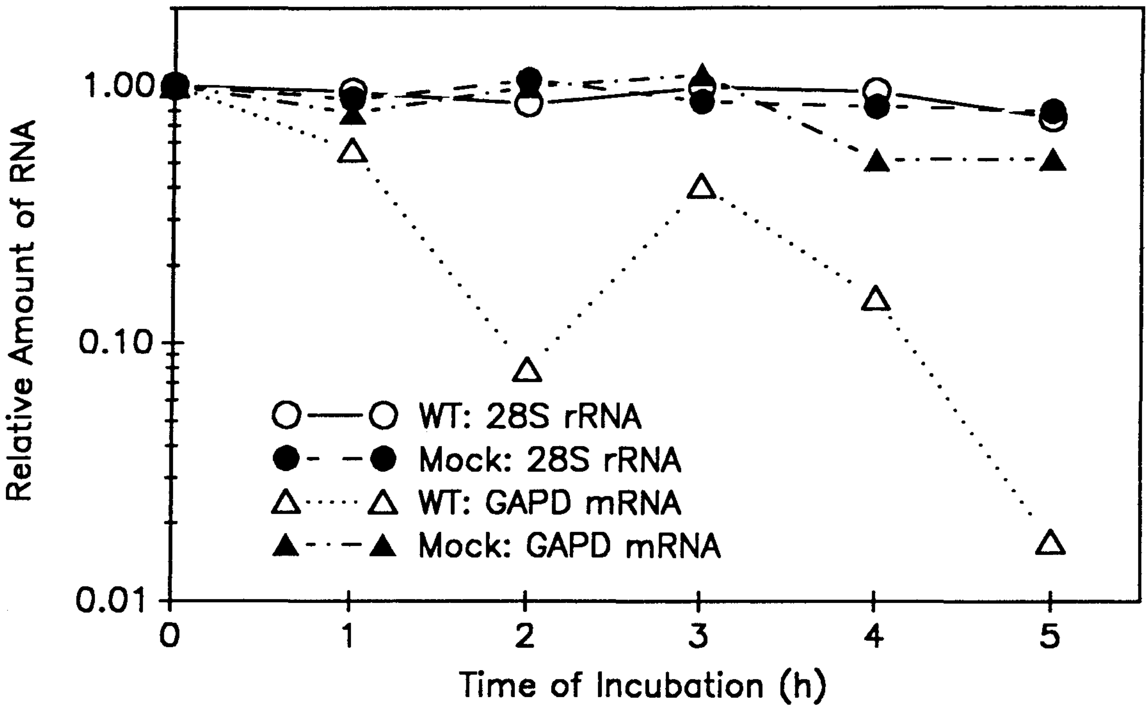
○——○ WT Control
●- -● WT + Heat
△·····△ WT + Proteinase K
Fraction of GAPD mRNA Remaining
1.00
0.50
0.10
0.05
0
1
2
3
4
5
Time of Incubation (h)

vitro degradation. These results are consistent with the involvement of one or more heat-labile proteins in vhs mediated message turnover.

Recently Brewer and Ross have shown that a factor that is present in the post-polysomal supernatant fraction from the cytoplasm of K652 cells, and which specifically accelerates the decay of c-myc and c-myb mRNAs, is inactivated by brief digestion with micrococcal nuclease (25). To determine whether similar micrococcal nuclease pretreatment of extracts from HSV-1 infected cells would inactivate the in vitro vhs activity, standard in vitro degradation extracts were prepared from HeLa cells five h after mock infection or infection with 20 pfu per cell of wild type HSV-1. The extracts were supplemented with micrococcal nuclease and $CaCl_2$, and then preincubated at 30°C for 10 min. EGTA was then added to chelate the Ca^{2+} and thereby inactivate the micrococcal nuclease, and the extracts were chilled briefly on ice. Micrococcal nuclease is routinely used to deplete in vitro translation extracts of endogenous mRNAs and to render translation dependent upon exogenously added message (147). Therefore, in order to provide target RNA for the vhs induced degradation activity after endogenous targets were depleted by micrococcal nuclease pretreatment, the extracts were supplemented with deproteinized total cytoplasmic RNA from an equivalent number of uninfected HeLa cells. The extracts were then incubated at 30°C and analyzed for in vitro decay of exogenous GAPD mRNA as described above. The results of this experiment are shown in Figure 15, indicating that micrococcal nuclease had no effect on the _vhs_ activity inducing mRNA degradation _in vitro_.

Three conclusions can by drawn form the results of this experiment. First, pretreatment of the extract form wild type virus infected cells with micrococcal nuclease did not inhibit the rapid degradation of exogenous GAPD mRNA. That this degradation was due to the _vhs_ activity and was not the results of residual micrococcal nuclease activity is indicated by the fact that GAPD mRNA was relatively stable in the micrococcal nuclease-treated extracts from mock infected cells. Second, the fact that exogenous GAPD mRNA was degraded in the wild type infected cell extracts suggests that the _in vitro_ mRNA degradation system will be useful for studying the decay of both exogenous and endogenous mRNAs. Third, 28S rRNA was stable in extracts from both mock infected and wild type infected cells. Since initially the total amount of 28S rRNA was a 50:50 mixture of endogenous and deproteinized exogenous 28S rRNA, the results indicated that in wild type infected cell extracts deproteinized exogenous GAPD mRNA was degraded much more rapidly than deproteinized exogenous 28S rRNA. This result is an additional indication that the RNase activity seen in wild type infected cell extracts was specific for mRNAs and was not the result of a nonspecific RNase.

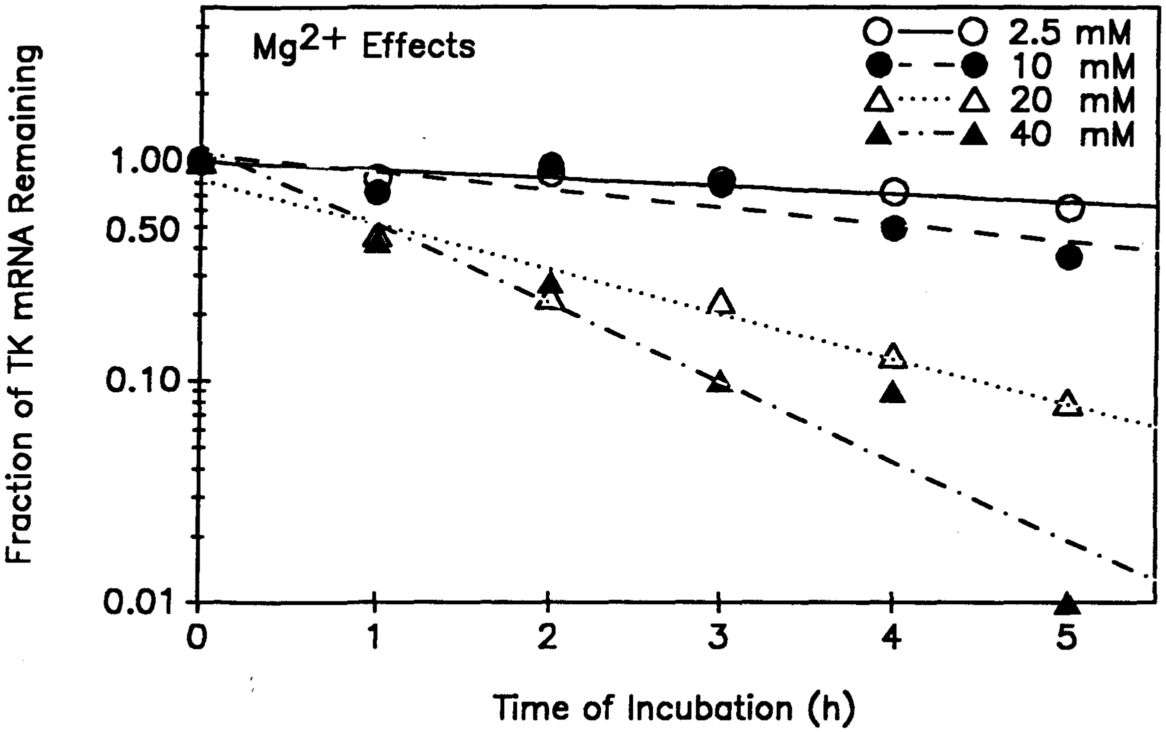
Figure 15. Decay of Exogenous GAPD mRNA and 28S rRNA in Micrococcal Nuclease-treated In Vitro Degradation Extracts. Standard in vitro mRNA degradation extracts were prepared from cells 5 h after infection with 20 pfu of wild type HSV-1 per cell. The extracts were pretreated as described in the Materials and Methods with micrococcal nuclease in the presence of added Ca^{2+}, and deproteinized total cytoplasmic RNA from an equivalent number of cells was added to each extract. The extracts were incubated at 30°C for the indicated times. Samples were withdrawn and total RNAs were extracted and analyzed for GAPD mRNA and 28S rRNA by Northern blotting. The relative amount of 28S rRNA in mock infected and wild type infected cell extracts is plotted as a fraction of the amount present at 0 h. In contrast to other figures in this report, the relative amount of GAPD mRNA detected in mock infected and wild type infected cell extracts is plotted without being normalized to the amount of 28S rRNA present in the sample.


Relative Amount of RNA
1.00
0.10
0.01
0
1
2
3
4
5
Time of Incubation (h)
○—○ WT: 28S rRNA
●- -● Mock: 28S rRNA
△·····△ WT: GAPD mRNA
▲-·-▲ Mock: GAPD mRNA

The next set of experiments was undertaken to examine the Mg^{2+} dependence of the *in vitro* degradation system. Parallel *in vitro* degradation extracts were prepared from HeLa cells five h after infection with 20 pfu per cell of wild type HSV-1. Individual reaction mixtures were supplemented with concentrated $Mg(OAc)_2$ to bring the Mg^{2+} concentration to the desired level. The extracts were incubated for five h and analyzed for the decay of the viral TK mRNA. As shown in Figure 16, *in vitro* degradation of TK mRNA showed a strong dependence upon the concentration of Mg^{2+} ion. While a significant difference could be seen between the rates of degradation in wild type and *vhs* 1 infected cell extracts at a concentration of 5 mM Mg^{2+} (see Figures 12 and 13), increasing the Mg^{2+} concentration to 20 mM or higher significantly increased the degradation rate in wild type extracts (Figure 16). That the effect of raising the Mg^{2+} concentration was not simply the result of inducing nonspecific changes in mRNP structure or the activation of nonspecific nucleases is indicated by the fact that a significant difference in the mRNA decay rates in wild type and *vhs* 1 infected cell extracts was still observed at a Mg^{2+} concentration of 20 mM (see Figure 18). This concentration of 20 mM Mg^{2+} was chosen as optimal for all experiments except that in Figure 11.

A similar experiment was undertaken to determine the effect of varying the K^+ concentration upon the rate of *vhs* mediated decay of TK mRNA (Figure 17). Parallel *in vitro*

Figure 16. Mg^{2+} Dependence of Vhs-induced mRNA Degradation. In vitro mRNA degradation extracts were prepared from cells 5 h after infection with 20 pfu of wild type HSV-1 per cell. The extracts were prepared in standard reaction buffer modified to contain 2.5 mM, 10 mM, 20 mM, or 40 mM $Mg(OAc)_2$. The extracts were incubated at 30°C for the indicated times. Samples were withdrawn and total RNAs were extracted and analyzed for TK mRNA and 28S rRNA by Northern blotting, and the amount of TK mRNA in each sample was normalized to the amount of 28S rRNA.


Mg^{2+} Effects
○—○ 2.5 mM
●- -● 10 mM
△····△ 20 mM
▲-·-▲ 40 mM
Fraction of TK mRNA Remaining
1.00
0.50
0.10
0.01
0
1
2
3
4
5
Time of Incubation (h)

degradation reaction were prepared from HeLa cells five h after infection with 20 pfu per cell of wild type HSV-1. In these reaction, the Mg^{2+} concentration was held at a constant 20 mM, while the K^+ ion concentration was varied from seven to 500 mM. As can be noted in Figure 17, efficient degradation of TK mRNA was observed at K^+ concentrations from seven to 200 mM, while increasing the K^+ concentration to 500 mM severely inhibited the degradation reaction. A K^+ concentration of seven mM was used in all the other experiments reported in this section.

The vhs mediated in vitro mRNA degradation system was next tested for its dependence upon the components of an energy generating system, namely ATP, GTP, creatine phosphate, and creatine phosphokinase. Parallel in vitro degradation extracts were prepared from HeLa cells at five h after infection with 20 pfu per cell of either wild type HSV-1 or the mutant vhs 1. Half of the reactions contained all of the components of the standard reaction, while the energy generating system components listed above were omitted from the other half. As shown in Figure 18, efficient vhs induced degradation of TK mRNA occurred in the presence (Figure 18A) and absence (Figure 18B) of the components of an energy generating system.

The final series of experiments was performed to begin to fractionate the in vitro degradation extract, to determine where the vhs induced degradation function was localized. An

Figure 17. K^+ Dependence of Vhs-induced mRNA Degradation. *In vitro* mRNA degradation extracts were prepared from cells 5 h after infection with 20 pfu of wild type HSV-1 per cell. The extracts were prepared in standard reaction buffer modified to contain 7 mM, 100 mM, 200 mM, or 500 mM KCl. The extracts were incubated at 30°C for the indicated times. Samples were withdrawn and total RNAs were extracted and analyzed for TK mRNA and 28S rRNA by Northern blotting, and the amount of TK mRNA in each sample was normalized to the amount of 28S rRNA.

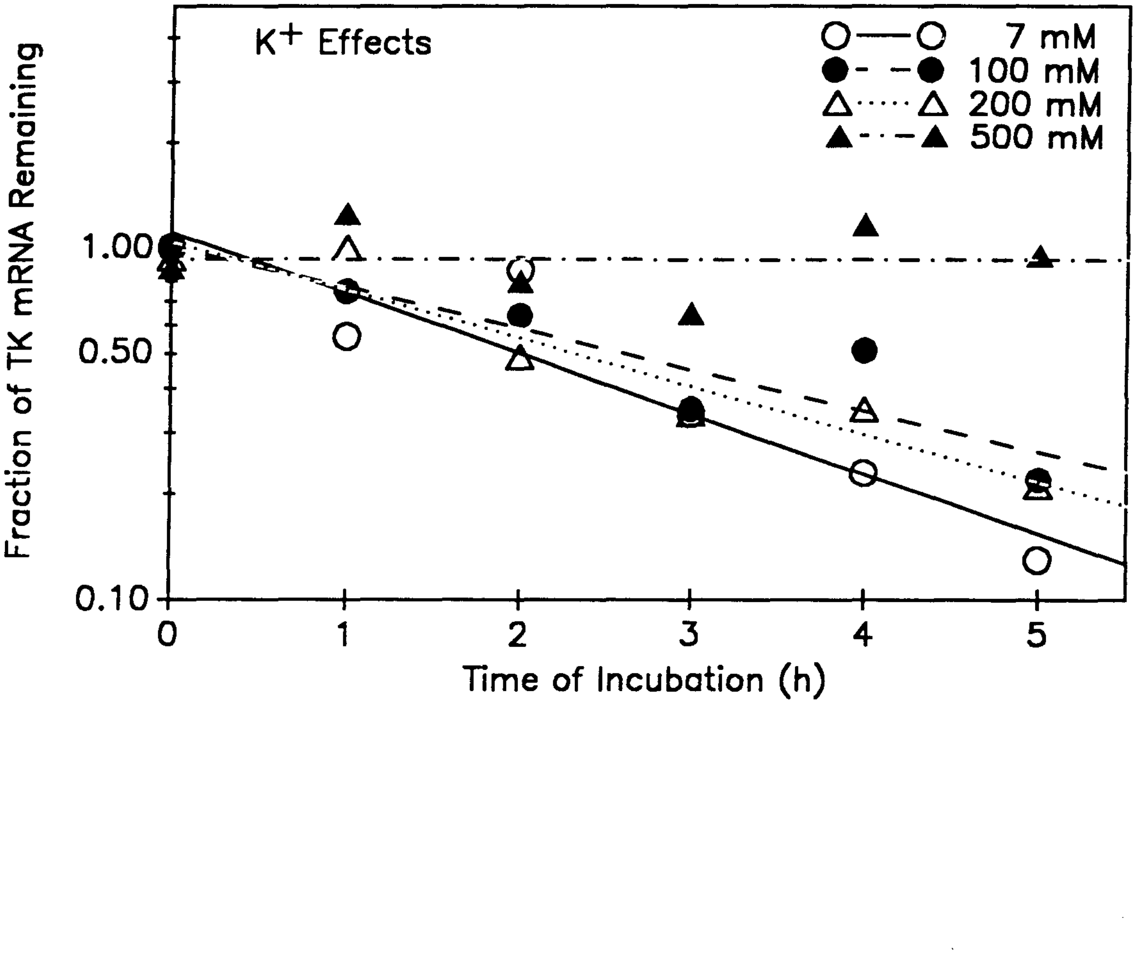
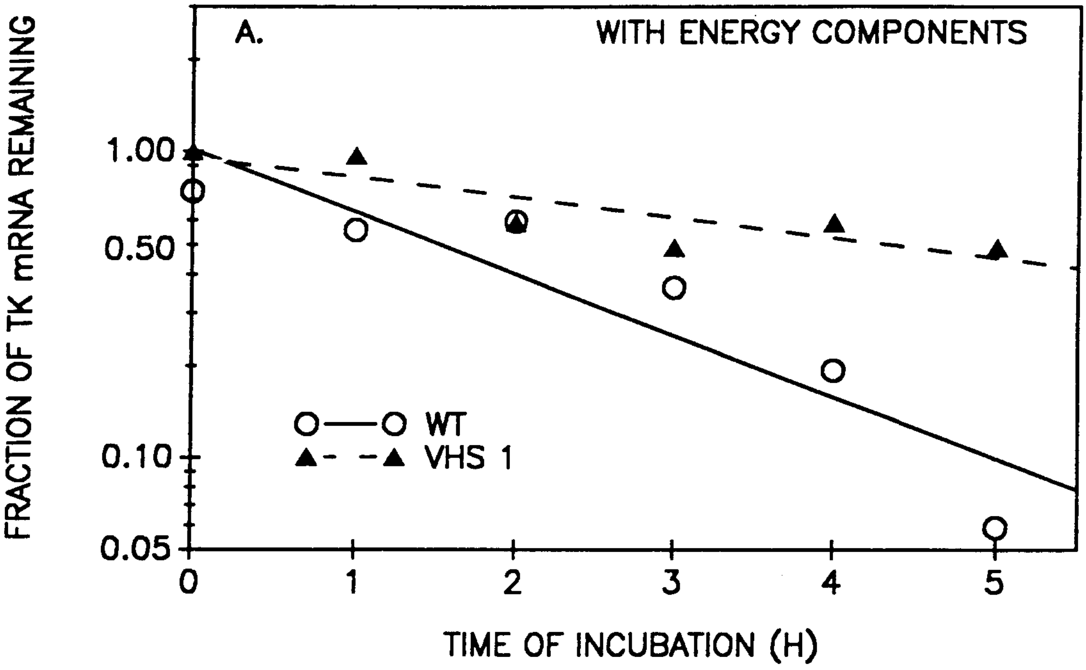
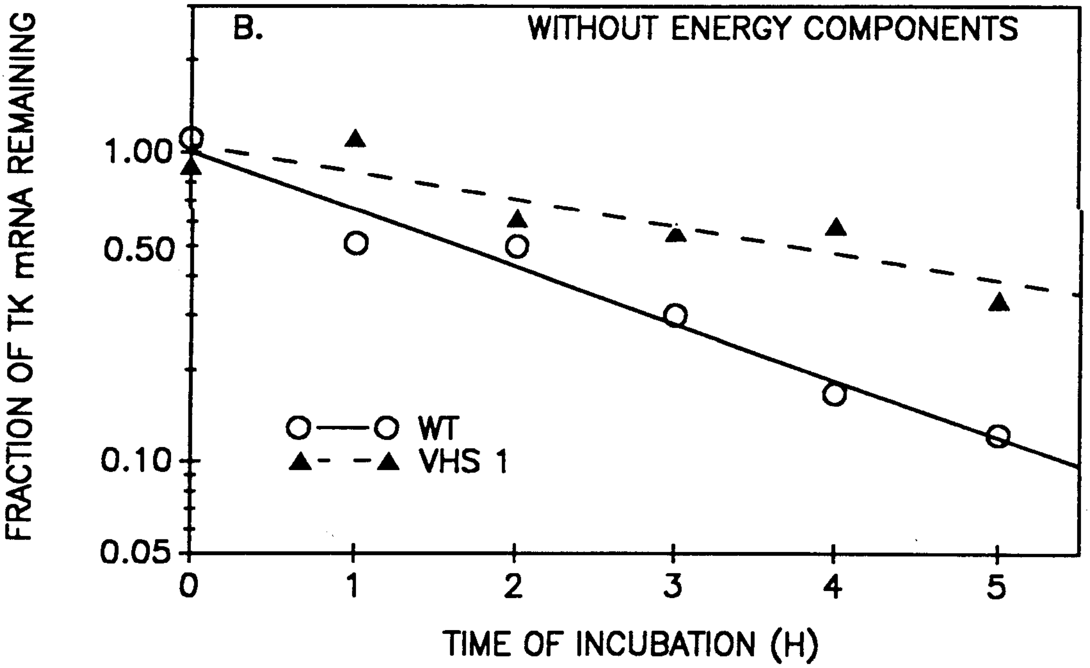

K+ Effects
○—○ 7 mM
●- -● 100 mM
△·····△ 200 mM
▲-·-▲ 500 mM
Fraction of TK mRNA Remaining
1.00
0.50
0.10
0
1
2
3
4
5
Time of Incubation (h)

Figure 18. Dependence of Vhs-induced mRNA Degradation upon the Components of an Energy-generating System. *In vitro* mRNA degradation extracts were prepared from cells 5 h after infection with 20 pfu of wild type HSV-1 or *vhs* 1 per cell. The extracts were prepared either in standard reaction buffer (A) or in standard reaction buffer from which ATP, GTP, creatine phosphate, and creatine phosphokinase had been omitted (B). The extracts were incubated at 30°C for the indicated times. Samples were withdrawn and total RNAs were extracted and analyzed for TK mRNA and 28S rRNA by Northern blotting, and the amount of TK mRNA in each sample was normalized to the amount of 28S rRNA.


A.
WITH ENERGY COMPONENTS
FRACTION OF TK mRNA REMAINING
1.00
0.50
0.10
0.05
0
1
2
3
4
5
TIME OF INCUBATION (H)
○—○ WT
▲- -▲ VHS 1


B.
WITHOUT ENERGY COMPONENTS
FRACTION OF TK mRNA REMAINING
1.00
0.50
0.10
0.05
0
1
2
3
4
5
TIME OF INCUBATION (H)
○—○ WT
▲- -▲ VHS 1

initial experiment was performed to see if crude solubilized virions had any inherent ribonuclease activity. Both wild type and _vhs_ 1 virion extracts were prepared using 0.5% NP-40, and were added to mock infected cell extracts. Figure 19 shows the results of such an experiment. Wild type virion extracts were no more able to degrade endogenous GAPD mRNA than were _vhs_ 1 extracts. Therefore, experiments turned away from fractionation of the virions and turned towards fractionation of infected cell extracts. Extracts were prepared from mock infected cells, or from cells two h after infection with 20 pfu per ml of wild type or _vhs_ 1. The extracts were layered onto small sucrose gradients and centrifuged to separate a polysomal precipitate from a soluble supernatant. Polysome pellets from all three infection types were re-solubilized in the standard reaction buffer and incubated as before. These polysomal fractions were examined for their ability to degrade GAPD mRNA. As shown in Figure 20, no degradation activity could be detected. Therefore, polysome pellets from all three infection types were mixed with soluble supernatants from all three infection types, making nine possible combinations. All combination extracts were then examined for their ability to mediate _in vitro_ degradation of the cellular GAPD mRNA. As can be seen in Figure 21, only those mixed extracts that contained a wild type soluble fraction were able to mediate the degradation of GAPD mRNA. It appears, then, that the wild type supernatant contains a factor that mediates the in vitro degradation of GAPD mRNA, and to a first approximation, all polysome pellets were functionally equivalent.

Figure 19. Extent of mRNA Degradation in Mock Infected Cell Extracts Supplemented with Viral Extracts. Standard in vitro mRNA degradation extracts were prepared from HeLa cells 5 h after mock infection. Crude viral extracts were prepared from both wild type and vhs 1 virus, using 0.5% NP-40, in a procedure described by Preston et al. (158). The viral extracts were then added to the mock infected cell extracts, and the extracts were incubated at 30°C for the indicated times, at which point samples were withdrawn and total RNAs were extracted and analyzed for GAPD mRNA and 28S rRNA by Northern blotting. Mock extract controls were treated with the NP-40 buffer used to prepare the viral extracts. Autoradiograms were quantitated as described in the Materials and Methods, and the fraction of GAPD mRNA is plotted here.

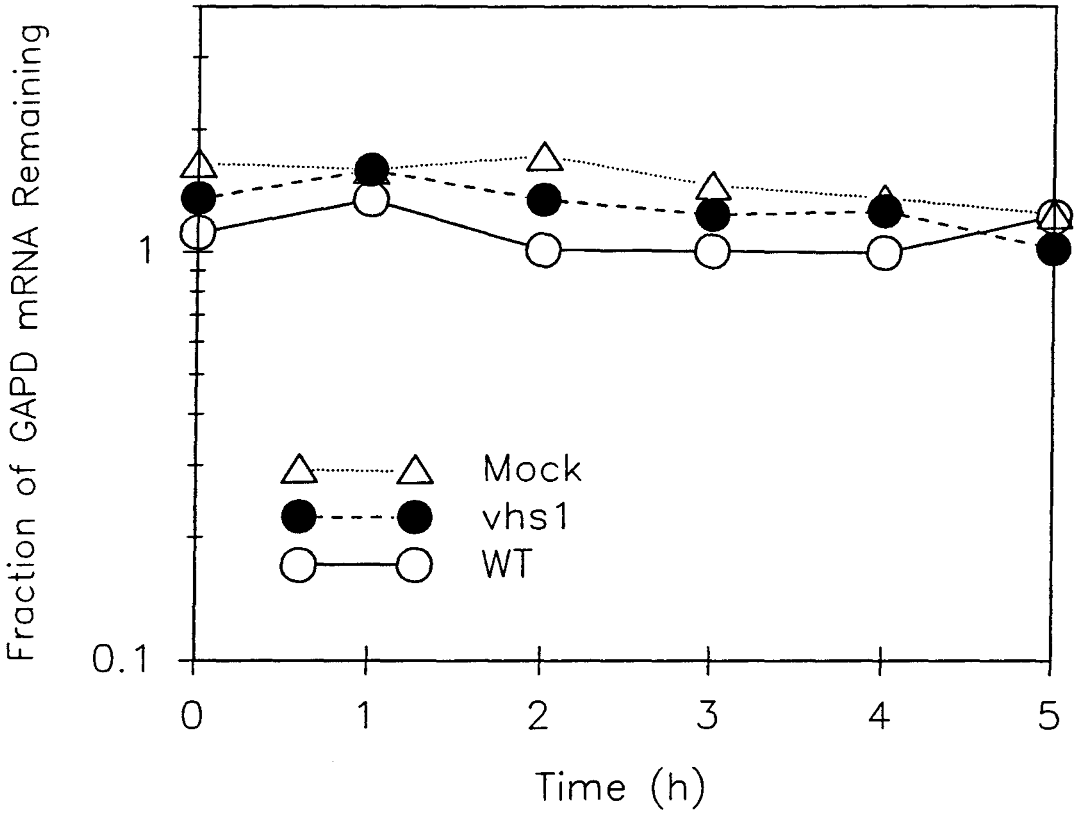

Fraction of GAPD mRNA Remaining
1
0.1
0
1
2
3
4
5
Time (h)
Mock
vhs1
WT

Figure 20. Extent of TK mRNA Degradation in Isolated Polysomes Derived from Infected Cells. Polysomal fractions were prepared from HeLa cells 5 h after infection with wild type or *vhs* 1 virus, as described in the Materials and Methods. The polysomal pellets were resuspended in standard *in vitro* mRNA degradation buffer, and the extracts were incubated at 30°C for the indicated times, at which point samples were withdrawn and total RNAs were extracted and analyzed for TK mRNA and 28S rRNA by Northern blotting. Autoradiograms were quantitated as described in the Materials and Methods, and the fraction of TK mRNA is plotted here.

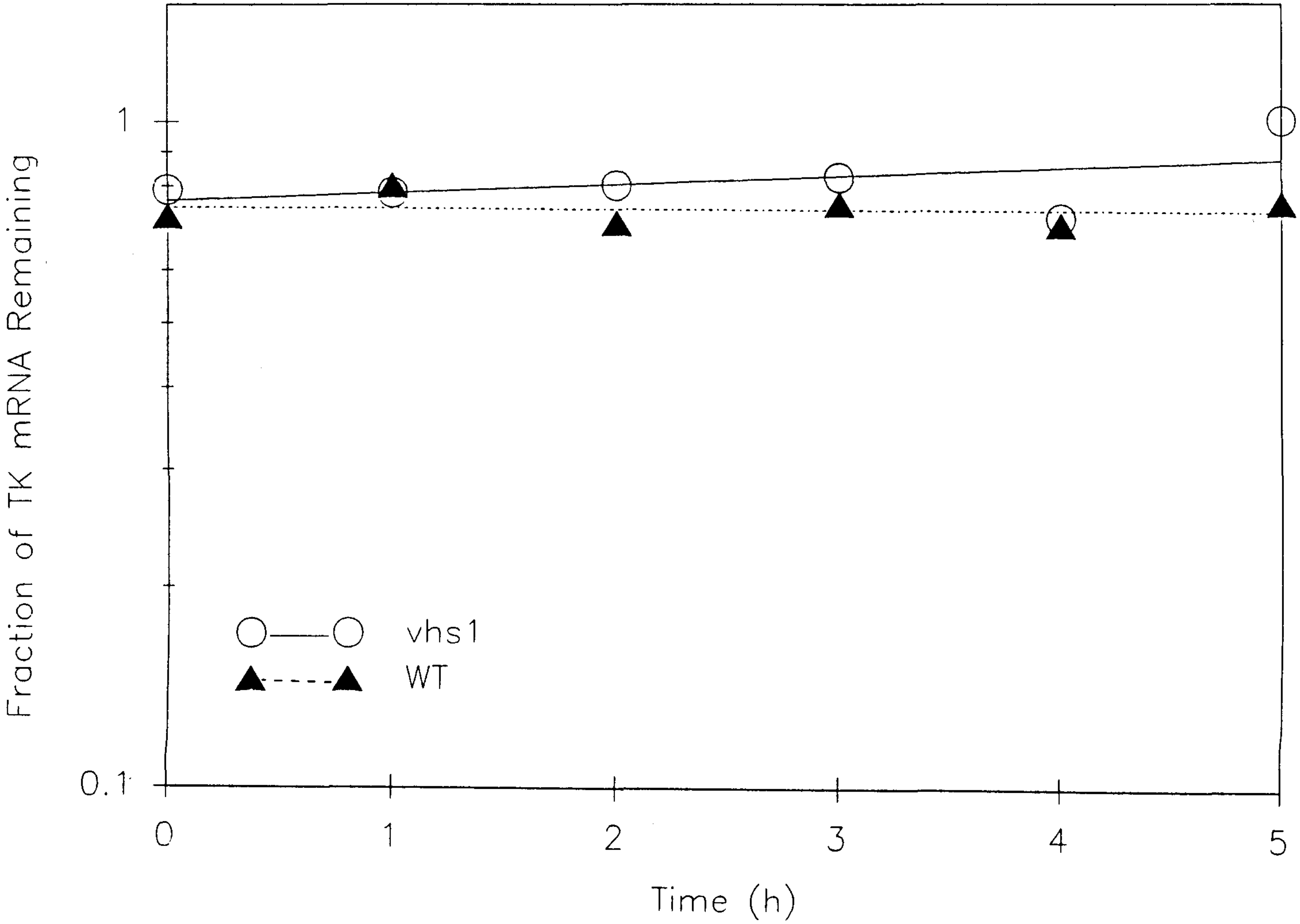
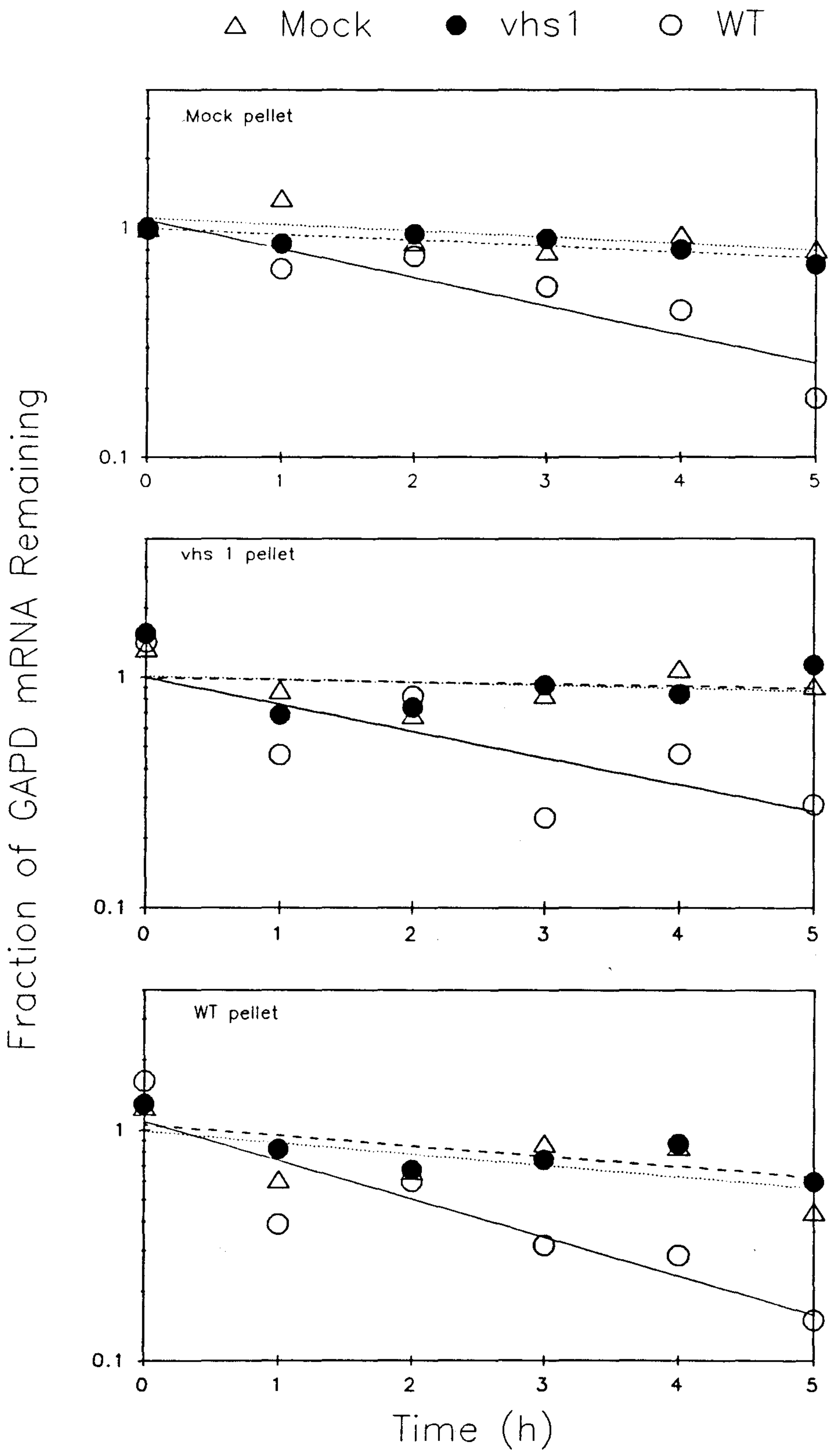

1
0.1
Fraction of TK mRNA Remaining
0
1
2
3
4
5
Time (h)
vhs1
WT

Figure 21. Extent of GAPD mRNA Degradation in Polysome Pellets Supplemented with Soluble Supernatants. Polysome pellets were prepared from HeLa cells 5 h after mock infection, or 5 h after infection with 20 pfu per ml of wild type or vhs 1 virus, as described in the Materials and Methods. Soluble supernatants from each infection type were saved, and supplemented with concentrated stock solutions to obtain the appropriate standard *in vitro* degradation buffer. The soluble supernatants were then added to the polysome pellets in all nine possible combinations, and the mixed extracts were incubated at 30°C for the indicated times, at which point samples were withdrawn and total RNAs were extracted and analyzed for TK mRNA and 28S rRNA by Northern blotting. Autoradiograms were quantitated as described in the Materials and Methods, and the fraction of TK mRNA is plotted here.


△ Mock
● vhs1
○ WT
Mock pellet
vhs 1 pellet
WT pellet
Fraction of GAPD mRNA Remaining
Time (h)
1
0.1
0
1
2
3
4
5

DISCUSSION

The experiments described herein were designed to study the genetic, molecular, and biochemical nature of the virion host shutoff (vhs) function of herpes simplex virus type 1. This function is responsible for the apparently indiscriminate degradation of both cellular and viral mRNAs, by an as yet undefined mechanism. The results described in this report have mapped the vhs function to a large EcoR1 restriction fragment, have shown that the vhs function can alter the structure of messenger ribonucleoprotein particles *in vivo*, and have led to the development of an *in vitro* degradation system to study the vhs function. These experiments have begun to unravel the mechanism of the vhs function, and suggest other experiments that can determine precisely what that mechanism is.

Marker Rescue of the Vhs Gene

The first set of experiments was designed to determine the genetic locus of the vhs function. The vhs mutants were generated by bromodeoxyuridine mutagenesis, which could lead to mutations in several genes, none of which are lethal. The vhs mutants were isolated by their inability to effect the shutoff of host cell protein synthesis. Further analysis of the mutants showed that the synthesis of viral α polypeptides was prolonged relative to wild type virus. Genetic mapping of the vhs function is one way to determine whether the same, or a closely mapping, locus is involved in the shutoff of cellular polypeptide synthesis as well as the shutoff of α polypeptide synthesis. Proof that one gene was responsible for the vhs phenotype would require cloning and sequencing the region identified by the marker rescue technique described here.

This technique depends on the fact that intact viral DNA is infectious and, by transfecting a permissive cell line, will eventually yield progeny virus. The intact viral DNA is derived from a virus carrying the mutation to be studied, and will upon transfection give rise to progeny with a mutant phenotype. By co-transfecting intact mutant virus DNA with cloned fragments derived from wild type viral DNA, a recombination event spanning the mutation in the intact viral genome may occur. This event would "rescue" the mutant marker and restore a wild type phenotype to the resulting progeny virus. This technique is most often used to rescue temperature-sensitive (ts) mutations, since a shift to the non-permissive temperature would inhibit the growth of the mutant progeny and allow only those progeny whose ts marker had been rescued to grow. This makes selecting the recombinants very easy, and allows a much larger number of progeny to be screened.

In the case of the vhs 1 mutant, there was no ts marker to facilitate selection. Therefore, all progeny virus had to be screened for the presence of the wild type host shutoff phenotype. This screen involved assaying small stocks of progeny derived from co-transfections for the ability to shutoff cellular polypeptide synthesis. A hallmark of wild type host shutoff is that it can occur in the absence of any viral gene expression. Therefore, the screen involved growing progeny virus in the presence of dactinomycin and assaying the amount of radioactivity incorporated into TCA-precipitable material as a measure of ongoing host translation. Progeny virus that maintained the vhs 1 phenotype would not be expected to reduce the amount of TCA-precipitable material to the level found in wild type infected cell controls. In contrast, progeny from the co-transfection whose mutant marker was rescued would be expected to reduce the amount of TCA-precipitable material to the same level as wild type controls.

Figure 2 demonstrates that, as expected, the vast majority of progeny derived from the co-transfection retained their mutant phenotype. The host shutoff activity is expressed as percent of mock infected control experiments. The average value of the amount of radioactivity incorporated into TCA-precipitable material from these controls was made 100 percent. The amount of incorporated radioactivity in the progeny virus was then expressed relative to this 100 percent value. A cutoff value of 70 percent of the mock level was chosen as representing mutant vhs activity. Progeny at or below that level were rescreened for their phenotype, to more precisely determine if the phenotype was wild type or mutant.

Table 1 shows that six progeny derived from the co-transfection experiment retained their wild type vhs phenotype upon rescreening, whereas rescreened progeny from the control transfection retained their mutant phenotype, as expected. This is because the only way progeny from the control transfection with intact vhs 1 DNA could have a wild type vhs phenotype is by reversion of the mutation, an event as yet unobserved in laboratory stocks of these virus. Therefore, a background reversion frequency was not observed. The co-transfection experiment was expected to yield some progeny with a wild type vhs phenotype. The co-transfected wild type DNA fragment was some 21 kb in length, presumably providing a sufficiently large region for recombination events to take place. Still, the recombination event had to be such that it spanned the vhs mutation, an occurrence judged to be infrequent. Therefore, the observed recombination frequency of 1.6×10^{-2} was thought to be reasonable.

Thus assured that the recombination event in these six rescreened progeny rescued the defective shutoff of host polypeptide synthesis in vhs 1, the next experiment showed that the recombination event also rescued the defective α shutoff phenotype in that mutant (Figures 3 and 4). In six out of six cases, rescue of the defective host shutoff phenotype led to rescue of the defective α shutoff phenotype. This result suggests that the two phenotypes are controlled either by the same gene, or two tightly linked genes. By using smaller and smaller cloned fragments derived from the EcoR1 fragment A of wild type HSV-1 in co-transfection experiments, it was hoped that further data could be obtained that demonstrated that the two phenotypes were controlled by the same gene.

In the course of those subsequent experiments, it was learned that another group had succeeded in mapping the vhs 1 mutation to a 256 bp fragment derived from the EcoR1 fragment A (108). This group also utilized the marker rescue technique described above, but dispensed with the laborious screening and rescreening of the progeny virus by exploiting the fact that wild type virus quickly outgrows vhs 1 in cell culture (160). Hence, the resulting progeny from a co-transfection were not plaque-purified, but rather were passaged twice in cell culture to enrich for those putative recombinants with wild type vhs activity. As a control, wild type virus, present in an initial mix of 1:100 relative to the mutant vhs 1, was shown to expand sufficiently in two passages to give the resulting mixed virus population a wild type vhs phenotype. Fine mapping studies, done by generating a variety of smaller subclones of the EcoR1 fragment A from wild type, eventually led to the conclusion that a 256 bp fragment could rescue the vhs 1 mutation. The progeny rescued with this small fragment were therefore wild type for both cellular and viral mRNA destabilization, leading the authors to conclude that the same viral function controls these events. Subsequent sequencing data has shown that the 256 bp fragment lies within the open reading frame UL 41, allowing this open reading frame to be identified as the vhs gene (126).

It is evident, then, that one viral gene, the vhs gene, mediates the shutoff of both cellular and viral polypeptide synthesis by regulating the indiscriminate degradation of both cellular and viral mRNA. Sequencing the remaining five vhs mutants can offer significant insight into the nature of the vhs function, especially since three of these mutants are temperature sensitive for host shutoff, and the nature of these mutations may be instructive in locating important regions of the protein. The sequence of the wild type vhs gene has not yet provided much information concerning the mechanism of vhs mediated mRNA degradation, as it does not appear to share any homology to known proteins. Still, the sequence has provided much useful information about restriction enzyme sites to exploit in developing deletion mutants and type 1 x type 2 fusion proteins, which may be very helpful in understanding the vhs mechanism. Likewise, regions of interest in the protein can be targeted for analysis by site-directed mutagenesis. Most intriguing would be the development of a vhs gene under the control of an inducible promoter, leading to experiments to prove that the vhs function is the only viral function required to cause the rapid degradation of cellular message. Transfection experiments with the intact vhs gene under the control of its own promoter indicate that the vhs gene can act to degrade target mRNA encoded by a transfected CAT plasmid (Schmidt and Read, unpublished results).

Studies on mRNPs in HSV-infected Cells

Much additional work on the genetic aspects of the vhs function must still be done. The experiments in this report, however, next focused on understanding the vhs function at the molecular level. As has often been mentioned, the target of the vhs function is mRNA. In eukaryotic cells, mRNA exists complexed with a set of proteins, in a structure called messenger ribonucleoprotein particles (mRNP). One possible role for these proteins is the protection of the mRNA moiety. Since the vhs function apparently attacks this mRNA, in an undefined manner, it is possible that the protective mRNP proteins are altered in some way to make the mRNA more susceptible to that attack.

The process by which the proteins in mRNPs were studied involved the ultraviolet light irradiation of intact cells. This procedure covalently crosslinks intimately associated proteins to the mRNA, thereby greatly reducing artifactual mRNA-protein interactions after cell lysis. Indeed, the cytoplasmic material was heated to 90°C in the presence of SDS to prevent those kinds of nonspecific interactions. Therefore, the proteins eventually resolved on polyacrylamide gels were considered to be genuinely associated with mRNA in vivo. By isolating these proteins from mock, wild type, or vhs 1 infected cells, it was possible to demonstrate what changes, if any, occurred in the protein composition of mRNPs.

Figure 5A demonstrates that the majority of the proteins associated with mRNPs from mock infected cells were also associated with mRNPs from cells infected with the wild type virus. This is not surprising, as most of these proteins are thought to be important in translation. However, mRNPs isolated from wild type infected cells showed a decrease in the amount of a 52 kda major mRNP protein, and an increase in the amount of a minor 49 kda protein. These changes did not occur when cells were infected with the mutant vhs 1 (Figure 6). Likewise, these changes occurred in the absence of viral gene expression (Figure 7). These data taken together implicate the wild type vhs function as somehow responsible for these changes (103).

It is important to reiterate that the changes in the proteins associated with mRNA in cells infected with wild type infected cells can occur in the absence of any viral gene expression (Figure 7). That is, the population of mRNA examined under these conditions derives entirely from cellular genes. The only viral gene products present are those associated with the incoming virion. Yet the structure of these cellular mRNPs matches the structure of mRNPs derived from cells productively infected with wild type virus, when the mRNA population is largely viral in origin. This points out a common mechanism of the vhs protein on both cellular and viral mRNPs, and strengthens the contention that the vhs function is nonspecific.

At issue is whether these changes are caused by the vhs function, or are an effect of the vhs function mediating the degradation of these mRNAs. It could be argued that the altered profile of mRNPs from cells infected with wild type virus was simply a characteristic of mRNPs that were in the process of being degraded. While this possibility cannot be excluded, all of the mRNPs that were isolated were sufficiently intact to have poly(A) tails. In uninfected cells, mRNA turnover involves progressive shortening of the poly(A) tail followed by rapid 3′ to 5′ degradation of the body of the message. If this is also the case for vhs induced message decay, the mRNPs isolated in this report must be either undegraded or at a very early stage of degradation.

A previous report by Bartkoski also demonstrated that the protein profile of mRNPs in cells infected with wild type HSV are altered (10). These experiments, described in the Introduction, showed a decrease in abundance of a 52 kda protein as well as in a 74 kda protein, thought to be the poly(A) binding protein. An increase in a 47 kda protein was also noted. Several caveats about this experiments were noted in the Introduction, but the changes in the protein profile are in general agreement with the work in this report. The decrease in the 52 kda protein was observed in both cases, and an increase in a 47 kda protein in Bartkoski's report (10) presumably correlated with an increase of a 49 kda protein in this report. The major difference is in the presence or absence of the 74 kda poly(A) binding protein. One possibility is to suggest that both observations are correct. In both reports, only poly(A)$^+$ mRNPs were selected. In the case of UV crosslinked mRNPs, perhaps sufficient poly(A) must remain on the 3′ end for interactions with both oligo(dT) and the covalently bound poly(A) binding protein. In Bartkoski's system, proteins were not covalently bound (10). This may allow mRNAs with shorter poly(A) tails to interact with oligo(dT). In other words, the protocol used in this report may select mRNAs with longer poly(A) tails. These mRNPs may not be as far along in the degradation pathway as those in Bartkoski's report, although in both cases the presence of a poly(A) tail was assumed.

Indeed the pool of poly(A)$^+$ mRNA may be quite small in wild type infected cells, as evidenced by the lower amounts of mRNP material recovered from these cells noted in this report (Figure 6A), and by previous reports showing that HSV infection led to a decrease in the ratio of poly(A)$^+$ to poly(A)$^-$ mRNA in the cells (136,205). Presumably, as active degradation takes place fewer mRNA species exist that can bind to oligo(dT) columns. The mRNP material recovered appeared to be intact, and, for the most part, carried the same complement of proteins as mock infected cells. In other words, the recovered material did not represent small degradation products. Therefore, the conclusions in this report concerning the proteins profiles of intact mRNPs appear to be valid.

In Vitro mRNA Decay Mediated by the Vhs Function

In any case, the elucidation of the exact mechanism of the vhs protein is perhaps best accomplished by examining a vhs mediated *in vitro* mRNA degradation system. The final set of experiments involves the development and preliminary biochemical characterization of just such a system. As with any *in vitro* system, the initial concern was that the *in vitro* system approximate events known to occur *in vivo*. In this case, the *in vitro* system was required to reproduce the levels of mRNA stability in mock, wild type, and vhs 1 infected cells.

The *in vitro* system shares several important parallels with *in vivo* observations on the vhs function. First, host messages were degraded rapidly in extracts prepared from cells infected with wild type HSV-1, but not in extracts from mock infected cells or cells infected with the mutant vhs 1.

Second, the accelerated turnover of host mRNAs occurred in extracts from cells infected with wild type virus in the presence of dactinomycin, indicating that the degradation was induced by a component of the infecting virions and was not dependent upon de novo viral gene expression. Third, accelerated turnover of viral mRNAs was observed in extracts from cells productively infected with wild type HSV-1, but not in extracts from vhs 1 infected cells. In each of the above cases, the most important observation supporting the fidelity of the in vitro system was the striking difference between the mRNA decay rates in extracts from cells infected with wild type virus and in extracts from vhs 1 infected cells. This indicates that the accelerated in vitro degradation of mRNAs was dependent upon infection of the cells with virions containing a functional vhs polypeptide, and was not simply the consequence of a nonspecific ribonuclease liberated during cell fractionation or induced as a consequence of viral infection per se. Finally, although the wild type vhs function induced accelerated in vitro turnover of both viral and cellular mRNAs, endogenous 28S ribosomal RNA was equally stable in extracts from mock infected cells and in extracts from cells infected with either wild type virus or vhs 1. This lends further support to the conclusion that the degradative activity seen in wild type infected cell extracts was specific for mRNAs and was not due to a contaminating nonspecific nuclease that should have been detected in all three kinds of extract.

Analysis of the crude *in vitro* system showed that one or more factors necessary for *in vitro* *vhs* activity was inactivated by heating the extracts to 90°C or by brief proteinase K digestion. These data are consistent with the involvement of one or more heat labile proteins in *vhs* induced degradation. In contrast, pretreatment of wild type extracts with micrococcal nuclease did not inhibit the subsequent degradation of added exogenous mRNA, indicating that the factors required for *in vitro* *vhs* activity are apparently insensitive to treatments known to inactivate a number of small RNAs and ribonucleoproteins (97) as well as a factor that accelerates *in vitro* decay of c-*myc* and c-*myb* mRNAs (25). Furthermore, the finding that exogenous mRNAs were rapidly degraded in extracts from cells infected with wild type virus, but were relatively stable in extracts from mock infected cells suggests that the *in vitro* degradation system will be suitable for studying the *vhs* induced decay of exogenous as well as endogenous mRNAs. It is also worth noting that in these experiments the source of the exogenous mRNA was total deproteinized cytoplasmic RNA containing a mixture of mRNA and ribosomal RNA. Thus, the results shown in Figure 15 indicate that added deproteinized mRNA was degraded more rapidly in wild type extracts than added deproteinized ribosomal RNA. This adds further support to the conclusion that the *in vitro* *vhs* activity observed in wild type infected cells was specific for mRNA, and did not simply result from a contaminating nonspecific ribonuclease.

Preliminary biochemical characterization of the in vitro mRNA degradation system from HSV infected cells indicates that it is similar in a number of respects to in vitro mRNA degradation systems from uninfected cells described previously by Ross (reviewed in 173) and others (146, 208). In particular, the vhs induced mRNA degradation activity was not inhibited by the placental ribonuclease inhibitor RNasin, and was dependent upon added divalent cation. Efficient vhs induced degradation occurred at K^+ ion concentrations of up to 200 mM, but was inhibited by 500 mM K^+. Also, mRNA degradative activity was not dependent upon the addition of ATP, GTP, creatine phosphate or creatine phosphokinase. In each of these respects, the vhs induced ribonuclease activity was similar to the exonuclease shown by Ross and coworkers to induce degradation of histone mRNAs in extracts from K562 erythroleukemia cells (175).

Although both systems required added divalent cation, the in vitro vhs activity reported here and the exonuclease described by Ross (175) apparently differ somewhat in the nature of their dependence upon added Mg^{2+}. While the exonuclease that degrades histone mRNA exhibited a broad optimum ranging from 5 to 20 mM Mg^{2+}, the vhs mediated activity in the extracts described here was more strongly dependent upon added Mg^{2+}. Thus, while a striking difference was observed between mRNA decay rates in extracts from wild type virus infected and vhs 1 infected cells at Mg^{2+} concentrations ranging from 2.5 to 20 mM (Figures 9-13, and 16), the rate of mRNA degradation in wild type extracts increased continuously as the Mg^{2+} concentration was raised to 20 or 40 mM. At present, the reason for this difference in the Mg^{2+} dependence of the two in vitro systems is unclear. It is possible that one or more of the proteins involved in vhs induced degradation may have an increased Mg^{2+} dependence. Or, higher Mg^{2+} concentrations may favor a conformational change in mRNP structure that renders the mRNA more susceptible to vhs induced degradation.

Besides providing the groundwork for future experiments, the preliminary biochemical characterization of the vhs induced ribonuclease activity allows it to be distinguished from several previously characterized RNases that are commonly found in cell extracts. Pancreatic ribonuclease is resistant to boiling, but is inhibited by RNasin (146). The fact that the vhs mediated activity is sensitive to heating to 90°C but is not inhibited by RNasin therefore indicates that it does not involve a pancreatic-type ribonuclease. A nucleolar exonuclease activity has been described that, like the vhs induced ribonuclease activity, is dependent upon added Mg^{2+} (111). However, unlike the vhs induced enzyme, the nucleolar ribonuclease is inactive at K^+ ion concentrations above 90 mM. A lysosomal acid ribonuclease has been described (182). However, unlike the vhs induced enzyme, it does not require added Mg^{2+}.

In several previously characterized in vitro degradation systems, the ribonucleases responsible for mRNA turnover were found to be polysome associated. This was the case for the nucleases responsible for the in vitro degradation of histone and c-myc mRNAs, although in both cases the decay rate was greatly accelerated by soluble factors present in a post-polysomal supernatant fraction of the cytoplasm (25,152). In addition, Brawerman and coworkers have recently reported a ribonuclease that is associated with polysomes as well as free messenger ribonucleoprotein particles in a variety of mammalian cells (9).

The existence of a polysome associated ribonuclease was examined in the vhs mediated in vitro degradation system. The in vitro extracts from HSV infected HeLa cells were separated by centrifugation into a polysome pellet and a post-polysomal supernatant. Polysomal mRNAs from cells infected with wild type virus or vhs 1 were both found to be as stable as polysomal mRNA from mock infected cells when those polysomes were resuspended and incubated in the standard reaction buffer (Figure 20). However, readdition of the post-polysomal supernatant from wild type infected cells extracts to polysomes from any source restored the rapid degradation of the mRNAs (Figure 21). These data indicate that one or more factors required for vhs induced mRNA degradation is found in the post-polysomal supernatant fraction of wild type infected cell extracts. This suggests either that the post-polysomal supernatant alone is sufficient for in vitro mRNA degradation, or that this supernatant contains a trans-acting factor that modulates a polysome associated nuclease. Experiments by other workers using in vitro degradation systems derived from uninfected cells suggests that the degradation activity resides in the polysomal fraction, but that it is modulated by unknown components in the supernatant fraction.

Dominance, Secondary Shutoff, and the Vhs Function

One issue as yet unaddressed is that of the dominance of the vhs 1 mutation. It was previously noted that in mixed infections where the ratio of vhs 1 to wild type virus was on the order of 100:1 (108), or even 1:1 (G. S. Read, unpublished data), the dominant phenotype was that of vhs 1, that is, there was no detectable host shutoff. These observations led to the proposal that the active vhs function is composed of a multimer of two or more vhs proteins. Coupling one mutant copy of the vhs protein from vhs 1 to a wild type copy abrogates vhs activity. This issue could be easily examined in the in vitro system, initially by mixing various amounts of vhs 1 and wild type infected cell extracts, and ultimately by mixing amounts of purified vhs protein from the two sources derived by use of antibody against the vhs protein (see below), and examining the extent of in vitro degradation of a given message. The antibody would also be useful in glycerol gradients or gel exclusion chromatography followed by Western blotting of the fractions, to see if the native state of the protein is as a multimer of the approximately 58 kda monomer vhs protein.

A second unexamined issue is that of secondary shutoff, which remains a poorly understood phenomenon. Work done in the late 1970s suggested that a second stage of host shutoff existed, and that this secondary shutoff required viral protein synthesis (56,82,139,196). Secondary shutoff was revisited by Frenkel and Read in 1983 (160). Experiments with the vhs mutants showed that, although these viruses were defective in virion associated host shutoff, they were capable of secondary shutoff if infection was allowed to proceed past the expression of α polypeptides. The conclusion was that these events were separable and controlled by separate viral functions.

A simple hypothesis for secondary shutoff is that the host protein synthetic apparatus is overwhelmed by viral mRNA, and that this overabundance of viral mRNA does not exist until somewhat later in infection. Exclusion of cellular mRNA from polysomes by this large quantity of viral mRNA would lead to shutoff of host polypeptide synthesis. This secondary shutoff is accompanied by degradation of host mRNA in both wild type and vhs 1 infected cells (206). This may suggest that there is a viral function(s) that controls this event, as the authors hypothesize, or that an indiscriminate cellular RNase is activated at late times after infection. Degradation of host mRNAs at late times after infection with _vhs_ 1 could easily be examined both _in vivo_ and _in vitro_. An _in vitro_ secondary shutoff system could be analyzed in the same way as the initial _vhs_ mediated mRNA degradation system examined in this report to determine the degree of similarity between these two shutoff events. Putative mutants in viral functions that control secondary shutoff could also be examined in such an _in vitro_ system.

It is difficult, however, to postulate the existence of a second viral function controlling mRNA degradation at late times in _vhs_ 1 infected cells. None of the experiments described here demonstrated any accelerated mRNA degradation in _vhs_ 1 infected cells at any time during infection. If secondary host shutoff is a virally controlled phenomenon, acceptable hypotheses about the nature of that shutoff must account for discrimination between host and viral mRNAs. Cells infected with _vhs_ 1 contain a jumble of mRNAs, both cellular and viral, all competing for a limited translational capacity. The volume of messenger RNA late in infection in cells infected with _vhs_ 1 must be overwhelming, as all of the positive control elements of HSV-1 function unchecked by the _vhs_ protein, normally acting to actively degrade both cellular and viral messages. This may be what makes the simple hypothesis first proposed above the most plausible hypothesis, namely that secondary shutoff is the result of uncontrolled viral transcription leading to the cessation of cellular translation. Since productive infection by HSV invariably leads to cell death, perhaps the degradation of mRNAs during secondary shutoff is a consequence of cell destruction, mediated by one or more indiscriminate cellular RNase activities, rather than by the elaboration of a new viral degradation function late in infection.

Vhs Mediated Degradation: Hypotheses and Experiments

Genetic and sequencing data prove that host and α shutoff are mediated by the same gene, the vhs gene. A variety of supplementary data indicate that the gene product, the vhs protein, is responsible for the overall destabilization of cellular mRNA and viral mRNA of all kinetic classes. This destabilization is accomplished by the active degradation of both cellular and viral mRNAs, detected both in vivo and in vitro. The alteration in the mRNP structure in cells infected with wild type HSV-1, notably the loss of a 52 kda protein, may be a consequence of this active degradation, a hypothesis which can be tested by experiments proposed below.

One way to begin to examine this issue is by determining the protein profile of mRNPs isolated from the in vitro degradation system described in the Results. It has previously been shown that the mRNPs derived from in vitro translation extracts carry the same protein complement as mRNP complexes from intact cells. Therefore, by exposing the vhs mediated in vitro degradation system to UV light, thereby crosslinking proteins to mRNA, it would be very easy to compare in vivo and in vitro protein profiles. Cell-free extracts derived from wild type infected cells would be expected to contain mRNPs with decreased levels of the 52 kda protein as observed in vivo, while vhs 1 infected cell extracts would yield mRNP protein profiles identical to those from mock infected cell extracts, and identical to comparable mRNP complexes extracted in vivo.

To demonstrate that this alteration is an effect of vhs mediated mRNA degradation would also involve the examination of the in vitro degradation system. In this case, the proteins associated with a specific mRNA would be examined. The choice of the message is important, with the emphasis being on a message with a short half-life. Therefore, a message like the one encoding c-myc would be appropriate. If the amount of 52 kda protein were reduced in this short-lived message, that might suggest that part of the degradation pathway involves removal of that protein from the body of the mRNA. To test this hypothesis, the c-myc mRNA would be transcribed in vitro, and would be uniformally labeled with [^{32}P]ribonucleotides. This labeled mRNA would then be added to an uninfected cell-free mRNA degradation extract, and at appropriate times after incubation, aliquots would be removed for UV crosslinking. The proteins would be covalently linked to one or more labeled nucleotides, allowing the proteins to be visualized after polyacrylamide gel electrophoresis. An appropriate control for such an experiment would be to repeat the analysis using globin mRNA, a message with a long half-life.

A more direct analysis of the role of the 52 kda protein would require the development of an antibody against the protein. A cell-free mRNA degradation system derived from uninfected cells could be depleted of the 52 kda protein using that antibody. If removal of the 52 kda protein from mRNPs leads to a generalized increase in message instability _in vitro_, this may explain why the removal of this protein by the wild type _vhs_ function causes the same to occur _in vivo_. Mechanistically, the _vhs_ function may affect the affinity of the 52 kda protein for the mRNA, thereby exposing the mRNA to endoribonuclease attack. Or, the _vhs_ function may activate a normal cellular mRNA degradation pathway, part of which involves a progressive 3′ to 5′ degradation of the mRNA, starting with the poly(A) tail.

This 3′ to 5′ degradation may then lead to loss of the 52 kda protein. By synthesizing transcripts with varying poly(A) tail lengths _in vitro_, and adding them to the _in vitro_ mRNA degradation system, the influence of the length of the poly(A) tail on the binding of the 52 kda and other mRNP proteins can be examined by the UV crosslinking protocol. An unresolved question is whether the poly(A) tail itself is affected by the presence of the wild type vhs function.

This issue could be approached by examining the average poly(A) tail length of specific mRNAs derived from cells infected with wild type and vhs 1. The viral thymidine kinase transcript would be an ideal choice for such an analysis, as it exhibits a marked difference in half-life in cells infected with wild type or mutant virus (144). Examination of the average poly(A) tail length of a specific message involves the use of a short single strand DNA probe that hybridizes to a known internal position of the transcript (400 to 500 nucleotides 5′ of the poly(A) site; 127). The hybrid is treated with RNase H, which recognizes and cleaves DNA-RNA hybrids. The cleavage products are resolved by electrophoresis in agarose gels, and the fragments are then transferred to a membrane for Northern blotting with a 3′-specific probe. Upon autoradiography, the range of poly(A) tails will appear as a smear. The change over time of the size of this smear gives an indication of the extent of poly(A) tail loss. Presumably, there would be little change in the poly(A) tails of TK mRNA isolated from vhs 1 infected cells, whereas TK mRNA from wild type infected cells would be expected to show a rapid loss of the poly(A) tails. This same analysis could be performed on mRNAs derived from the *in vitro* degradation system described here.

The loss of the poly(A) tail in wild type infected cells, and wild type infected cell extracts, may support the contention that mRNA degradation in cells infected with wild type HSV occurs from a 3′ to 5′ direction, as has been reported in other systems (24). By using probes specific for the 3′ and 5′ ends, the directionality of degradation of a specific message like the viral thymidine kinase or the cellular GAPD could be studied. The extent of degradation at each end can be determined by using these probes in S1 nuclease mapping studies. By comparing the rate of disappearance of signal at the 3′ end with the rate of signal disappearance at the 5′ end, the directionality of the degradation could be determined. If the signal detected by the 3′ probe disappears with time, while the signal detected by the 5′ probe remains relatively constant, a 3′ to 5′ directionality is presumed. Indeed, the presence of a small 5′ fragment that remains undegraded has been reported in two separate in vitro mRNA degradation systems (25,208). These same experiments can be performed both in the in vitro system and in vivo, where unconflicting results would be expected.

Notwithstanding the results on the directionality of the degradation, studies could also be performed on the in vitro degradation of in vitro synthesized RNA targets. Vhs mediated degradation of mRNAs may require specific targets. These targets can be prepared by in vitro transcription of cloned cDNAs, either of cellular or viral origin. And, because these transcripts can be uniformally labeled with

[^{32}P]ribonucleotides in vitro, the need for Northern blots is eliminated. The degradation products, if any, can be visualized by direct autoradiography of the RNA glyoxal gels. Such experiments could be performed on RNA targets with and without poly(A) tails, to see if the rate of in vitro degradation were influenced by this structure. Likewise, the in vitro synthesized transcripts can be treated with an in vitro capping extract to add a 5′ cap, again to ascertain if such a structure influenced the rate of in vitro degradation. For the sake of completeness, chimeric transcripts, consisting of noncoding regions derived from viral mRNAs and coding sequences from cellular genes, and vice versa, could be generated and tested in vitro to reaffirm the nonspecificity of vhs mediated mRNA degradation.

The in vitro data presented in this report, taken together, suggest that some cellular mRNA turnover system, perhaps polysome associated, is activated or modified by the vhs protein, present free in the cytoplasm. The cellular location of the vhs protein could be determined using a biochemical purification scheme based upon following the degradation activities of various fractions, but this might be complicated by the requirement of factors from several cellular compartments for full activity. The best way to examine the vhs function alone would be to raise antibodies to purified virions, or to a fusion protein derived from the cloned vhs gene. Indeed, antibodies to a vhs fusion protein have been developed and are currently being analyzed. These antibodies could be used in several ways in the in vitro degradation system. For instance, the antibody could be added directly to a wild type infected cell extract to precipitate the vhs protein. Conceivably, if the vhs function acts by activating a normal cellular mRNA turnover pathway, the vhs function may not be needed continuously. Removal of the vhs protein with antibody at various times after incubation would show whether message degradation continued unimpaired.

Such an antibody could also be used to prepare a purified source of vhs protein, either from infected cells or from solubilized virions. The purified vhs protein could be used to determine if the protein has inherent ribonuclease activity, or if it requires factors present in cell-free extracts for its activity. Obviously, one attractive hypothesis concerning the need for both soluble and polysome-associated factors in the in vitro degradation system is that the soluble factor is the vhs protein. This can easily be tested with purified vhs protein, by preparing polysome pellets from mock infected cells and adding the purified vhs protein to the resolubilized polysomes. The data presented in this report suggests that the source of the polysome pellet is irrelevant; it is the existence of a soluble factor in wild type infected cells that is important in activating the degradation activity.

Indeed, the entire polysome pellet may not be required. If polysome associated factors are required, it may be possible to wash off these factors by addition of high salt buffers to the polysome pellets. The post-polysomal supernatant from wild type infected cell extracts would be added to these salt washed polysomes to determine if the degradation activity remains. If the degradation activity is abrogated, readdition of the polysome salt wash fraction may restore activity, demonstrating that both a polysomal and post-polysomal function are required for _vhs_ mediated mRNA degradation. In experiments with the purified _vhs_ protein, the same type of polysome salt washing procedures can be undertaken in an effort to determine what polysomal factors are required.

In theory, then, a minimal _in_ _vitro_ degradation system could be established consisting of the purified _vhs_ protein, polysome salt washes (if required), and messenger ribonucleoproteins. This minimal system would further simplify the identification of ancillary factors in the degradation of mRNA, and may lead to a better understanding of the pathway of that degradation. Most importantly, this system will allow an unambiguous determination of the way in which targeted mRNA is degraded.

This issue points to the central unanswered question concerning the _vhs_ function, which is whether the _vhs_ protein is itself a ribonuclease or activates a cellular nuclease, perhaps one involved in the normal turnover of mRNAs in uninfected cells. Attempts to demonstrate a ribonuclease activity in preparations of disrupted wild type virions were unsuccessful (Figure 19). The reason for this may simply be that methods of virion disruption that preserve the ribonuclease activity have yet to be found. Alternatively, one or more cellular macromolecules may be required for vhs activity. The similarity of the biochemical characteristics of the vhs induced activity to those of in vitro degradation systems from uninfected cells (175) is consistent with this second possibility. Fractionation of the vhs mediated degradation system as described above may resolve this issue.

These proposed experiments will hopefully begin to answer some of the central questions about the vhs function. The vhs function is one of the few trans-acting factor that affects the stability of messenger RNA identified to date. As such, it provides a very useful tool in the examination of the role that mRNA stability plays in both cells and viruses. Whether it functions directly or indirectly, the vhs function is the first major avenue available for the fine dissection of this important issue.

NOTES

1. Adam, S. A., Y. D. Choi, and G. Dreyfuss. 1986. Interaction of mRNA with proteins in vesicular stomatitis virus-infected cells. J. Virol. 57:614-622.

2. Adam, S. A., and G. Dreyfuss. 1987. Adenovirus proteins associated with mRNA and hnRNA in infected HeLa cells. J. Virol. 61:3276-3283.

3. Adam, S. A., T. Nakagawa, M. S. Swanson, T. K. Woodruff, and G. Dreyfuss. 1986. mRNA polyadenylate-binding protein: gene isolation and sequencing and identification of a ribonucleoprotein consensus sequence. Mol. Cell. Biol. 6:2932-2945.

4. Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson. 1983. Molecular biology of the cell. Garland Publishing, Inc. New York.

5. Aviv, K., and P. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA 69:1408-1412.

6. Baer, B. W., and R. D. Kornberg. 1980. Repeating structure of cytoplasmic poly(A)-ribonucleoprotein. Proc. Natl. Acad. Sci. USA 77:1890-1892.

7. Baer, B. W., and R. D. Kornberg. 1983. The protein responsible for the repeating structure of cytoplasmic poly(A)-ribonucleoprotein. J. Cell Biol. 96:717-721.

8. Baltimore, D., and A. S. Huang. 1970. Interaction of HeLa cell proteins with mRNA. J. Mol. Biol. 47:263-273.

9. Bandyopadhyay, R., M. Coutts, A. Krowczynska, and G. Brawerman. 1990. Nuclease activity associated with mammalian mRNA in its native state: possible basis for selectivity in mRNA decay. Mol. Cell. Biol. 10:2060-2069.

10. Bartkoski, M. J. 1982. Polysome-associated proteins in herpes simplex virus-infected cells. J. Virol. 43:357-360.

11. 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.

12. 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.

13. Becker, Y., H. Dym, and I. Sarov. 1968. Herpes simplex virus DNA. Virology *36*:184-192.

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

15. Ben-Porat, T., and S. Tokazewski. 1977. Replication of herpesvirus DNA. II. Sedimentation characteristics of newly synthesized DNA. Virology *79*:292-301.

16. Bergman, I., and G. Brawerman. 1977. Control of breakdown of the polyadenylate sequence in mammalian polyribosomes: role of poly(adenylic acid)-protein interactions. Biochemistry *16*:259-264.

17. Bernstein, P., S. W. Peltz, and J. Ross. 1989. The poly(A)-poly(A)-binding protein complex is a major determinant of mRNA stability *in vitro*. Mol. Cell. Biol. *9*:659-670.

18. Bernstein, P. and J. Ross. 1989. Poly(A), poly(A) binding protein and the regulation of mRNA stability. Trends Biochem. Sci. *14*:373-377.

19. Beswick, T. S. L. The origin and the use of DAO herpes. 1962. Med. Hist. *6*:214-232.

20. Blobel, G. 1972. Protein tightly bound to globin mRNA. Biochem. Biophys. Res. Comm. *47*:88-95.

21. Blobel, G. 1973. A protein of molecular weight 78,000 bound to the polyadenylate region of eukaryotic messenger RNAs. Proc. Natl. Acad. Sci. USA *70*:924-928.

22. Brawerman, G. 1981. The role of the poly(A) sequence in mammalian messenger RNA. CRC Crit. Rev. Biochem. *8*:1-38.

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

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

25. Brewer, G., and J. Ross. 1989. Regulation of c-myc mRNA stability in vitro by a labile destabilizer with an essential nucleic acid component. Mol. Cell. Biol. 9:1996-2006.

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

27. 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. 23:14309-14314.

28. Buckmaster, E. A., U. Gompels, and A. Minson. 1984. Characterisation and physical mapping of an HSV-1 glycoprotein of approximately 115 x 10^3 molecular weight. Virology 139:408-413.

29. Butcher, P. D., and R. V. Arnstein. 1985. Efficient translation and polyribosome binding of ^{125}I labeled rabbit globin messenger ribonucleoprotein. FEBS Lett. 153:119-124.

30. 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. 62:2596-2604.

31. 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 of immediate early transcription. J. Mol. Biol. 180:1-19.

32. Caron, J. M., A. L. Jones, and M. W. Kirschner. 1985. Autoregulation of tubulin synthesis in hepatocytes and fibroblasts. J. Cell Biol. 101:1763-1772.

33. Caron, J., A. L. Jones, L. B. Rall, and M. W. Kirschner. 1985. Autoregulation of tubulin synthesis in enucleated cells. Nature 317:648-650.

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

35. Choi, Y. D., P. J. Grabowski, P. A. Sharp, and G. Dreyfuss. 1986. Heterogeneous nuclear ribonucleoproteins: role in RNA splicing. Science 231:1534-1539.

36. Clements, J. B., R. J. Watson, and N. M Wilkie. 1977. Temporal regulation of herpes simplex virus type 1 transcription: location of transcripts on the viral genome. Cell 12:275-285.

37. Cleveland, D. W. 1988. Autoregulated instability of tubulin mRNAs: a novel eukaryotic regulatory mechanism. Trends Biochem. Sci. 13:339-343.

38. Cleveland, D. W., M. A. Lopata, P. Sherline, and M. W. Kirschner. 1981. Unpolymerized tubulin modulates the level of tubulin mRNAs. Cell 25:537-546.

39. Cleveland, D. W., M. F. Pittenger, and J. R. Feramisco. 1983. Elevation of tubulin levels by microinjection suppresses new tubulin synthesis. Nature 305:738-740.

40. 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. J. Virol. 37:191-206.

41. Costa, R. H., B. G. 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.

42. 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.

43. Deb, S., and M. Doelberg. 1988. A 67-base-pair segment from the ori-S region of herpes simplex virus type 1 encodes origin function. J. Virol. 62:2516-2519.

44. Deiss, L. P., J. Chou, and N. Frenkel. 1986. Functional domains within the *a* sequence involved in the cleavage and packaging of herpes simplex virus DNA. J. Virol. 59:605-618.

45. Delius, H., and J. B. Clements. 1976. A partial denaturation map of herpes simplex virus type 1 DNA: evidence for inversions of the unique DNA regions. J. Gen. Virol. 33:125-133.

46. DeLuca, N. A., M. A. Courtney, and P. A. Schaffer. 1984. Temperature-sensitive mutants in herpes simplex virus

type 1 ICP4 permissive for early gene expression. J. Virol. 52:767-777.

47. DeLuca, N. A., and P. A. Schaffer. 1988. Physical and functional domains of the herpes simplex virus transcriptional regulatory protein ICP4. J. Virol. 62:732-743.

48. Dreyfuss, G. 1986. Structure and function of nuclear and cytoplasmic ribonucleoprotein particles. Annu. Rev. Cell. Biol. 2:459-498.

49. Epstein, M. A. 1962. Observation on the mode of release of herpes virus from infected HeLa cells. J. Cell Biol. 12:589-597.

50. Etchison, D., S. Milburn, I. Edery, N. Sonenberg, and J. W. B. Hershey. 1982. Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with proteolysis of a 220,000 dalton polypeptide associated with eIF3 and a cap binding protein complex. J. Biol. Chem. 257:14806-14810.

51. Everett, R. D. 1986. The products of herpes simplex virus type 1 (HSV-1) immediate early genes 1, 2, and 3 can activate HSV-1 gene expression in trans. J. Gen. Virol. 67:2507-2513.

52. Everett, R. D. 1987. A detailed mutational analysis of Vmw 110, a trans-acting transcriptional activator encoded by herpes simplex virus type 1. EMBO J. 6:2069-2076.

53. 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.

54. Fenwick, M., L. S. Morse, and B. Roizman. 1979. Anatomy of herpes simplex virus DNA. XI. Apparent clustering of functions effecting rapid inhibition of host DNA and protein synthesis. J. Virol. 29:825-827.

55. 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.

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

57. Fernandez-Munoz, R., and J. Darnell. 1976. Structural differences between the 5′-termini of viral and cellular mRNA

in poliovirus infected cells: possible basis for the inhibition of host protein synthesis. J. Virol. 18:719-726.

58. Fields, B. N., and D. M. Knipe (eds.). 1990. Fields Virology, 2nd. Ed. Raven Press. New York.

59. Frenkel, N., H. Locker, W. Batterson, G. Hayward, and B. Roizman. 1976. Anatomy of herpes simplex DNA. VI. Defective DNA originated from the S component. J. Virol. 20:527-531.

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

61. Gaedgigk, R., S. Oehler, K. Kohler, and B. Setyono. 1985. In vitro reconstitution of messenger ribonucleoprotein particles from globin messenger RNA and cytosol proteins. FEBS Lett. 179:201-207.

62. Gander, E. S., A. S. Stewart, C. M. Morel, and K. Scherrer. 1973. Isolation and characterization of ribosome-free cytoplasmic messengerribonucleoprotein complexes from avian erythroblasts. Eur. J. Biochem. 38:443-452.

63. 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.

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

65. Gerster, T., and R. G. Roeder. 1988. A herpesvirus trans-activating protein interacts with transcription factor OTF-1 and other cellular proteins. Proc. Natl. Acad. Sci. USA 85:6247-6251.

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

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

68. 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. USA 83:256-260.

69. 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.

70. Greenberg, J. R. 1977. Isolation of messenger ribonucleoproteins in cesium sulfate density gradients: evidence that polyadenylated and nonpolyadenylated messenger RNAs are associated with protein. J. Mol. Biol. 108:403-416.

71. Greenberg, J. R. 1979. Ultraviolet light-induced crosslinking of mRNA to proteins. Nucleic Acids Res. 6:715-732.

72. Greenberg, J. R., and V. E. Burn. 1988. Proteins associated with rabbit reticulocyte mRNA caps during translation investigated by photocrosslinking. Nucleic Acids Res. 16:3437-3454.

73. Greenberg, J. R., and E. Carroll. 1985. Reconstitution of functional mRNA-protein complexes in a rabbit reticulocyte cell-free translation system. Mol. Cell. Biol. 5:342-351.

74. Grubman, M. J., and D. A. Shafritz. 1977. Identification and characterization of messenger ribonucleoprotein complexes from vesicular stomatitis virus-infected HeLa cells. Virology 81:1-16.

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

76. 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 segments. Proc. Natl. Acad. Sci. USA 72:4243-4247.

77. 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.

78. Henshaw, E. C. 1968. Messenger RNA in rat liver exists as ribonucleoprotein particles. J. Mol. Biol. 56:401-411.

79. Henshaw, E. C., and J. Loebenstein. 1970. Rapidly labeled, polydisperse RNA in rat liver cytoplasm: evidence that it is contained in ribonucleoprotein particles of heterogeneous size. Biochim. Biophys. Acta 199:405-420.

80. Holland, L. E., K. P. Anderson, C. Shipman, and E. K. Wagner. 1980. Viral DNA synthesis is required for the efficient expression of specific herpes virus type 1 mRNA species. Virology 101:10-24.

81. Honess, R. W., and B. Roizman. 1973. Proteins specified by herpes simplex virus. XI. Identification and relative molar rates of synthesis of structural and non-structural herpesvirus polypeptides in infected cells. J. Virol. 12:1346-1365.

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

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

84. Huez, G., G. Marbaix, D. Gallwitz, E. Weinberg, R. Devos, E. Hubert, and Y. Cleuter. 1978. Functional stabilization of HeLa cell histone messenger RNAs injected into Xenopus oocytes by 3′-OH polyadenylation. Nature 271:572-573.

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

86. Irwin, D., A. Kumar, and R. A. Malt. 1975. Messenger ribonucleoprotein complexes isolated with oligo(dT)-cellulose chromatography from kidney polysomes. Cell 4:157-165.

87. Jackson, R. J., and N. Standart. 1990. Do the poly(A) tail and 3′ untranslated region control mRNA translation? Cell 62:15-24.

88. Jacob, R. J., L. S. Morse, and B. Roizman. 1979. Anatomy of herpes simplex virus DNA. XIII. Accumulation of head to tail concatamers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. J. Virol. 29:448-457.

89. Jacob, R. J., and B. Roizman. 1977. Anatomy of herpes simplex virus DNA. VIII. Properties of the replicating DNA. J. Virol. 23:394411.

90. Jacobsen, A., and M. Favreau. 1983. Possible involvement of poly(A) in protein synthesis. Nucleic Acids Res. 11:6353-6368.

91. Jain, S. K., M. G. Pluskal, and S. Sarkar. 1979. Thermal chromatography of eukaryotic messenger ribonucleoprotein particles on oligo(dT)-cellulose. FEBS Lett. 97: 84-90.

92. Jenkins, F. J., and B. Roizman. 1986. Herpes simplex virus recombinants with non-inverting genomes frozen in different isomeric arrangements are capable of independent replication. J. Virol. 59:494-499.

93. Johnson, D. C., and M. W. Ligas. 1988. Herpes simplex viruses lacking glycoprotein D are unable to inhibit viral penetration: quantitative evidence for virus-specific cell surface receptors. J. Virol. 43:4605-4612.

94. Johnson, D. C., and P. G. Spear. 1983. Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. J. Virol. 51:389-394.

95. Joklik, W. K., and Y. Becker. 1965. Studies on the genesis of polyribosomes. II. The association of nascent messenger RNA with the 40S subribosomal particle. J. Mol. Biol. 13:511-520.

96. Jones, T. R., and M. D. Cole. 1987. Rapid cytoplasmic turnover of c-myc mRNA: requirement of the 3′ untranslated sequences. Mol. Cell. Biol. 7:4513-4521.

97. Kass, S., K. Tyc, J. A. Steitz, and B. Sollner-Webb. 1990. The U3 small nucleolar ribonucleoprotein functions in the first step of preribosomal RNA processing. Cell 60: 897-908.

98. Katze, M. G., D. DeCorato, B. Safer, J. Galabru, and A. G. Hovanessian. 1987. Adenovirus VAI RNA complexes with the 68,000 Mr protein kinase to regulate its autophosphorylation and activity. EMBO J. 6: 689-697.

99. Kelly, J. M., and R. A. Cox. 1982. Periodicity in the length of 3′-poly(A) tails from native globin mRNA of rabbits. Nucleic Acids Res. 10:4173-4179.

100. 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. 8:125-129.

101. King, T. C., R. Sirdeskmukh, and D. Schlessinger. 1986. Nucleolytic processing of ribonucleic acid transcripts in prokaryotic cells. Microbiol. Rev. 50:428-451.

102. Kozak, M. 1986. Regulation of protein synthesis in virus-infected animal cells. Adv. Virus Res. 1:229-292.

103. Krikorian, C. R., and G. S. Read. 1989. Proteins associated with mRNA in cells infected with herpes simplex virus. Biochem. Biophys. Res. Comm. 164:355-361.

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

105. Kristie, T. M., and B. Roizman. 1984. Separation of sequences defining basal expression from those conferring gene recognition within the regulatory domains of herpes simplex virus 1 genes. Proc. Natl. Acad. Sci. 81:4065-4069.

106. Kwan, S.-W., and G. Brawerman. 1972. A particle associated with the polyadenylate segment in mammalian messenger RNA. Proc. Natl. Acad. Sci. USA 69:3247-3250.

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

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

109. Landin, B. F., M. L. Blankenship, and T. Ben-Porat. 1980. Replication of herpesvirus DNA. V. The maturation of concatameric DNA of pseudorabies virus to genome length is related to capsid formation. J. Virol. 33:1151-1164.

110. Langeland, N., L. J. Moore, H. Holmesen, and L. Haarr. 1987. Interaction of polylysine with the cellular receptor for herpes simplex virus type 1. J. Gen. Virol. 69:1137-1145.

111. Lasater, L. S., and D. C. Eichler. 1984. Isolation and properties of a single-strand 5′ to 3′ exoribonuclease from Ehrlich ascites tumor cell nucleoli. Biochemistry 23:4367-4373.

112. Lee, W. M., M. Schwab, D. Wesyway, and H. E. Varmus. 1985. Augmented expression of normal c-*myc* is sufficient for cotransfection of rat embryo cells with a mutant *ras* gene. Mol. Cell. Biol. 5:3345-3356.

113. Levine, B. J., N Chodchoy, W. F. Marzluff, and I. Skoultchi. 1987. Coupling of replication type histone mRNA levels to DNA synthesis requires the stem-loop sequence at

the 3′ end of the mRNA. Proc. Natl. Acad. Sci. USA 84:6189-6193.

114. Leder, A., P. K. Pattengale, A. Kuo, T. A. Stewart, and P. Leder. 1986. Consequences of widespread deregulation of the c-*myc* gene in transgenic mice: multiple neoplasms and normal development. Cell 45:485-495.

115. Lindberg, U., and B. Sundquist. 1974. Isolation of messenger ribonucleoproteins from mammalian cells. J. Mol. Biol. 86:451-468.

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

117. Locker, H., N. Frenkel, and I. Halliburton. 1982. Structure and expression of class II defective herpes simplex virus genomes encoding infected cell polypeptide 8. J. Virol. 43:574-593.

118. Lockshon, D., and D. A. Galloway. 1986. Cloning and characterization of oriL2, a large palindromic DNA replication origin of herpes simplex virus type 2. J. Virol. 62:513-521.

119. Lodish, H. F., and M. Porter. 1980. Translational control of protein synthesis after infection by vesicular stomatitis virus. J. Virol. 36:719-733.

120. Maniatis, T. M., E. F. Fritsch, and J. Sambrook (eds.). 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York.

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

122. Martin, T. E., J. M. Pullman, and M. D. McMullen. 1980. Structure and function of nuclear and cytoplasmic ribonucleoprotein complexes. *In* D. M. Prescott and L. Goldstein (eds.), Cell biology: a comprehensive treatise. Academic Press, New York.

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

124. 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.

125. McConkey, E. H., and J. W. Hopkins. 1965. Subribosomal particles and the transport of messenger RNA in HeLa cells. J. Mol. Biol. 14:257-270.

126. 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.

127. Mercer, J. F. B., and S. A. Wake. 1985. An analysis of the rate of metallothionein poly(A)-shortening using RNA blot hybridization. Nucleic Acids Res. 13:7929-7943.

128. Mocarski, E. S., and B. Roizman. 1981. Site specific inversion sequence of herpes simplex virus genome: domain and structural features. Proc. Natl. Acad. Sci. USA 78:7047-7051.

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

130. Mocarski, E. S., and B. Roizman. 1982. Herpesvirus-dependent amplification and inversion of a cell-associated viral thymidine kinase gene flanked by viral a sequence and linked to an origin of viral DNA replication. Proc. Natl. Acad. Sci. USA 79:5626-5630.

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

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

133. Mullner, E. W., B. Neupert, and L. D. Kuhn. 1989. A specific mRNA binding factor regulates the iron-dependent stability of cytoplasmic transferrin receptor mRNA. Cell 58:373-382.

134. Munroe, D., and A. Jacobson. 1990. mRNA poly(A) tail, a 3′ enhancer of translational initiation. Mol. Cell. Biol. 10:3441-3455.

135. Nahmias, A. J., and W. R. Dowdle. 1968. Antigenic and biologic differences in herpesvirus hominis. Prog. Med. Virol. 10:110-159.

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

137. Nevins, J. R. 1983. The pathway of eukaryotic mRNA formation. Annu. Rev. Biochem. 52:441-466.

138. Nii, S., C. Morgan, H. M. Rose, and K. C. Hsu. 1968. Electron microscopy of herpes simplex virus. IV. Studies with ferritin conjugated antibodies. J. Virol. 2:1172-1184.

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

140. Nudel, U., H. Soreq, U. Z. Littauer, G. Marbaix, G. Huez, M. Leclercq, E. Hubert, and H. Chantrenne. 1976. Globin mRNA species containing poly(A) segments of different lengths. Their functional stability in Xenopus oocytes. Eur. J. Biochem. 64:115-121.

141. 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.

142. O'Hare, P., and G. S. Hayward. 1984. Evidence for a direct role for both the 175,000 and 110,000 molecular weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. 53:751-760.

143. 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.

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

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

146. Pei, R., and K. Calame. 1988. Differential stability of c-myc mRNAs in a cell free system. Mol. Cell. Biol. 8:2860-2868.

147. Pelham, H. R. B., and R. J. Jackson. 1976. An efficient mRNAdependent translation system from reticulocyte lysates. Eur. J. Biochem. 67:247-256.

148. Pellegrini, S., and C. Basilico. 1986. Rat fibroblasts expressing high levels of human c-myc transcripts are anchorage-independent and tumorigenic. J. Cell Physiol. 126:107-114.

149. Pelletier, J., and N. Sonenberg. 1988. Internal initiation of translation on eukaryotic mRNA directed from poliovirus mRNA. Nature 334:320-325.

150. Pelletier, J., and N. Sonenberg. 1989. Internal binding of eukaryotic ribosomes on poliovirus mRNA: translation in HeLa cell ex-tracts. J. Virol. 63:441-444.

151. 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.

152. Peltz, S. W., and J. Ross. 1987. Autogenous regulation of histone mRNA decay by histone proteins in a cell-free system. Mol. Cell. Biol. 7:4345-4356.

153. Pereira, L., M. Wolff, M. Fenwick, and B. Roizman. 1977. Regulation of herpesvirus synthesis. V. Properties of polypeptides specified by HSV-1 and HSV-2. Virology 77:733-749.

154. Philipson, L., U. Pettersson, U. Lindberg, C. Tibbetts, B. Vennstron, and T. Persson. 1975. RNA synthesis and processing in adenovirus infected cells. Cold Spring Harbor Symp. Quant. Biol. 39:1203-1207.

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

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

157. Poffenberger, K. L., and B. Roizman. 1985. Studies on non-inverting genome of a viable herpes simplex virus 1.

Presence of head-to-tail linkages in packaged genomes and requirements for circularization after infection. J. Virol. 53:589-595.

158. 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.

159. Rabbitts, P. H., A. Forster, M. A. Stinson, and T. H. Rabbits. 1985. Truncation of exon 1 from the c-myc gene results in prolonged c-myc mRNA stability. EMBO J. 4:3727-3733.

160. 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.

161. 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.

162. Rice, A. P., and B. E. Roberts. 1983. Vaccinia virus induces cellular mRNA degradation. J. Virol. 47:529-539.

163. Rice, S. A., L. Su, and D. M. Knipe. 1989. Herpes simplex virus alpha protein ICP27 possesses separable positive and negative regulatory activities. J. Virol. 83: 3899-3907.

164. Roizman, B. 1969. The herpesviruses - a biochemical definition of the group. Curr. Top. Microbiol. Immunol. 49:1-79.

165. Roizman, B. 1979. The organization of the herpes simplex virus genomes. Annu. Rev. Genet. 13:25-57.

166. Roizman, B., L. Aurelian, and P. R. Roane, Jr. 1963. The multiplication of herpes simplex virus. I. The programming of viral DNA duplication in HEp-2 cells. Virology 21:482-498.

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

168. Roizman, B., and D. Furlong. 1974. The replication of herpesviruses. In H. Fraenkel-Conrat, and R. R. Wagner, (eds.), Comprehensive Virology. Plenum Press, New York.

169. Roizman, B, and P. R. Roane, Jr. 1964. Multiplication of herpes simplex virus. II. The relation between protein synthesis and the duplication of viral DNA in infected HEp-2 cells. Virology 22:262-269.

170. Rose, K. M., S. T. Jacob, and A. Kumar. 1979. Poly(A) polymerase and poly(A)-specific mRNA binding protein are antigenically related. Nature 279:260-262.

171. Rosen, C. A., H. L. Ennis, and P. S. Cohen. 1982. Translational control of vesicular stomatitis virus protein synthesis: isolation of an mRNA-sequestering particle. J. Virol. 44:932-938.

172. Rosen, C. A., J. Siekierka, H. L. Ennis, and P. S. Cohen. 1984. Inhibition of protein synthesis in vesicular stomatitis virus infected Chinese hamster ovary cells: role of virus mRNA-ribonucleoprotein particle. Biochemistry 23:2407-2411.

173. Ross, J. 1988. Messenger RNA turnover in eukaryotic cells. Mol. Biol. Med. 5:1-14.

174. 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.

175. Ross, J., G. Kobs, G. Brewer, and S. W. Peltz. 1986. Properties of the exonuclease activity that degrades H4 histone mRNA. J. Biol. Chem. 19:9374-9381.

176. 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.

177. Rouault, T. A., M. W. Hentze, D. J. Haile, J. B. Harford, and R. D. Klausner. 1989. The iron-responsive element binding protein: a method for the affinity purification of a regulatory RNA-binding protein. Proc. Natl. Acad. Sci. USA 86:5678-5772.

178. Ryner, L. C., Y. Takagaki, and J. L. Manley. 1990. Multiple forms of poly(A) polymerases purified from HeLa cells function in specific mRNA 3′-end formation. Mol. Cell. Biol. 9:4229-4238.

179. Sachs, A. B., M. W. Bond, and R. D. Kornberg. 1986. A single gene from yeast for both nuclear and cytoplasmic polyadenylate-binding protein: domain structure and expression. Cell 45:827-835.

180. Sachs, A. B., R. W. Davis, and R. D. Kornberg. 1987. A single domain of yeast poly(A)-binding protein is necessary and sufficient for RNA binding and cell viability. Mol. Cell. Biol. 7:3268-3276.

181. 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.

182. Saha, B. K., M. Y. Graham, and D. Schlessinger. 1979. Acid ribonuclease from HeLa cell lysosomes. J. Biol. Chem. 254:5951-5957.

183. Saito, H., A. C. Hatday, K. Wiman, W. S. Hayward, and S. Tonegawa. 1983. Activation of the c-myc gene by translocation: a model for translational control. Proc. Natl. Acad. Sci. USA 80:7476-7480.

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

185. Schmid, H.-P., K. Kohler, and B. Setyono. 1982. Possible involvement of messenger RNA-associated proteins in protein synthesis. J. Cell Biol. 93:893-898.

186. Schmid, H.-P., M. Schonfelder, B. Setyono, and K. Kohler. 1982. 76-kDA poly(A)-protein is involved in the formation of 48S initiation complexes. FEBS Lett. 157:105-110.

187. Schneider, R. J., B. Safer, S. M. Munemitsi, C. E. Samuel, and T. Shenk. 1985. Adenovirus VAI RNA prevents phosphorylation of the eukaryotic initiation factor 2 subunit subsequent to infection. Proc. Natl. Acad. Sci. USA 82:1959-1963.

188. Schuler, G. D., and M. D. Cole. 1988. GM-CSF and oncogene mRNA stabilities are independently regulated in trans in a mouse monocytic tumor. Cell 55:1115-1122.

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

190. Schwartz, H., and J. E. Darnell. 1976. The association of protein with the polyadenylic acid tail of HeLa cell messenger RNA: evidence for a "transport" role of a 75,000 molecular weight polypeptide. J. Mol. Biol. 104:833-851.

191. Sears, A. E., I. W. Halliburton, B. Meignier, S. Silver, and B. Roizman. 1985. Herpes simplex virus mutant deleted in the α22 gene: growth and gene expression in permissive and restrictive cells, and establishment of latency in mice. J. Virol. 55:338-346.

192. Setyono, B., and J. R. Greenberg. 1981. Proteins associated with poly(A) and other regions of mRNA and hnRNA molecules as investigated by crosslinking. Cell 24:775-783.

193. 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.

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

195. Siekierka, J., T. M. Mariano, P. A. Riechel, and M. B. Matthews. 1985. Translational control by adenovirus: lack of virus-associated RNAI during adenovirus results in phosphorylation of initiation factor eIF-2 and inhibition of protein synthesis. Proc. Natl. Acad. Sci. USA 82:1959-1963.

196. Silverstein, S., and E. L. Englehardt. 1979. Alterations in the protein synthetic apparatus of cells infected with herpes simplex virus. Virology 95:324-342.

197. Smith, K. C. 1969. Photochemical addition of amino acids to ^{14}C-uracil. Biochem. Biophys. Res. Comm. 34:354357.

198. Spaete, R. R., and N. Frenkel. 1982. The herpes simplex virus amplicon: a new eukaryotic defective-virus cloning-amplifying vector. Cell 30:295-304.

199. Spaete, R. R., and N. Frenkel. 1985. The herpes simplex virus amplicon: analysis of cis-acting replication functions. Proc. Natl. Acad. Sci. USA 82:694-698.

200. Spirin, A. S. 1969. Informosomes. Eur. J. Biochem. 10:20-35.

201. Spirin, A. S., and M. Nemer. 1965. Messenger RNA in early seaurchin embryos: cytoplasmic particles. Science 150:214-217.

202. Stow, N. D., and E. C. McMonagle. 1983. Characterization of the TRs/IRs origin of DNA replication of herpes simplex virus type 1. Virology 130:427-438.

203. Stow, N. D., and E. C Stow. 1985. Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw 110. J. Gen. Virol. 67:2571-2585.

204. Stow, N. D., J. H. Subak-Sharpe, and N. M. Wilkie. 1978. Physical mapping of herpes simplex virus type 1 mutations by marker rescue. J. Virol. 28:182-192.

205. 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.

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

207. Sundquist, B., T. Persson, and U. Lindberg. 1977. Characterization of mRNA-protein complexes from mammalian cells. Nucleic Acids Res. 4:899-915.

208. Sunitha, I., and L. I. Slobin. 1987. An in vitro system derived from Friend erythroleukemia cells to study messenger RNA stability. Biochem. Biophys. Res. Comm. 144:560-568.

209. Sydiskis, R. J., and B. Roizman. 1966. The disaggregation of host polyribosomes in productive and abortive infection with herpes simplex virus. Virology 32:678-686.

210. Tasseron-de Jong, J. G., J. Brouwer, K. Rietveld, C. E. M. Zoetmelk, and L. Bosch. 1979. Messenger ribonucleoprotein complexes in human KB cells infected with adenovirus type 5 contain tightly bound viral-coded '100K' protein. Eur. J. Biochem. 100:271-283.

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

212. Triezenberg, S. J., K. L. LaMarco, and S. L. McKnight. 1988. Evidence of DNA:protein interactions that mediate HSV-1 immediate early gene expression. Genes Dev. 2:730-742.

213. Tso, J. Y., X.-H. Sun, T.-H. Kao, K. S. Knight, and R. Wu. 1985. Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. Nucleic Acids Res. 13:2485-2502.

214. Van der Marel, P., J. G. Tasseron-de Jong, and L. Bosch. 1975. The proteins associated with mRNA from uninfected and adenovirus type 5-infected KB cells. FEBS Lett. 51:330-334.

215. Van Venrooij, W. J., T. Riemen, and C. A. G. van Eekelen. 1982. Host proteins are associated with adenovirus specific mRNA in the cytoplasm. FEBS Lett. 145:62-66.

216. Van Venrooij, W. J., C. A. G. van Eekelen, R. T. P. Jansen, and J. M. G. Princen. 1977. Specific poly-A-binding protein of 76,000 molecular weight in polyribosomes is not present on poly A of free cytoplasmic mRNP. Nature 70:189-191.

217. Vincent, A., S. Goldenberg, and K. Scherrer. 1981. Comparisons of proteins associated with duck-globin mRNA and its polyadenylated segment in polyribosomal and repressed free messenger ribonucleoprotein complexes. Eur. J. Biochem. 114:179-193.

218. Vlazny, D. A., and N. Frenkel. 1981. Replication of herpes simplex virus DNA: location of replication recognition signals within defective virus genomes. Proc. Natl. Acad. Sci. USA 78:742-746.

219. 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.

220. Wagenmakers, A. J. M., R. J. Reinders, and W. J. van Venrooij. 1980. Cross-linking of mRNA to proteins by irradiation of intact cells with ultraviolet light. Eur. J. Biochem. 114:323-330.

221. Wagner, E. K., and B. Roizman. 1969. RNA synthesis in cells infected with herpes simplex virus. I. The patterns of RNA synthesis in productively infected cells. J. Virol. 4:36-46.

222. 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.

223. 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.

224. Weller, S. K., A. Spadoro, J. E. Schaffer, A. W. Murray, A. M. Maxam, and P. A. Schaffer. 1985. Cloning, sequencing, and functional analysis of oriL, a herpes simplex

virus type 1 origin of DNA synthesis. Mol. Cell. Biol. 5:930-942.

225. Wildy, P. 1973. Herpes: history and classification. In A. S. Kaplan, (ed.), The herpesviruses. Academic Press, New York.

226. Wilson, R., and R. Treisman. 1988. Removal of poly(A) and consequent degradation of c-fos mRNA facilitated by 3′ AU-rich sequences. Nature 366:396-399.

227. Wudunn, D., and P. G. Spear. 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. 63:52-58.

228. Yen, T. J., D. A. Gay, J. S. Patcher, and D. W. Cleveland. 1988. Autoregulated changes in stability of polyribosome-bound beta-tubulin mRNA are specified by the first 13 translated nucleotides. Mol. Cell. Biol. 8:1224-1235.

229. 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.

230. Zarkower, D., P. Stephenson, M. Sheets, and M. Wickens. 1986. The AAUAAA sequence is required both for cleavage and for polyadenylation of simian virus 40 pre-mRNA in vitro. Mol. Cell. Biol. 6:2317-2323.

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