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A herpesvirus genetic element which affects translation in the absence of the viral GADD34 function

Ian Mohr[1,2] and Yakov Gluzman

Wyeth-Ayerst Research, Lederle Laboratories, Pearl River, NY 10965, USA
[1]Present address: NYU School of Medicine, Department of Microbiology, 550 First Avenue, New York, NY 10016, USA

[2]Corresponding author

Novel suppressor variants of conditionally lethal HSV-1 γ34.5 deletion mutants have been isolated which exhibit restored ability to grow on neoplastic neuronal cells. Deletion of the viral γ34.5 genes, whose products share functional similarity with the cellular GADD34 gene, renders the virus non-neurovirulent and imposes a block to viral replication in neuronal cells. Protein synthesis ceases at late times post-infection and the translation initiation factor eIF2α is phosphorylated by the cellular PKR kinase [Chou *et al.* (1990) *Science*, 252, 1262–1266; (1995) *Proc. Natl Acad. Sci. USA*, 92, 10516–10520]. The suppressor mutants have overcome the translational block imposed by PKR. Multiple, independent isolates all contain rearrangements within a 595 bp element in the HSV-1 genome where the unique short component joins the terminal repeats. This alteration, which affects the production of the viral mRNA and protein from the Us11 and Us12 genes, is both necessary and sufficient to confer the suppressor phenotype on γ34.5 mutant viruses. HSV-1 thus encodes a specific element which inhibits ongoing protein synthesis in the absence of the viral GADD34-like function. Since this inhibition involves the accumulation of phosphorylated eIF2α, the element identified by the suppressor mutations may be a discrete PKR activator. Activation of the PKR kinase thus does not proceed through a general, cellular 'antiviral' sensing mechanism. Instead, the virus deliberately activates PKR and encodes a separate function which selectively prevents the phosphorylation of at least one PKR target, eIF2α. The nature of this potential activator element, and how analogous cellular elements could affect PKR pathways which affect growth arrest and differentiation are discussed.

Keywords: GADD34/herpesvirus/PKR/translational control

Introduction

A hallmark among herpetic viruses is the successful, life-long colonization of specific host cell types. Herpes simplex virus type-1 persists in latent, immunologically cloaked form in the neuronal cell bodies of the trigeminal ganglion. Periodically, in response to stress or ultraviolet (UV) irradiation, a lytic infection initiates in these neurons, virus again emerges at the oral epithelia and a fever blister forms. However, with a frequency of approximately 1 in 250 000, HSV-1 virions can infect the CNS and cause fatal encephalitis. A number of viral genes have been demonstrated to affect neurovirulence (for review see Roizman and Sears, 1993). While some affect the ability of the virus to replicate in a variety of cells and tissues, others affect the virus' ability to spread through the CNS.

Mutants in the γ34.5 gene are profoundly non-neurovirulent upon intracranial injection into mice (Chou *et al.* 1990; MacLean *et al.*, 1991; Bolovan *et al.*, 1994). In cultured cells, γ34.5 mutant viruses display a host-range phenotype and fail to grow in many malignant neuronal cells. Viral DNA replication appears to trigger the complete cessation of protein synthesis, preventing the production of viral late proteins (Chou and Roizman, 1992). The block in translation coincides with phosphorylation of the translation initiation factor eIF2α by the cellular PKR protein kinase (Chou *et al.*, 1995), a known tumor suppressor gene (Koromilas *et al.*, 1992; Lengyel, 1993; Meurs *et al.*, 1993).

Many classes of viruses encode functions which prevent the cellular PKR from phosphorylating eIF2α (for review, see Katze, 1995). Poliovirus induces PKR degradation, reoviruses and vaccinia virus encode double-strand RNA binding proteins which are presumed to sequester any potential activators, adenovirus VARNA, and perhaps the EBERs encoded by Epstein–Barr virus, bind PKR and prevent its activation, and influenza virus induces a cellular PKR inhibitor (Bhat and Thimmappaya, 1983; Kitajewski *et al.*, 1986; O'Malley *et al.*, 1986; Lee *et al.*, 1990; Beattie *et al.*, 1991; Mathews and Shenk, 1991; Chang *et al.*, 1992; Lloyd and Shatkin, 1992; Black *et al.*, 1993; Davies *et al.*, 1993; Sharp *et al.*, 1993). While these methods of circumventing an activated PKR have been identified, the nature of the events which lead to PKR activation and culminate in the phosphorylation of eIF2α remain ambiguous. It has been speculated that RNA produced from multiple, overlapping transcription units on opposite strands of DNA activate the kinase in adenovirus-infected cells, but this hypothesis is difficult to test directly in infected cells. The potential for producing double-stranded RNA in the course of RNA virus replication has also been offered as an explanation. While synthetic and natural RNA structures can stimulate the kinase *in vitro* (Maran and Mathews, 1988; Bischoff and Samuel, 1989; Gunnery *et al.*, 1992; Maitra *et al.*, 1994), it remains to be seen if discrete effectors are responsible for PKR activation, or if activation is the result of a general process, for example viral replication or transcription.

The carboxy-terminus of the γ34.5 gene product shares extensive homology with the human and rodent GADD34 gene product (McGeoch and Barnett, 1991; Chou and Roizman, 1994; Zhan *et al.*, 1994). These cellular gene products are induced in response to growth

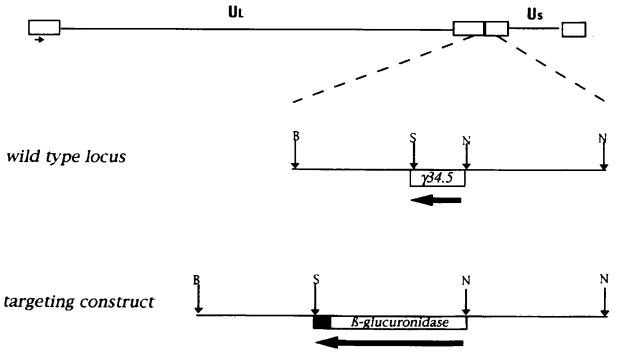
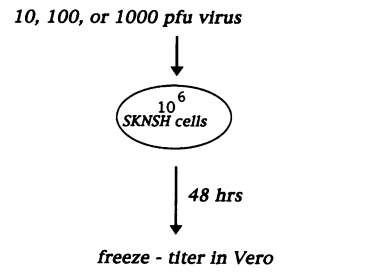


Fig. 1. Replacement of HSV-1 γ34.5 coding sequences with the β-glucuronidase gene. The schematic outlines the construction of the recombinant virus SPBg5e. A targeting vector, pSPBg, was constructed to 'surround' the β-glucuronidase gene with sequences which normally flank the HSV-1 γ34.5 gene. β-glucuronidase expression is thus driven by the endogenous γ34.5 promoter. The flanking sequences direct homologous recombination with HSV regions which contain the γ34.5 genes. The second copy of the γ34.5 gene is denoted by an arrow beneath the map. After co-transfection of this plasmid with wild-type HSV-1 DNA into Vero cells, recombinant plaques were identified by staining the agar overlay with an indicator dye specific for β-glucuronidase.



	input pfu	pfu/ml yield SKNSH
Δ 34.5	10	--
	100	10
	1000	125
REV 4	10	40
	100	1040
	1000	6400
wt	10	12,000
	100	5.6×10^5

Fig. 2. Growth properties of a suppressor isolate. Either 10, 100 or 1000 p.f.u. of WT HSV-1, Δγ34.5 mutant (SPBg5e), or REV4 (a suppressor isolate) were used to infect SKNSH cells. A lysate was prepared at 48 h post-infection by freeze–thawing, and the viral titer was determined on Vero cells.

arrest, DNA damage and differentiation signals (for review see Fornace, 1992; Hoffman and Liebermann, 1994). Although little is known about the function of GADD34, the cellular GADD34 gene can complement an HSV-1 γ34.5 deletion mutant and enables the virus to grow in neuroblastoma cells (He *et al.*, 1996). The GADD34 function plays a pivotal role in the viral life-cycle, as it fosters the survival of infected cells and the dissemination of infectious progeny. It is thus formally possible that cells which growth arrest, differentiate, sustain DNA damage, or become infected with herpes simplex require a GADD34-like function to overcome a protein synthesis checkpoint guarded by the cellular PKR. The types of signals the virus generates to activate the cellular PKR could be relevant to understanding the cellular signals to which PKR normally responds. For example, is an indirect sensing mechanism involved which responds to deregulated replication or transcription, or are specific elements involved? To determine if discrete viral components participate in this signaling cascade, we sought to isolate second-site suppressors which regained the ability to grow on non-permissive neuronal cells. The existence of these isolates proves that mutants which overcome the host restriction imposed by the activated PKR kinase can be obtained, and suggests that a specific viral element may potentiate PKR activation.

Results

Isolation of suppressor mutants

Both copies of the HSV-1 γ34.5 gene were replaced with sequences encoding β-glucuronidase in the recombinant virus SPBg5e. The expression of β-glucuronidase from the endogenous 34.5 promoter facilitated the identification of recombinant plaques in the presence of the indicator dye X-gluc (Figure 1). As the Patton strain was employed in these studies, we confirmed that these mutants exhibited the same phenotypes as the γ34.5 deletions originally described in strain F (Chou *et al.*, 1990; Chou and Roizman, 1992). These isolates were not capable of sustained late protein synthesis in neoplastic neuronal cells (see Figure 3 for example) and were non-neurovirulent upon intracranial inoculation into mice (not shown). To obtain suppressor isolates capable of restored growth on non-permissive cells, cultures of SKNSH cells were infected with SPBg5e virus at multiplicities of infection (m.o.i.) ranging from 10^{-1} to 10^{-4}. While significant cytopathic effect occurred at higher m.o.i., at the lower m.o.i. the neuroblastoma cells would continue to proliferate and eventually die by 5–7 days post-infection. Uninfected, control cultures also deteriorated in this time frame. A lysate was prepared by freeze–thawing the culture, and 0.1 ml was used to infect a second culture. This process was repeated four or five times in succession until some of the cultures were capable of eliciting a viral-specific cytopathic effect on SKNSH cells. Multiple, independent isolates were then plaque-purified on permissive monkey kidney (Vero) cells.

To quantitate the difference in growth of a selected suppressor isolate, low multiplicity infections of SKNSH cells were harvested at 48 h post-infection and titered on Vero cells. At all the m.o.i. examined, the suppressor isolate grew 50- to 100-fold better than the 34.5 mutant parent virus (Figure 2). In the course of these studies, we discovered that U373 human glioblastoma cells also failed to support the growth of γ34.5 mutant viruses. U373 cells produce a nicer monolayer, maintain this monolayer for longer times in a healthy state and are generally easier to work with than the SKNSH line. To document that the enhanced growth properties of these isolates was due to their ability to sustain protein synthesis at late times post-infection, U373 glioblastoma cells were infected at high multiplicity, and pulse-labeled with ^{35}S-labeled amino acids. The labeled proteins were fractionated on SDS–polyacrylamide gels and visualized on autoradiograms.

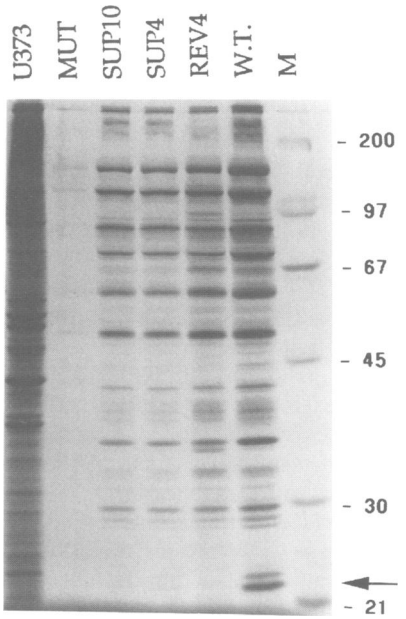


Fig. 3. The suppressor mutants are capable of sustained protein synthesis on neoplastic neuronal cells. Autoradiograph of an SDS–polyacrylamide gel which displays viral proteins produced at late times post-infection in U373 human glioblastoma cells infected with either the Δγ34.5 deletion mutant SPBg5e (MUT), WT HSV-1, or suppressor isolates SUP10, SUP4 or REV4. The lane marked U373 displays proteins synthesized in uninfected cells. The arrow off-set to the lower right denotes the position of the Us11 polypeptide present only in cells infected with wild-type virus. The molecular weight, in kDa, of the protein markers displayed in lane M appears to the right.

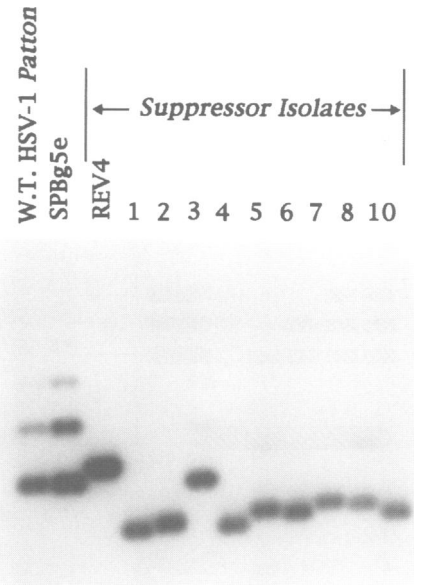


Fig. 4. Multiple, independent suppressor isolates all contain rearranged *Bam*HI Z fragments. Viral DNA from suppressor isolates (SUP1–8, 10 and Rev4), Δγ34.5 mutant (SPBg5e) and WT HSV-1 was digested with *Bam*HI, fractionated by agarose gel electrophoresis, transferred to a nylon membrane and hybridized to a ^{32}P-labeled *Bam*HI–*Bst*EII probe corresponding to the unique portion of the *Bam*HI Z fragment.

Figure 3 demonstrates that while cells infected with the parental SPBg5e virus failed to synthesize substantial amounts of host cell or virus-encoded proteins at late times post-infection, all of the cells infected with the suppressor isolates were capable of sustained viral protein synthesis and efficiently blocked host protein synthesis. As both the parental virus SPBg5e and the suppressor isolates lacked the γ34.5 genes, the suppressor viruses had sustained a mutation at one or more novel sites and were thus second-site suppressors. Similar results were obtained on the SKNSH cell line (data not shown).

Identification of a DNA rearrangement

Molecular cloning of the viral genome as a series of *Eco*RI fragments and their subsequent digestion with several restriction enzymes revealed a single DNA rearrangement (data not shown). Southern analysis of the viral chromosome revealed that all of the isolates had suffered a rearrangement in the *Bam*HI Z fragment (Figure 4). This fragment contains a small portion of unique sequences and a repetitive component (Figure 5). Heterogeneity in the *Bam*HI Z fragment in the wild-type and SPBg5e lanes is due to variation in a repetitive component. Following isolation of the *Bam*HI Z fragments by molecular cloning techniques, restriction analysis indicated that one boundary of the rearrangement was located between the unique *Bst*E2 site (nt #145 316) and the short terminal repeats in all of the isolates (not shown). A sequencing primer was then designed to read from just upstream of the *Bst*E2 site towards the terminal repeats. The points at which the sequences of the suppressor isolates deviated from wild-type were thus identified and are displayed

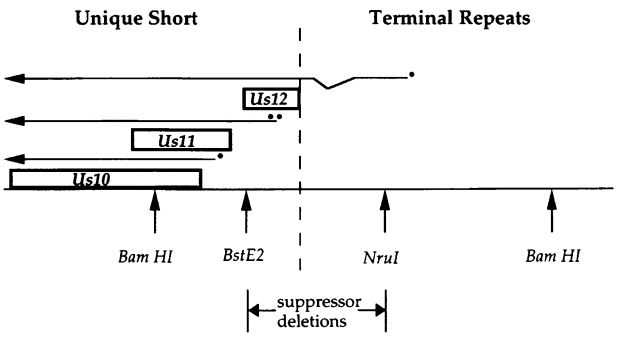


Fig. 5. Map of the *Bam*HI Z fragment highlighting the junction region where Us joins terminal repeats (TRs). The Us10, 11, and 12 open reading frames are shown in the unique short component along with restriction enzyme cleavage sites for *Bam*HI, *Bst*E2 and *Nru*I. Open reading frames are denoted as heavy boxes. mRNAs appear as arrows terminating at a common polyadenylation site. The *cis*-elements which direct transcription of the various open reading frames are denoted as solid circles (●) above their respective transcript. The vertical dashed line denotes the transition from sequences in the unique short component and the terminal repeats. The region deleted in the suppressor viruses is also shown.

in Figure 6. The rearrangements fall into two broad categories. The first class, typified by SUP1, SUP10, SUP5 and SUP8, consist of simple deletions. Both SUP1 and SUP10 harbor 583 bp deletions between nucleotide numbers 145 415 and 145 999. SUP5 and SUP8 have suffered a 497 bp deletion between nt 145 421 and nt 145 919. In the second class of rearrangements, typified by SUP2, SUP6 and SUP3, net deletions accompanied by additional alterations to the *Bam*HI Z fragment have occurred. In SUP2 and SUP6, multiple copies of a unique viral sequence normally present between 145 462 and 145 477 have been reiterated at nt 145 477, followed by a 12-nt stretch normally present between 145 481 and

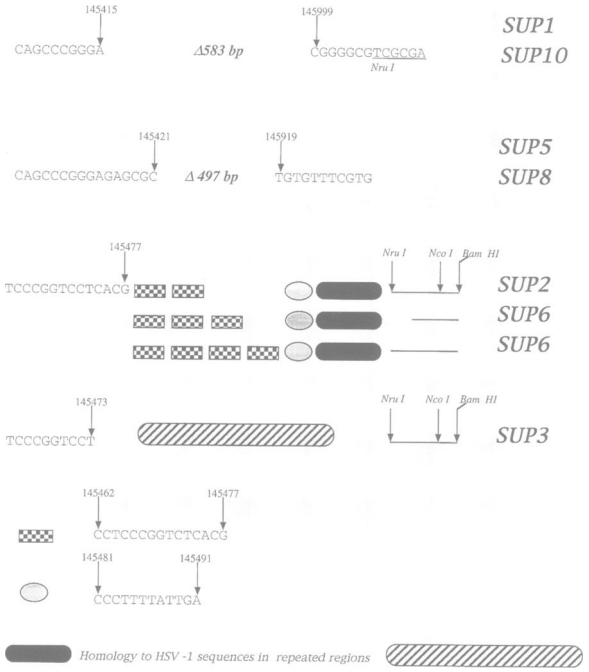


Fig. 6. The DNA rearrangements disrupt the Us12 ORF and effect *cis*-elements which direct Us11 expression. The nucleotide sequences in the *Bam*HI Z fragment at which point the suppressors deviate from the wild-type sequence of the Patton strain are presented. (Note: all of the nucleotide coordinates which follow refer to the published sequence of strain 17, as the Patton strain has not been completely sequenced and thus it is not possible to assign nucleotide numbers.) SUP1, SUP10, SUP5 and SUP8 are simple deletions with precisely established boundaries. SUP2, SUP3 and SUP6 are more complex, involving insertions of repetitive DNA elements along with the deletion of some genetic material. The structure of this class is thus illustrated in schematic form. The nucleotide sequence in the unique short component where SUP2, SUP6 and SUP3 deviate from wild-type is shown. SUP2 and two SUP6 clones depart from the wild-type sequence at nucleotide 145 477, while the breakpoint for SUP3 falls at 145 473. The checkered rectangles each represent an iteration of HSV-1 sequences normally present from nucleotides 145 462 to 145 477. The small, light gray oval represents HSV-1 sequences from 145 481 to 145 491. Both elongated ovals (shaded and striped) are sequences which share homology with HSV-1 sequences contained in the repetitive portion of the viral genome. The location of the more distal junction, located in the repeats, is not known at the nucleotide level. The solid black line identifies conserved restriction sites retained in the various clones. As the Patton strain has not been completely sequenced, and the repetitive non-coding regions may exhibit sequence variation, it is not possible to localize an exact region of homology within the repetitive component of strain 17.

145 491. This sequence is then followed by a region which shares homology with sequences within the repetitive regions of the viral chromosome. In SUP3, the sequence at 145 473 immediately diverges from wild-type and directly enters a region which shares homology with repetitive regions. Although the nucleotide sequence at which SUP2, SUP3 and SUP6 deviate from wild-type have been determined for the boundary which lies in the unique short component, the nucleotide at which the normal *Bam*HI Z sequence resumes in the terminal repeats has not been determined. In the case of SUP2, SUP3 and one SUP6 clone, restriction mapping appears normal from the *Nru*I site to the distal *Bam*HI site. The existence of multiple points at which these isolates deviate from the wild-type nucleotide sequence proves that they are truly independent isolates. These mutations share the common theme of affecting *cis*-acting sequences which direct the transcription of the Us11 ORF and disrupt the coding region of the Us12 ORF. The effect on Us11 expression is apparent in Figure 3, as the levels of Us11 protein produced in a 1-h pulse with ^{35}S-labeled amino acids are dramatically reduced.

Both Us11 and Us12 are non-essential for lytic growth in culture and the central nervous system of mice (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987; Nishiyama *et al.*, 1993). The Us12 ORF encodes the α47 immediate-early gene product which binds to the TAP transporter and prevents cellular MHC class I molecules from reaching the cell surface (Hill *et al.*, 1995). The Us11 gene encodes an RNA binding protein which is expressed at late times post-infection, and binds to its own mRNA and the mRNA of the viral UL34 gene (Roller and Roizman, 1990, 1991). Although the function of Us11 is not known, it is found associated with rRNA, both in cytoplasmic ribosomes and in the nucleolus, when expressed in uninfected cells. We will return in detail to the role that these proteins may play in producing the suppressor phenotype in the Discussion.

Marker rescue analysis

As multiple, independent isolates were obtained with breakpoints clustering in the same region, it suggested that this rearrangement was a necessary component of the suppressor phenotype. To prove that it was both necessary and sufficient to confer this phenotype on γ34.5 mutant viruses, marker rescue experiments were performed. The rearranged regions in all but one SUP6 clone were contained completely between the *Bst*E2 and *Nru*I sites. These rearranged *Bst*E2–*Nru*I fragments were then used to replace the wild-type *Bst*E2–*Nru*I fragment in the targeting vector pSXZY. This vector contains HSV-1 sequences from nt 143 481 to 147 040 and has unique *Bst*E2 and *Nru*I sites to facilitate the exchange of *Bst*E2–*Nru*I fragments from the *Bam*HI Z region. The flanking sequences, which extend in either direction from the internal *Bst*E2–*Nru*I fragment, function to direct homologous recombination within this region of the viral chromosome. The cartoon in Figure 7A illustrates the experimental approach employed to rescue the suppressor phenotype. HSV-1 DNA from γ34.5 mutant viruses was transfected either alone, or co-transfected with a specific rescue plasmid, into permissive Vero cells. Each rescue plasmid contained the *Bst*E2–*Nru*I fragment from a specific suppressor isolate and could be recognized by its altered electrophoretic mobility relative to the wild-type *Bam*HI Z fragment specified in the parental HSV-1 γ34.5 mutant (Figure 7B; compare the Δ34.5 lane marked C with the SUP1, SUP3, SUP5 and SUP6 C lanes). Each transfection resulted in the appearance of ~90–200 plaques on permissive Vero cells. Cell-free lysates prepared from these transfections by freeze–thawing were then used to infect non-permissive U373 glioblastoma cells. At the first appearance of cytopathic effect, a freeze–thaw lysate was again prepared and used to infect a second set of U373 cells. At this juncture, the U373 cells which were infected with lysates derived from transfections of only 34.5 viral DNA appeared as uninfected monolayers. However, U373 cells infected with lysates derived from co-transfections of 34.5 viral DNA and each specific rescue plasmid all

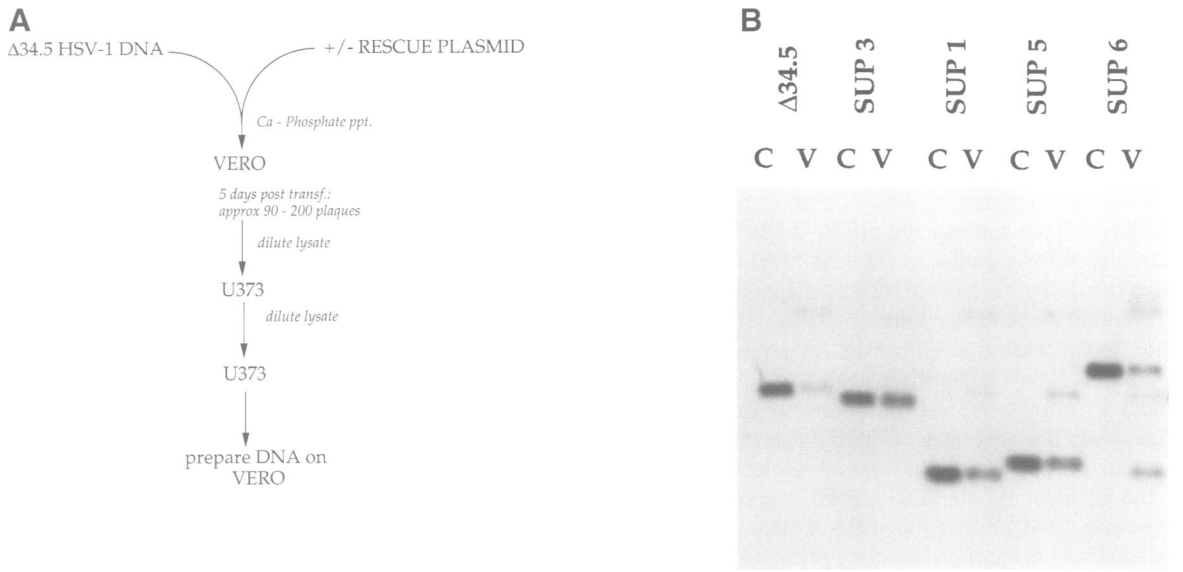


Fig. 7. The rearrangements identified in the suppressor isolates are both necessary and sufficient for γ34.5 mutant viruses to grow on glioblastoma cells. (**A**) Schematic illustrating the experimental approach. γ34.5 mutant viral DNA was transfected either alone, or co-transfected with a specific rescue plasmid, into permissive Vero cells. The rescue plasmids were wild-type except for the internal *Bst*E2–*Nru*I mutant fragments specified by the different suppressor viruses. After the appearance of cytopathic effect, a cell-free lysate was prepared by freeze–thawing and used to infect non-permissive U373 cells. The final lysate from U373 cells was used to infect permissive Vero cells to prepare DNA. (**B**) Rescue plasmid DNA clones (lanes marked C) harboring *Bst*E2–*Nru*I fragments isolated from various viral strains (SUP1, SUP3, SUP5, SUP6, Δ34.5), and the corresponding population of rescued viral DNA isolated from Vero cells (SUP1, SUP3, SUP5, SUP6; lanes marked V) were digested with *Bam*HI, fractionated on a 1% agarose gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled *Bam*HI–*Bst*E2 DNA fragment which contains the unique portion of the *Bam*HI Z fragment (see Figure 5 map). The filter was washed, and the autoradiogram is shown. As Δ34.5 mutant viruses fail to grow on U373 cells, the viral DNA digested was prepared from stocks propagated only on Vero cells. The *Bam*HI Z fragment of Δ34.5 HSV-1 mutant viruses co-migrates with the wild-type HSV-1 *Bam*HI Z fragment (see Figure 4).

exhibited substantial cytopathic effect (data not shown). DNA was prepared from these rescued cultures to analyze the genotype of the resulting viruses. Figure 7B presents a Southern analysis of the *Bam*HI Z region of the rescued isolates. The lanes marked C in Figure 7B contain cloned plasmid DNA used in the marker rescue transfection which was subsequently digested with *Bam*HI, while the lanes marked V contain *Bam*HI-digested viral DNA recovered after passage of the transfection lysate on U373 cells. In four separate instances, the rescued viruses had acquired the *Bam*HI Z fragment specified by the suppressor plasmid present in the transfection (Figure 7B, compare SUP1 C versus V, SUP3 C versus V, SUP5 C versus V, SUP6 C versus V). Only the *Bst*E2–*Nru*I fragments in each of these plasmids differed from wild-type, and in the case of SUP1 this minimal fragment is 109 bp. This Southern analysis proves that the viruses which have acquired the suppressor *phenotype*, assessed by cytopathic effect on U373 cells, have acquired the *genotype* specified by the specific plasmid which was co-transfected with γ34.5 mutant HSV-1 DNA. Viruses harboring rearranged *Bam*HI Z fragments have thus overtaken the entire population of γ34.5 mutant viruses in a single passage on non-permissive cells. This high-frequency generation of the suppressor phenotype in a single pass contrasts to the multiple passes needed to select these isolates.

The additional bands hybridizing in the Δ34.5, SUP1, SUP5 and SUP 6 lanes are due to variation in the repetitive component of the *Bam*HI Z fragment. Similar alterations can be observed in the wild-type and SPBg5e lanes in Figure 4. The slower mobility of the cloned SUP6 *Bam*HI fragment in Figure 7B is also due to variation in a reiterated component.

Discussion

In neoplastic neuronal cells infected with HSV-1 γ34.5 mutants, the onset of viral DNA synthesis is associated with the activation of PKR, phosphorylation of eIF2α and the complete cessation of protein synthesis (Chou and Roizman, 1992, 1995). The translational block imposed by the cell could be either a general response to uncontrolled viral replication, or a precise event triggered by a specific viral component. The isolation of second-site suppressor mutants which rescue the growth of HSV-1 γ34.5 deletions in non-permissive, neoplastic neuronal cells proves that discrete viral components, in addition to the γ34.5 gene product, participate in a signaling pathway which involves PKR activation and translation. Multiple independent isolates have been obtained, all of which contain rearrangements in a 595 bp element which spans the region where the unique short component of the genome joins the terminal repeats. Deletions which disrupt the Us12 ORF and sever the Us11 ORF from upstream transcriptional control elements are both necessary and sufficient to confer the suppressor phenotype to γ34.5 mutant viruses. The simplest hypothesis which takes all of this information into account is that in the absence of the 34.5 gene product, the signaling determinant identified in the suppressor viruses can constitutively activate—either directly or via other cellular components—PKR. As the resulting phosphorylation of eIF2α inhibits polypeptide chain initiation, protein synthesis ceases (Chou and Roizman, 1995). The suppressor isolates may have inactivated or removed this signal and are thus capable of sustaining viral protein synthesis in the absence of the viral GADD34 homolog. Consistent with this proposal,

agents which inhibit the PKR kinase (2-amino-purine and theophylline) restore the ability of γ34.5 mutant viruses to synthesize late viral proteins on U373 cells (I.Mohr and Y.Gluzman, in preparation). Furthermore, cells infected with the suppressor mutant viruses do not contain activated PKR and phosphorylated eIF2α, while those infected with the parental γ34.5 mutant virus contain activated PKR and phosphorylated eIF2α (J.Chou, I.Mohr, Y.Gluzman and B.Roizman, unpublished observations).

Although the deletions have identified a specific genetic element, defining the precise nature of the determinants responsible for the inhibition of translation requires further experimentation. Four possibilities need to be considered with respect to known HSV-1 functions in this region. The first involves the synthesis of the Us12 protein which is involved in preventing MHC class I molecules from reaching the surface. As the Us12 gene is expressed at immediate-early time points, the mRNA which encodes Us12, and the Us12 protein product, are present in infected cells at peak levels hours before protein synthesis is shut off. The Us11 gene product is more interesting in this regard, as it is expressed after the onset of DNA replication and as such is a true late gene. Furthermore, the Us11 protein is an RNA binding protein of unknown function and can associate with ribosomes. It thus fulfills two requirements of being expressed at the proper time and localized to a key spot which could affect protein synthesis. However, the Us11 protein is also present in virions. As a virion component, it is introduced into the cytosol of infected cells and associates with ribosomes at times which precede immediate-early gene expression (Roller and Roizman, 1992). Limiting quantities of either Us11 or Us12 (acting alone or synergistically) may not be sufficient to trigger the inhibition of protein synthesis. This scenario would require these polypeptides to possess a novel function or modification at late times post-infection in order for them to inhibit translation. It also does not rule out the possibility that the Us11 protein must accumulate beyond certain threshold levels before it can inhibit translation. In this regard, although the rate of Us11 protein synthesis is dramatically reduced in the suppressor isolates (Figure 3), significant amounts of the protein accumulate in infected cells, albeit not to wild-type levels (data not shown). Thus, the suppressor phenotype cannot simply be attributed to either the Us11 or Us12 polypeptide.

The third possibility involves the mRNA which encodes the Us11 gene product. This species is the only *identified* product of the suppressor region whose appearance is confined to the period in which protein synthesis ceases. Specifically, all of the rearrangements affect the lengthy 5′ untranslated region (5′UTR) of the Us11 mRNA and the protein-coding region of the Us12 mRNA which overlaps with the Us11 5′UTR, but leave the Us11 ORF intact. The gradual decline in protein synthesis observed between 5 and 12 h post-infection parallels the kinetics of Us11 mRNA accumulation (Johnson *et al.*, 1986). Moreover, synthesis of the Us11 transcript is dependent on DNA replication. As the PKR kinase contains an RNA binding domain which renders it highly responsive to RNA harboring double-stranded regions, it is conceivable that a specific RNA structure is part of the signal which triggers the shutdown of protein synthesis (for review, see Williams, 1995).

Finally, it is entirely possible that the suppressor phenotype cannot be explained in terms of viral functions which have been identified previously. In marker rescue experiments where the SUP1 deletion can create the suppressor phenotype, mutations which affect the ATG of either the Us11 or Us12 ORFs, or deletions which remove all of the unique short coding sequences from the *Bst*E2 site (nt 145 316) to the boundary of the terminal repeats (145 481), do not produce the suppressor phenotype (I.Mohr and Y.Gluzman, unpublished observations). This is consistent with the proposal that a novel, complex herpes simplex virus genetic element is defined by these mutations. Further investigation of this element is in progress and will determine if it acts by producing a protein, an RNA, or functions as a *cis*-acting structural element.

While previous studies have established that viruses must have methods to prevent eIF2α phosphorylation by PKR, the scenario which generates an activated PKR kinase has remained obscure. In particular, it was not clear if the mechanism by which PKR became activated proceeded through an indirect sensing mechanism, or if specific effector components were involved. The isolation of HSV-1 γ34.5 second-site suppressor mutants proves that discrete viral species participate in the activation of PKR. This raises an interesting question, since the suppressor viruses have effectively bypassed the PKR control circuit by removing both the activator and the inhibitor. One must ask what advantage is conferred on HSV-1 to maintain a potent PKR pathway activator in the viral genome. HSV—and perhaps all viruses which encode PKR inhibitors—may derive a benefit from having PKR phosphorylate substrates other than eIF2α. Indeed, wild-type HSV-1 infection leads to PKR activation, and 30% of eIF2α is phosphorylated in cells infected with wild-type adenovirus (O'Malley *et al.*, 1989; Chou *et al.*, 1995). In some viral systems, it is conceivable that a transient accumulation of phosphorylated eIF2α could attenuate the translation of certain host mRNAs.

The activation of PKR in infected cells must now be re-evaluated, as it is not a defense response enacted by the cell in the wake of a destructive invader. On the contrary, it is deliberately activated by the virus, which selectively prevents the phosphorylation of at least one PKR target, eIF2α. To our knowledge, the element identified in the suppressor mutants may represent the first genetic definition of a *specific* PKR activator. It is thus quite conceivable that specific RNA, RNP or protein structures activate PKR in a diverse array of viral and cellular settings. The parasitism of the GADD34 response by herpes simplex may reflect the prominent role that terminally differentiated neurons play in its life-cycle. It is intriguing that African swine fever virus, an unrelated large DNA virus which infects cells of the monocyte/macrophage lineage, also encodes a GADD34 homolog which plays a prominent role in pathogenicity (Sussman *et al.*, 1992; D.L.Rock, personal communication).

Although the translational apparatus is poised to respond to minute environmental cues, little is known about how protein synthesis is linked to the cell cycle. Signals which trigger differentiation, growth arrest and DNA damage

induce the GADD genes (for review, see Fornace, 1992; Hoffman and Liebermann, 1994). As the GADD34 gene product permits HSV-1 γ34.5 mutants to synthesize late proteins in neuroblastoma cells (He *et al.*, 1996), the function of GADD34 may be to permit passage through a PKR translational control point. Cellular counterparts of this discrete viral element may exist. Activation of PKR in response to specific effectors would make translation dependent on a GADD34-like function and create a powerful translational checkpoint. As PKR may play a role in growth and differentiation (Petryshyn *et al.*, 1988), activation of this kinase and modulation of its ability to phosphorylate eIF2α could potentially determine various aspects of cell fate. In addition, PKR and eIF2α are tumor suppressor elements, raising the possibility that PKR signaling pathways may also contribute to the malignant transformation of cultured cells (Koromilas *et al.*, 1992; Donze *et al.*, 1995).

HSV-1 γ34.5 mutants display a host-range phenotype, and may be a useful probe for the status of the PKR pathway in a given cell. Any effector which influences the activation of PKR, or inhibits its activity, can be viewed as a permissivity determinant for the growth of γ34.5 mutants. Tissue-specific expression of various RNA binding proteins, in concert with PKR and PKR inhibitor levels, may contribute to this (e.g. see Park *et al.*, 1994). Pinpointing the exact nature of the function deleted in the suppressor viruses, coupled with biochemical studies aimed at deciphering its mechanism of action, will provide a foothold from which to explore the roles of GADD34-like proteins, PKR and PKR activators in growth control and differentiation.

Materials and methods

Replacement of the HSV-1 γ34.5 coding sequences with the β-glucuronidase gene

The plasmid pBg10pA was a gift from Dr T.Jones. This plasmid, based in pT7-1 (USB), contains the 2.1 kb fragment harboring the β-glucuronidase gene (from Clontech, San Diego pRAJ275b) fused to the human cytomegalovirus Us10 polyadenylation site (an *Apa*I–*Sma*I fragment corresponding to hHCMV AD169 nt 13 527–13 782) at its 3′ border. The HSV-1 Patton strain *Bam*HI SP fragment (obtained from T.Jones as a *Bam*HI insert into pBR322) was digested with *Nco*I. The 1.8 kb *Nco*I fragment (corresponding to nt 127 666–125 855 in the published sequence of strain 17) which contains the promoter and the first ATG codon of the 34.5 gene was isolated and cloned into *Nco*I-digested pBg10pA. This plasmid (p5′γ34.5Bγ10pA) was digested with *Eco*RI, end-filled with Klenow polymerase, and then digested with *Sac*I. The HSV-1 *Bam*HI SP clone was digested with *Bam*HI, end-filled with Klenow polymerase, and digested with *Sac*I. The 1.6 kb terminal *Sac*I–*Bam*HI (Klenow-filled) fragment (corresponding to nucleotide numbers 123 459–125 066 in the published strain 17 sequence) was isolated and ligated into *Eco*RI (Klenow-filled)/*Sac*I-digested p5′γ34.5Bg10pA to create p5′γ34.53′γ34.5, which has been abbreviated as pSPBg. This plasmid places the β-glucuronidase gene under the control of the endogenous 34.5 promoter and surrounds the β-glucuronidase gene with HSV-1 sequences which normally flank the 34.5 gene. Five μg of *Hin*dIII-linearized pSPBg and 2 μg of pure HSV-1 DNA (Patton strain) were assembled with sonicated salmon sperm DNA and introduced into Vero cells by the calcium phosphate technique. Once the cytopathic effect had progressed through the monolayer, a lysate was obtained by freeze–thawing, followed by a 1-min burst in a water bath sonicator. Dilutions were prepared and isolates were subjected to two rounds of plaque purification on Vero cells in the presence of the indicator dye X-gluc. The isolate used in these studies was designated SPBg5e. Restriction digestion and Southern analysis demonstrated that the gene for β-glucuronidase had replaced both copies of the 34.5 gene (not shown).

Selection procedure to obtain suppressor mutants

SKNSH neuroblastoma cells (ATCC) which were either just confluent, or approaching confluence (60 mm dishes) were placed in DMEM + 2% FBS and infected with SPBg5e at an m.o.i. ranging from 10^{-1} to 10^{-4}. Cultures were examined each day for signs of cytopathic effect. Generally, at 5–7 days post-infection, the cultures displayed either complete cytopathic effect (at higher m.o.i.), or the SKNSH cultures completely exhausted the medium. A lysate was then prepared by freeze–thawing, sonicated in a water bath for 1 min, and 0.1 ml of this lysate was used to infect a second 60 mm dish. This process was repeated four times in succession. At this point, some of the lysates were capable of generating substantial cytopathic effect on SKNSH cells prior to the cells exhausting the media. Isolates were then subjected to two rounds of plaque purification in Vero cells, and large-scale stocks were prepared in SKNSH cells. Staining of the plaques with X-gluc revealed that the isolated plaques retained the β-glucuronidase gene of the parental virus and were capable of growth in both Vero and SKNSH cells.

Analysis of viral DNA from suppressor isolates

Stocks of isolates grown in SKNSH cells were used to infect Vero cells at an m.o.i. of 1. Infected cell cultures were maintained in Media 199 (m199) plus 1% calf serum and incubated at 34°C until maximum cytopathic effect was observed. Cells were harvested, suspended in 10 mM Tris, pH 8.4, 140 mM NaCl, 10 mM $MgCl_2$, and lysed by the addition of an equal volume of the same buffer containing 1% Triton X-100. After 5 min on ice, the extracts were centrifuged at 14 000 *g* for 2 min. The supernatant (removed with a wide-bore pipet tip) was adjusted to a final concentration of 0.4% SDS, 10 mM EDTA, 25 μg/ml RNase A, 1 mg/ml Pronase and incubated for at least 1 h at 37°C. DNA was purified by two extractions with phenol:chloroform, one extraction with chloroform, and then precipitated with ethanol. Following a wash with 70% ethanol, the pellet was allowed to air-dry at room temperature and resuspended in 10 mM Tris, pH 8.0, 0.1 mM EDTA.

To isolate *Bam*HI Z fragments, viral DNA was digested with *Bam*HI and fragments that migrated between 1 and 3 kb were size-selected by agarose gel electrophoresis, purified and cloned into *Bam*HI-digested pGEM2. Clones containing *Bam*HI Z inserts were identified by colony hybridization and confirmed by Southern blotting.

The sequencing primer 5′CCCTCCGCCCAGAGACTCG3′ corresponds to nt 145 270–145 288 in the published strain 17 sequence. DNA sequencing was performed using a USB sequenase kit according to the manufacturer's instructions.

Analysis of total viral protein synthesis

Confluent wells of U373 glioblastoma cells (ATCC) seeded in a 6-well dish were infected with either SPBg5e, various SUP isolates or the wild-type HSV-1 Patton strain virus at high m.o.i. (1–10) for 1 h at 37°C. The cells were then re-fed with DMEM plus 2% FBS and allowed to incubate overnight. At any point after 12 h post-infection (usually 15.5 h), the infected cells were overlaid with 1 ml of DMEM containing 50–70 μCi/ml [^{35}S]Express (commercial mixture of methionine and cysteine from DuPont NEN) and the incubation continued for 1 h at 37°C. Total cellular protein was solubilized in 1× Läemmli buffer, boiled for 3 min, and a portion was fractionated on 12.5% SDS–polyacrylamide gels. Gels were fixed in 25% methanol, 10% acetic acid, dried, and exposed to Kodak XAR film.

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References

Beattie,E., Tartaglia,J. and Paoletti,E. (1991) Vaccinia virus encoded eIF-2 alpha homolog abrogates the antiviral effect of interferon. *Virology*, **183**, 419–422.

Bhat,R. and Thimmappaya,B. (1983) Two small RNAs encoded by Epstein–Barr virus can be functionally substituted for the virus-associated RNAs in the lytic growth of adenovirus 5. *Proc. Natl Acad. Sci. USA*, **80**, 4789–4793.

Bischoff,J.R. and Samuel,C.E. (1989) Activation of the human PI/eIF2 protein kinase by individual reovirus s-class mRNAs: s1 mRNA is a potent activator relative to s4 mRNA. *Virology*, **172**, 106–115.

Black,T.L., Barber,G.N. and Katze,M.G. (1993) Degradation of the interferon-induced 68,000 Mr protein kinase by poliovirus requires RNA. *J. Virol.*, **67**, 791–800.

Bolovan,C.A., Sawtell,N.M. and Thompson,R.L. (1994) ICP34.5 mutants of herpes simplex virus type 1 strain 17syn+ are attenuated for neurovirulence in mice and for replication in confluent primary mouse embryo cell cultures. *J. Virol.*, **68**, 48–55.

Brown,S.M. and Harland,J. (1987) Three mutants of herpes simplex virus type 2: one lacking the genes US10, US11, and US12 and two in which Rs has been extended by 6 kb to 0.91 map units with loss of Us sequences between 0.94 and the Us/TRs junction. *J. Gen Virol.*, **68**, 1–18.

Chang,H.W., Watson,J.C. and Jacobs,B.L. (1992) The E3L gene of vaccinia virus encodes an inhibitor of the interferon induced, dsRNA dependent protein kinase. *Proc. Natl Acad. Sci. USA*, **89**, 4825–4829.

Chou,J. and Roizman,B. (1992) The γ34.5 gene of Herpes Simplex Virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. *Proc. Natl Acad. Sci. USA*, **89**, 3266–3270.

Chou,J. and Roizman,B. (1994) Herpes simplex virus 1 γ34.5 gene function, which blocks the host response to infection, maps in the homologous domain of the genes expressed during growth arrest and DNA damage. *Proc. Natl Acad. Sci. USA*, **91**, 5247–5251.

Chou,J., Kern,E.R., Whitley,R.J. and Roizman,B. (1990) Mapping of Herpes Simplex virus 1 neurovirulence to γ34.5, a gene nonessential for growth in cell culture. *Science*, **252**, 1262–1266.

Chou,J., Chen,J.J., Gross,M. and Roizman,B. (1995) Association of a M_r 90,000 phosphoprotein with protein kinase PKR in cells exhibiting enhanced phosphorylation of translation initiation factor eIF2α and premature shutoff of protein synthesis after infection with γ34.5$^-$ mutants of herpes simplex virus 1. *Proc. Natl Acad. Sci. USA*, **92**, 10516–10520.

Davies,M.V., Chang,H.W., Jacobs,B.L. and Kaufman,R.J. (1993) The E3L and K3L vaccinia virus gene products stimulate translation through inhibition of the double-stranded RNA dependent protein kinase by different mechanisms. *J. Virol.*, **67**, 1688–1692.

Donze,O., Jagus,R., Koromilas,A.E., Hershey,J.W.B. and Sonenberg,N. (1995) Abrogation of translation initiation factor eIF-2 phosphorylation causes malignant transformation of NIH 3T3 cells. *EMBO J.*, **14**, 3828–3834.

Fornace,AJ.,Jr (1992) Mammalian genes induced by radiation: activation of genes associated with growth control. *Annu. Rev. Genet.*, **26**, 507–526.

Gunnery,S., Green,S.R. and Mathews,M.B. (1992) Tat-responsive region RNA of human immunodeficiency virus type 1 stimulates protein synthesis *in vivo* and *in vitro*: relationship between structure and function. *Proc. Natl Acad. Sci. USA*, **89**, 11557–11561.

He,B., Chou,J., Liebermann,D.A., Hoffman,B. and Roizman,B. (1996) The carboxyl terminus of the murine MyD116 gene substitutes for the corresponding domain of the γ34.5 gene of Herpes Simplex to preclude the premature shutoff of total protein synthesis in infected human cells. *J. Virol.*, **70**, 84–90.

Hill,A., Jugovic,P., York,I., Russ,G., Bennick, J., Yewdell,J., Ploegh,H. and Johnson,D. (1995) Herpes simplex virus turns off the TAP to evade host immunity. *Nature*, **375**, 411–415.

Hoffman,B. and Liebermann,D.A. (1994) Molecular controls of apoptosis: differentiation/growth arrest primary response genes, proto-oncogenes, and tumor suppressor genes as positive and negative modulators. *Oncogene*, **9**, 1807–1812.

Johnson,P.A., MacLean,C., Marsden,H.S., Dalziel,R.G. and Everett,R.D. (1986) The product of gene Us11 of Herpes Simplex Virus Type 1 is expressed as a true late gene. *J. Gen. Virol.*, **67**, 871–883.

Katze,M.G. (1995) Regulation of the interferon-induced PKR: can viruses cope? *Trends Microbiol.*, **3**, 75–78.

Kitajewski,J., Schneider,R.J., Safer,B., Munemitsu,S., Samuel,C.E., Thimmappaya,B. and Shenk,T. (1986) Adenovirus VAI RNA antagonizes the antiviral action of interferon-induced eIF2α kinase. *Cell*, **45**, 195–200.

Koromilas,A.E., Roy,S., Barber,G.N., Katze,M.G. and Sonenberg,N. (1992) Malignant transformation by a mutant of the IFN-inducible dsRNA dependent protein kinase. *Science*, **257**, 1685–1689.

Lee,T.G., Tomita,J., Hovanessian,A.G. and Katze,M.G. (1990) Purification and partial characterization of a cellular inhibitor of the interferon induced protein kinase of Mr 68,000 from influenza virus infected cells. *Proc. Natl Acad. Sci. USA*, **87**, 6208–6212.

Lengyel,P. (1993) Tumor-suppressor genes: news about the interferon connection. *Proc. Natl Acad. Sci. USA*, **90**, 5893–5895.

Lloyd,R.M. and Shatkin,A.J. (1992) Translational stimulation by reovirus polypeptide s3: substitution for VAI RNA and inhibition of phosphorylation of the α subunit of eukaryotic initiation factor 2. *J. Virol.*, **66**, 6878–6884.

Longnecker,R. and Roizman,B. (1986) Generation of an inverting herpes simplex virus 1 mutant lacking the L-S junction a sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and the a47 gene. *J. Virol.*, **58**, 583–591.

MacLean,A.R., Ul-Fareed,M., Robertson,L., Harland,J. and Brown,S.M. (1991) Herpes simplex virus type 1 deletion variants 1714 and 1716 pinpoint neurovirulence-related sequences in Glasgow strain 17+ between immediate early gene 1 and the 'a' sequence. *J. Gen. Virol.*, **72**, 631.

Maitra,R.K., McMillan,N.A., Desai,S., McSwiggen,J., Hovanessian, A.G., Sen,G., Williams,B.R. and Silverman,R.H. (1994) HIV-1 TAR RNA has an intrinsic ability to activate interferon-inducible enzymes. *Virology*, **204**, 823–827.

Maran,A. and Mathews,M.B. (1988) Characterization of the double-stranded RNA implicated in the inhibition of protein synthesis in cells infected with a mutant adenovirus defective for VARNAI. *Virology*, **164**, 106–113.

Mathews,M.B. and Shenk,T. (1991) Adenovirus virus-associated RNA and translational control. *J. Virol.*, **65**, 5657–5662.

McGeoch,D.J. and Barnett,B.C. (1991) Neurovirulence factor. *Nature*, **353**, 609.

Meurs,E.F., Galabru,J., Barber,G.N., Katze,M.G. and Hovanessian,A.G. (1993) Tumor suppression function of the interferon induced double-stranded RNA-activated protein kinase. *Proc. Natl Acad. Sci. USA*, **90**, 232–236.

Nishiyama,Y., Kurachi,R., Daikoku,T. and Umene,K. (1993) The US9, 10, 11, 12 genes of Herpes Simplex Type 1 are of no importance for its neurovirulence and latency in mice. *Virology*, **194**, 419–423.

O'Malley,R.P., Mariano,T.M., Siekiera,J. and Mathews,M.B. (1986) A mechanism for the control of protein synthesis by adenovirus VA RNA_I. *Cell*, **44**, 391–400.

O'Malley,R.P., Duncan,R.F., Hershey,J.W.B. and Mathews,M.B. (1989) Modification of protein synthesis initiation factors and the shut-off of host protein synthesis in adenovirus infected cells. *Virology*, **168**, 112–118.

Park,H. *et al.* (1994) TAR RNA binding protein is an inhibitor of the interferon-induced protein kinase PKR. *Proc. Natl Acad. Sci. USA*, **91**, 4713–4717.

Petryshyn,R., Chen,J. and London,I. (1988) Detection of activated double-stranded RNA dependent protein kinase in 3T3-F442A cells. *Proc. Natl Acad. Sci. USA*, **85**, 1427–1431.

Roizman,B.R. and Sears,A.E. (1993) Herpes Simplex Viruses and their replication. In Roizman,B., Whitley,R.J. and Lopez,C. (eds), *The Human Herpesviruses*. Raven Press, New York, pp. 11–68.

Roller,R.J. and Roizman,B. (1990) The herpes simplex virus Us11 open reading frame encodes a sequence-specific RNA binding protein. *J. Virol.*, **64**, 3463–3470.

Roller,R.J. and Roizman,B. (1991) The herpes simplex virus 1 RNA binding protein Us11 negatively regulates the accumulation of a truncated viral mRNA. *J. Virol.*, **65**, 5873–5879.

Roller,R.J. and Roizman,B. (1992) The herpes simplex virus RNA binding protein Us11 is a virion component and associates with ribosomal 60S subunits. *J. Virol.*, **66**, 3624–3632.

Sharp,T.V., Schwemmle,M., Jeffrey,I., Laing,K., Mellor,H., Proud,C.G., Hilse,K. and Clemens,M.J. (1993) Comparative analysis of the regulation of the interferon-inducible protein kinase PKR by Epstein–Barr virus RNAs EBER-1 and EBER-2 and adenovirus VAI RNA. *Nucleic Acids Res.*, **21**, 4483–4490.

Sussman,M.D., Lu,Z., Kutish,G., Alfonso,C.L., Roberts,P. and Rock,D.L. (1992) Identification of an African swine fever virus gene with similarity to a myeloid differentiation primary response gene and a neurovirulence associated gene of herpes simplex virus. *J. Virol.*, **66**, 5586–5589.

Umene,K. (1986) Conversion of a fraction of the unique sequence to part of the inverted repeats in the S component of the herpes simplex type 1 genome. *J. Gen. Virol.*, **67**, 1035–1048.

Williams,B.R.G. (1995) The role of the dsRNA-activated kinase, PKR, in signal transduction. *Semin. Virol.*, **6**, 191–202.

Zhan,Q. *et al.* (1994) The gadd and MyD genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. *Mol. Cell. Biol.*, **14**, 2361.

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