To replicate or not to replicate: achieving selective oncolytic virus replication in cancer cells through translational control

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To ensure that their mRNAs are translated and that the viral proteins necessary for assembling the next generation of infectious progeny are produced, viruses must effectively seize control of the translational machinery within their host cells. In many cases, the ability to productively engage host translational components can determine if a given cell type can support viral replication, illustrating the critical importance of this task in the viral life cycle. Failure to interface properly with the host translational apparatus can compromise the productive growth cycle, resulting in an abortive infection and radically restricting viral replication. Not only have viruses become facile at commandeering this machinery, they are also particularly adept at manipulating cellular translation control pathways for their own ends. In this review, the mechanisms by which numerous viruses manipulate host translational control circuits are discussed. Furthermore, particular attention is devoted to understanding how interfering with the ability of a virus to properly regulate translation in its host can be exploited to generate oncolytic strains that selectively replicate in cancer cells.

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Throughout the course of their evolution as obligate intracellular parasites, viruses have successfully woven a complex array of interactions with critical host cell functions. At the molecular level, these often signify whether a given cell type or host is capable of supporting productive viral growth. In some cases, productive replication is intimately linked with pathogenicity and disease, as altering the association of specific viral components with host cell functions can severely restrict viral growth, resulting in an abortive infection and a viral strain that is attenuated. Significantly, the multitude of interactions between viruses and their hosts manifests itself on numerous levels, ranging from the recognition of an appropriate cell surface protein required for entry to appropriating control of intracellular signaling pathways important for viral replication. It is particularly striking, however, that while certain animal viruses do in fact encode a limited subset of factors involved in macro-

molecular synthesis such as RNA and DNA polymerases, together with transcription and replication factors, none of them encode any components of the protein synthesis apparatus, underscoring their complete dependence upon host cell functions to produce viral polypeptides. Thus, virus–host interactions that regulate translation are poised to play a fundamental role in determining if the intracellular milieu is in fact capable of supporting viral replication. Indeed, understanding the basis behind how viruses effectively commandeer the cellular machinery required to produce proteins, with the goal of manipulating this property at the genetic level, can result in the creation of viral strains with a modified host range. An important application of this concept lies in exploiting differences between normal cells and cancer cells to isolate attenuated viral strains that selectively replicate in neoplastic cells.

Over the past century, accumulated anecdotal observations in humans and experimental evidence garnered from animal models supports the notion that viral infection is capable of inducing regression of existing tumors (Sinkovics and Horvath, 1993). The destruction of the tumor is thought to be driven, in part, by active viral replication within the cancer cells and is referred to as viral oncolysis. Initially, viruses whose oncolytic potential was investigated, while capable of replicating in human tumor cells, were naturally occurring, and either nonpathogenic in humans or capable of producing mild disease. Within the past 15 years, however, the advent of genetic engineering techniques has made it possible to modify selectively human viruses such that they are nonpathogenic, but retain the capability to replicate in cancer cells. In this review, the importance of translational control pathways in viral virulence is illustrated, and their importance in the development of new oncolytic viral strains is explored.

On the front lines of host defense: eIF2 α phosphorylation and translational control

As viruses are completely dependent upon the translational machinery resident in their host cells, it is not surprising that a major host defense component centers on impeding viral mRNA translation (reviewed in Pe'ery and Mathews, 2000). Indeed, one of at least four stress responsive eIF2 α kinases present in mammalian cells, the double stranded (ds) RNA-dependent protein

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kinase PKR accumulates in response to the antiviral cytokines interferon α/β . It has been proposed that abundant dsRNA, a replicative intermediate formed in the replication of RNA viruses and a by-product of overlapping transcription units on opposite DNA strands of DNA viruses, is a signature of viral infection. PKR binds dsRNA and in the presence of this activating ligand forms a dimer whereupon each subunit phosphorylates the other. The cellular protein PACT can also activate PKR in the absence of dsRNA (Patel and Sen, 1998). It is this activated, phosphorylated form of PKR that then goes on to phosphorylate other substrates, including eIF2 α , the regulatory subunit of eIF2 (reviewed in Kaufman, 2000).

eIF2 is a heterotrimeric G protein, forming a ternary complex composed of its α , β , and γ subunits bound to GTP along with the initiator tRNA, that is responsible for chaperoning the initiator tRNA to the 40S ribosomal subunit, forming what is known as the 48S complex (Figure 1a; reviewed in Schneider and Mohr, 2003). Like many G proteins, a GTPase activating protein (eIF5) and a guanine nucleotide exchange factor (eIF2B) regulate its activity. Normally, eIF5 promotes GTP hydrolysis following the joining of the 60S ribosomal subunit to form an 80S ribosome, releasing eIF2 bound to GDP. To participate in subsequent rounds of polypeptide chain initiation, the GDP form of eIF2 requires the activity of eIF2B in order to exchange GDP for GTP. However, phosphorylation of eIF2 α leads to a strong association between eIF2B and eIF2, effectively preventing eIF2B from catalysing the nucleotide exchange reaction (Figure 1b). As the quantity of eIF2B present in cells is limiting, phosphorylation of small amounts of eIF2 α by PKR can have relatively large effects on translation by sequestering eIF2B, hindering nucleotide exchange, and inhibiting translation. Unchecked, PKR activated by dsRNA in virus-infected cells would therefore effectively deplete active eIF2, inhibit ternary complex formation, and prevent viral and cellular protein synthesis. Should cells initially infected succeed in inhibiting translation, the viral invader would effectively be stopped in its tracks, denied access to the cellular translational apparatus it needs to complete its life cycle. This arm of the innate host response then is designed to kill the initially infected cells for the benefit of the larger population. However, numerous viruses, including herpes simplex virus-1 (HSV-1), have captured a variety of functions that operate through diverse mechanisms to counter this cellular response (Figure 2; reviewed in Schneider and Mohr, 2003). This struggle for control of the translational machinery is often an integral component of viral pathogenesis (Leib *et al.*, 1999, 2000; reviewed in Mohr, 2003, 2004).

Keeping eIF2 α phosphate free: the multiple functions encoded by HSV-1 and their role in neuropathogenesis

Herpes simplex virus is a large, enveloped DNA virus capable of replication and spread in a variety of cell

Figure 1 Regulation of translation by phosphorylation of eIF2, a critical translation initiation factor. (a) Composed of α , β , and γ subunits, eukaryotic translation initiation factor 2 (eIF2) forms a ternary complex with GTP and the initiator tRNA (tRNAⁱ). This complex associates with the 40S ribosomal subunit bound to eIF3, and recognizes the 5' end of the mRNA through an association with eIF4F. Once the AUG codon in the mRNA has been identified by a unidirectional translocation process termed scanning, GTP hydrolysis stimulated by eIF5 and the subsequent release of the eIF2-GDP complex facilitate the joining of the 60S ribosome subunit and translation elongation commences. The guanine nucleotide exchange factor eIF2B is required to exchange the GDP bound to eIF2 and replace it with GTP, thus recycling the active form of eIF2. (b) After phosphorylation of eIF2 on its α subunit by PKR, an eIF2 α kinase, eIF2B remains tightly bound and cannot exchange the GDP bound to eIF2 for GTP. This failure to recycle eIF2 to its active, GTP bound form inhibits the initiation of translation

types in culture and in animals. In its human host, the virus establishes a latent infection in sensory neurons characterized by a highly restricted pattern of gene expression (reviewed in Roizman and Knipe, 2001). Periodically, in response to a variety of stimuli, these latent infections spontaneously 'reactivate', and the ensuing episodes of productive viral growth are characterized by expression of over 80 viral open reading frames (ORFs) distributed among two unique, single copy segments or within multiple repetitive loci of the

Figure 2 Strategies implemented by oncolytic viruses to prevent the accumulation of phosphorylated eIF2 α . Activation of the cellular kinase PKR in virus-infected cells is believed to be triggered by the accumulation of dsRNA. Once two PKR monomers assemble a dimer on dsRNA, each subunit in turn phosphorylates the other and the activated enzyme subsequently phosphorylates the α subunit of eIF2, inactivating this critical translation initiation factor and arresting protein synthesis. The point at which effector molecules, encoded by select oncolytic viruses, intercede in this pathway is depicted. Adenovirus (Ad) VA RNA₁ binds PKR monomers and prevents assembly of PKR dimers. The herpes simplex virus (HSV) Us11, Vaccinia virus (VV) E3L, and reovirus (Reo) σ 3 proteins bind dsRNA, sequestering it from PKR. Us11 and E3L physically interact with PKR. While the vaccinia virus K3L gene product associates with PKR, it prevents eIF2 α phosphorylation by acting as a pseudosubstrate. The HSV γ _{134.5} gene product recruits the cellular protein phosphatase 1 α to oppose the action of PKR by promoting dephosphorylation of eIF2 α

large HSV-1 DNA genome (Figure 3). Activation of the productive or lytic gene expression program is associated with a profound inhibition of cellular mRNA and protein synthesis and results in the production of viral particles followed by the eventual death of the infected cell. Distinct mRNA populations accumulate at discrete times in the productive replication cycle, resulting in differential waves of viral gene expression in what has been termed a cascade pattern (Figure 4; reviewed in Roizman and Knipe, 2001). Four of the five immediate-early (IE) proteins produced in the first wave are global regulators important for the expression of subsequent classes of viral genes. The next class, viral early genes, primarily encode functions required for viral DNA synthesis, the initiation of which signals entry into the final late or γ phase of the viral life cycle. Two classes of late genes (γ ₁ and γ ₂) can be distinguished based upon their transcription in the presence of viral DNA synthesis inhibitors. While transcription of the γ ₂ class, requires viral DNA synthesis, expression of γ ₁ genes is not completely dependent upon viral DNA replication and is only modestly reduced in the presence of inhibitors. Included among the late gene products are polypeptides critical for assembling infectious virus. On rare occasions, HSV-1 can enter and replicate productively within the CNS, causing encephalitis. A major genetic determinant responsible for this viral neurovirulence is encoded by the γ _{134.5} gene, the product of which prevents the accumulation of phosphorylated eIF2 α and figures prominently in the development of safe, oncolytic HSV-1 strains.

Figure 3 Genetic structure of oncolytic HSV-1 γ _{134.5} mutant derivatives. The HSV-1 genome is 152 kb in length and is composed of a unique long segment (UL), a unique short segment (US), and several reiterated components (rectangles). The γ _{134.5} gene is contained within these repetitive components that flank the UL segment and is therefore diploid. (a) Δ 34.5 null mutant where both copies of the γ _{134.5} gene have been deleted. (b) A multimutated strain that contains a bacterial β -galactosidase (lacZ) expression cassette disrupting the viral UL39 gene in addition to a deletion affecting both copies of the γ _{134.5} gene. This Δ 34.5 null mutant is also unable to produce the large subunit of the viral ribonucleotide reductase, the UL39 gene product

Embedded within a repetitive genome component (Figure 3a), the γ _{134.5} gene is expressed with γ ₁ late kinetics and is not required for growth in cultured monkey kidney cells. Strikingly, its impact on viral neurovirulence is greater than any single HSV-1 gene identified to date (Chou *et al.*, 1990; Maclean *et al.*, 1991; Bolovan *et al.*, 1994). While the LD₅₀ of many WT HSV-1 strains is less than 300 PFU following intracranial delivery, it is not possible to measure accurately the LD₅₀ for γ _{134.5} mutant viruses. Indeed, upwards of 10⁶–10⁷ PFU of γ _{134.5} mutant viruses have been safely injected intracranially into mouse, non-human primate, and human brains (Chou *et al.*, 1990; Maclean *et al.*, 1991; Mineta *et al.*, 1995; Hunter *et al.*, 1999; Markert *et al.*, 2000; Rampling *et al.*, 2000; Sundaresan *et al.*, 2000). Although a standard line of monkey kidney cells were permissive or supported the replication of γ _{134.5} mutants, many human tumor cells were in fact nonpermissive, or did not support the growth of γ _{134.5} mutant derivatives. Thus, γ _{134.5} mutants actually behaved like classical viral host range mutants, exhibiting restricted growth in some lines of cultured cells but not others. Upon infection of a nonpermissive human tumor cell with a γ _{134.5} mutant strain, all of the events in the viral life cycle proceeded normally up to and including viral DNA replication and the accumulation of γ ₂ late mRNA transcripts. These viral late mRNAs encoding key structural proteins required to complete the viral life cycle and assemble the next generation of viral progeny, however, were never translated due to a block at the level of protein synthesis (Figure 5), effectively interrupting the viral life cycle prior to the assembly and release of viral particles (Chou and Roizman, 1992). Subsequent biochemical analysis demonstrated that the γ _{134.5} gene product was required to prevent accrual of phosphorylated eIF2, a critical translation initiation factor inactivated by phosphorylation of its α subunit (Chou *et al.*, 1995).

Figure 4 The HSV-1 productive or lytic replication cycle. Following fusion of the virion envelope with the host plasma membrane, the viral nucleocapsid is deposited into the cytosol along with numerous proteins contained within the virus particle. After the nucleocapsid docks at the nuclear membrane, viral DNA translocates through the nuclear pore into the nucleus, and transcription from five immediate-early (IE or α) viral genes commences. Several IE gene products are important for the subsequent expression of the second class of viral genes, the early genes, and return to the nucleus after being synthesized in the cytosol. Early (E or β) mRNAs predominately encode proteins involved in nucleotide metabolism and DNA synthesis. Replicating viral DNA accumulates as a large concatamer in the nucleus, where multiple genome segments are joined end to end, and is subsequently processed into unit length genomes concomitant with packaging into newly assembled nucleocapsids. In addition, DNA synthesis marks the transition from the early phase of the life cycle to the late or γ_2 phase, and is associated with an increase in late γ_2 mRNAs. Late genes (L or γ) encode virion structural proteins. Once assembled in the nucleus, capsids acquire an envelope by budding from the nuclear membrane

Normally, the $\gamma_134.5$ gene product prevents the accumulation of phosphorylated eIF2 α by recruiting a cellular phosphatase, protein phosphatase 1 α (PP1 α), to remove phosphate from eIF2 α (He *et al.*, 1997). Interestingly, the domain of the $\gamma_134.5$ protein that contains this activity is homologous to a domain in the GADD34 protein, a cellular PP1 α binding protein that promotes eIF2 α dephosphorylation in response to other forms of cell stress (Chou and Roizman, 1994; Novoa *et al.*, 2001). Besides their defect in protein synthesis, $\gamma_134.5$ mutant derivatives are hypersensitive to interferon α , and therefore more sensitive to this arm of innate host defenses that serve to limit viral replication in the host (Cheng *et al.*, 2001; Cervený *et al.*, 2003). This hypothesis is supported by reports demonstrating that $\gamma_134.5$ mutant viruses, while neuroattenuated in normal mice and mice with deficiencies in their acquired immune response, exhibit restored neurovirulence in mice with deficiencies in innate immunity (Leib *et al.*, 1999, 2000).

Ensuing genetic studies revealed that HSV-1 actually encodes multiple functions to control eIF2 α phosphorylation, as the dsRNA binding protein specified by the γ_2 or 'true late' *Us11* gene prevents PKR activation (Mohr and Gluzman, 1996; Mulvey *et al.*, 1999; Poppers *et al.*, 2000; Khoo *et al.*, 2002; Peters *et al.*, 2002). Analysis of a panel of $\gamma_134.5$ and *Us11* mutants established that both *Us11* and $\gamma_134.5$ gene products act at different times in the productive growth cycle to regulate eIF2 α phosphorylation in infected cells (Figure 6; Mulvey *et al.*, 2003). Importantly, *Us11*

Figure 5 Inhibition of late viral mRNA translation by activated PKR in cells infected with an HSV-1 $\gamma_134.5$ mutant virus. The temporal pattern of events in the HSV-1 productive growth cycle is depicted. Production of viral immediate-early (IE) gene products triggers expression of viral early (E) genes, leading to the initiation of viral DNA replication and the expression of viral late (L) genes. Two classes of viral late mRNAs, termed γ_1 and γ_2 , have been identified. Transcription of the γ_2 class is stringently dependent upon prior initiation of DNA synthesis. In cells infected with a $\gamma_134.5$ mutant virus, all of the events in the events in the productive growth cycle of HSV-1, up to and including the accrual of γ_2 late mRNAs proceed normally. However, late polypeptides are not produced as phosphorylated eIF2 α accumulates due to the unopposed action of the cellular kinase PKR. Activated, phosphorylated PKR is represented as PKR-P

expressed in its natural context as a late γ_2 protein is required to properly regulate PKR activation, eIF2 α phosphorylation, and viral translation. Thus, since late proteins are not produced in nonpermissive tumor cells infected with a $\gamma_134.5$ mutant virus, the $\gamma_134.5$ mutant is

Figure 6 Regulation of eIF2 α phosphorylation by different functions that act during discrete phases in the HSV-1 life cycle. Early in the HSV-1 life cycle, small quantities of dsRNA, or perhaps other effectors that remain to be identified activate the normally dormant cellular PKR kinase. After assembling a dimer of PKR on dsRNA, each subunit of the multimer phosphorylates the other (PKR-P). Since PKR is activated in cells infected with a γ_1 34.5 mutant virus, preventing the translation of γ_2 mRNAs, we propose that the γ_1 34.5 gene product, through its interaction with PP1 α , is able to adequately dephosphorylate the quantities of phosphorylated eIF2 α (eIF2 α -P) produced from the period preceding the initiation of viral DNA synthesis and extending into the initial segment of the late phase (designated as γ_1 L). However, late in the viral life cycle, synthesis of mRNA from γ_2 genes (γ_2 L), many of which are transcribed from ORFs located on opposing DNA strands, results in the production of large quantities of viral dsRNA (Jacquemont and Roizman, 1975). In the absence of Us11, the increase in dsRNA concentration generates more activated PKR, which in turn phosphorylates eIF2 α . The concentration of phosphorylated eIF2 α quickly rises beyond the capacity of the γ_1 34.5-PP1 α complex to effectively reverse the reaction, accounting for the observed reduction in viral translation rates in cells infected with a Us11 mutant virus. We suggest that while the γ_1 34.5 protein acts downstream of phosphorylated eIF2 α and therefore has the potential to counter a variety of eIF2 α kinases, Us11 acts late in infection to specifically antagonize PKR activation in response to the copious levels of dsRNA produced in virus-infected cells. In this drawing, relative concentrations of PKR-P, dsRNA, and eIF2 α -P at earlier, compared to later times in the viral life cycle, are represented by character size (reprinted from Mulvey *et al.* (2003) with permission)

actually doubly deficient in that it also fails to translate the γ_2 Us11 mRNA. Moreover, the interferon sensitivity of γ_1 34.5 mutants, previously attributed solely to the absence of γ_1 34.5 function, likewise results from the absence of the γ_1 34.5 protein and the failure to synthesize the Us11 polypeptide (Mulvey *et al.*, 2004).

Balancing oncolysis and safety: a translational block limits replication of γ_1 34.5 mutant derivatives

In studies designed to examine the efficacy with which γ_1 34.5 mutants were able to destroy human or murine gliomas implanted into mice, the treated animals survived longer than their untreated counterparts and did not develop encephalitis. Long-term surviving animals (usually in the vicinity of 60–80 days) were produced with efficiencies ranging from 10 to 50% of the treated animals depending on the tumor model and

treatment regimen (Markert *et al.*, 1993; Chambers *et al.*, 1995; Kesari *et al.*, 1995; Randazzo *et al.*, 1995; Andreansky *et al.*, 1996, 1997). γ_1 34.5 mutant viruses also retained a functional thymidine kinase gene and therefore retained their sensitivity to acyclovir, a safe, effective antiviral agent, which could be used, if necessary, to control viral encephalitis. Despite reports of γ_1 34.5 mutants replicating to varying degrees in CNS tissue depending on the viral strain and the immune status of the mice, encephalitis has not been observed in numerous studies performed with γ_1 34.5 mutants in different viral genetic backgrounds (Markovitz *et al.*, 1997; Kesari *et al.*, 1998; Lasner *et al.*, 1998). To further restrict viral replication to actively dividing cells and provide an additional layer of safety, additional mutations in the *UL39* ribonucleotide reductase gene (Figure 3b) or the *UL2* uracil DNA glycosidase gene were introduced into the γ_1 34.5 mutant background (Mineta *et al.*, 1995; Kramm *et al.*, 1997; Pyles *et al.*, 1997). Although each of these non-neurovirulent, multi-mutated viruses could still reduce subcutaneous tumor growth and extend the survival of mice with intracranial tumors, more than 80% of the treated subjects still succumbed, emphasizing a different problem limiting the efficacy and outcome of treatment. While encephalitis was not observed in animals treated with any of these γ_1 34.5 mutant derivatives, the γ_1 34.5 deletion, either alone or in conjunction with additional mutations, impaired the replicative ability of these viruses in many human tumor cells, allowing the growth of residual glioma cells that ultimately killed the animals. Thus, while viruses with engineered mutations in the γ_1 34.5 genes were sufficiently safe, they had lost a substantial amount of their replicative efficacy due to their inability to counter the host's efforts to incapacitate eIF2 successfully. As a result of this impediment to their productive growth, the oncolytic ability of these mutants is limited. In an effort to improve the growth of γ_1 34.5 mutant viruses, the oncolytic ability of derivatives that conditionally express the γ_1 34.5 gene (Chung *et al.*, 1999; Nakamura *et al.*, 2002) or recombinants that contain only one copy of the γ_1 34.5 gene, as opposed to the two copies present in the wild-type genome (Meignier *et al.*, 1988, 1990; Advani *et al.*, 1999), has been evaluated. While these viruses do indeed exhibit increased oncolysis, they are also more neurovirulent than the γ_1 34.5 parent virus, illustrating the potentially serious consequences of using viruses that carry wild-type alleles of the γ_1 34.5 gene. The experiences to date with these different HSV-1 mutant viruses in human clinical trials are reviewed elsewhere in this issue (Aghi and Martuza, 2005).

Crafting the next generation of γ_1 34.5 mutant derivatives through genetic selection in cancer cells

An alternative method for augmenting the oncolytic ability of an HSV-1 γ_1 34.5 mutant serendipitously emerged from further genetic analysis. To learn if other

viral components were important in regulating PKR activation and eIF2 α phosphorylation, a γ_1 34.5 deletion mutant was sequentially passed in nonpermissive cells to select for isolates with restored capacity to replicate (Mohr and Gluzman, 1996). These isolates had all sustained genetic rearrangements where the Us11 γ_2 late promoter and most of the Us12 ORF, including the AUG initiation codon, were deleted (Figure 7). This extragenic or second-site suppressor mutation resulted in the nearby Us12 promoter controlling expression of Us11, a dsRNA binding protein that inhibits PKR activation. Significantly, the Us12 promoter is active at IE times and directs the synthesis of Us11 mRNA, which is normally produced very late in the viral life cycle, as an IE mRNA. Thus, by altering the temporal pattern of Us11 expression, the suppressor mutation enables γ_1 34.5 mutants to replicate efficiently in what were previously nonpermissive cells (Mohr and Gluzman, 1996; Mulvey *et al.*, 1999; Poppers *et al.*, 2000; Khoo *et al.*, 2002). The considerable surprise was that although the suppressor mutant was capable of restored growth in cells that failed to support the replication of the γ_1 34.5 parent virus, it essentially remained as

Figure 7 Genetic structure of an attenuated, oncolytic HSV-1 γ_1 34.5 null virus with an additional growth enhancing mutation. The location of the γ_1 34.5 deletion (a) and the additional extragenic suppressor mutation (b) is shown on the viral genome. The unique long (UL) region and unique short (US) region are shown as solid lines. Repetitive regions appear as open rectangles. This neuroattenuated suppressor variant contains two mutations: (1) both copies of the γ_1 34.5 gene have been replaced with sequences encoding β -glucuronidase (a); and (2) a 583 bp deletion (Δ) that spans the junction region where the viral US segment joins the TRs component (b). The Us10, Us11, and Us12 ORFs are shown. The segment of the Us12 ORFs that is removed by the deletion is represented as a broken rectangle. The two RNAs that are synthesized appear as arrows above the ORFs. Promoter elements that direct the synthesis of these RNAs appear as stars and each promoter is normally associated with either the Us10, Us11, or Us12 ORF (denoted by the number 10, 11, or 12 at the lower right of each star). Note that the suppressor deletion removes the endogenous late Us11 promoter and a large segment of the Us12 ORFs, including the ATG codon. This allows the transcript initiating from the immediate-early (IE) Us12 promoter in the TRs to direct the synthesis of the Us11 protein. Accumulation of Us11 at IE times allows the suppressor mutant to sustain protein synthesis and thus replicate in nonpermissive cells that do not support the growth of γ_1 34.5 mutants. Furthermore, IE expression of Us11 also renders the virus resistant to interferon α , whereas simple Δ 34.5 mutants remain exquisitely sensitive

neuroattenuated as the parental γ_1 34.5 mutant at the doses examined (up to 2×10^7 PFU). This established that it was possible to introduce additional mutations into the genome of an HSV-1 γ_1 34.5 mutant that dramatically improve its replicative ability in cancer cells without increasing neurovirulence in mice (Mohr *et al.*, 2001).

The attenuated neurovirulence profile of the suppressor mutant coupled with its dramatically improved replication properties made it an ideal oncolytic virus candidate. In three studies, using independently constructed viruses in different tumor models, incorporating the suppressor mutation into a γ_1 34.5 mutant derivative resulted in a dramatic improvement in the ability of the virus to inhibit tumor growth (Figure 8; Taneja *et al.*, 2001; Todo *et al.*, 2001; Liu *et al.*, 2003). Finally, in addition to overcoming the block to protein synthesis seen in cells infected with a γ_1 34.5 mutant derivative, the suppressor mutation, by allowing the production of Us11 as an IE protein, confers interferon resistance and allows a γ_1 34.5 mutant virus to counteract this important arm of innate host defenses (Mulvey *et al.*, 2004).

Deciding where to start: recognizing the 5' end of the mRNA and its role in translational control

eIF2 is not the only translation factor whose activity can be exploited to achieve selective viral replication in cancer cells. As eIF4F recognizes the 7-methyl guanine cap at the mRNA 5' end and recruits the 40S ribosomal subunit bound to eIF3, it is a prime target for regulating translation initiation in response to a diverse array of stimuli including viral infection. Although some viruses, exemplified by poliovirus, whose mRNAs do not

Figure 8 Evaluation of γ_1 34.5 mutant derivatives that express Us11 at IE times as an antitumor agent in an animal model of human prostate cancer. Balb/c nu/nu mice ($n=5$ for each treatment group) harboring established, subcutaneous human prostate cancer tumors (produced from PC3 cells) measuring approximately 50 mm^3 received a single injection containing 2×10^6 PFU of either the γ_1 34.5 deletion mutant Δ 34.5 ($\blacktriangle$ -dotted line), the γ_1 34.5 suppressor mutant expressing Us11 as an IE protein ($\blacksquare$), or a virus free lysate prepared from mock infected cells ($\blacklozenge$). Tumors were measured every 2 days for 34 days and the average normalized values reflecting relative tumor size on each day were plotted. The initial tumor volume immediately prior to treatment was normalized to a relative size of 1.0. Error bars reflect the standard error of the mean. Reprinted from Taneja *et al.* (2001) with permission

contain a 5' cap, initiate translation through alternate means, others such as adenovirus and vesicular stomatitis virus (VSV) seek to inactivate the majority of eIF4F as a means of impairing the translation of host mRNAs, while members of the herpesvirus family go to great lengths to stimulate eIF4F activity in order to translate their mRNAs efficiently in quiescent cells. The translation initiation factor eIF4F (Figure 9a; reviewed in Gingras *et al.*, 1999) is a complex of cellular polypeptides whose core is composed of the cap binding protein (eIF4E), a large scaffolding subunit (eIF4G), and an RNA helicase (eIF4A). In addition, eIF4B or eIF4H stimulate the helicase activity of eIF4A, functioning as processivity factors for the helicase (reviewed in Rogers *et al.*, 2002). Depending upon their abundance, a family of small eIF4E binding proteins (4E-BPs) can sequester eIF4E in a phosphorylation-dependent manner (reviewed in Gingras *et al.*, 2001). In their hypophosphorylated form, 4E-BPs inhibit cap-dependent translation. Hyperphosphorylation of the 4E-BPs by the mTOR kinase in response to a variety of environmental cues results in the release of eIF4E, allowing the cap binding protein to associate with eIF4G and assemble an active eIF4F complex (Figure 9b; Haghighat *et al.*, 1995; Mader *et al.*, 1995; Marcotrigiano *et al.*, 1999). In addition, the cap binding protein eIF4E is phosphorylated by the eIF4G-associated kinase mnk-1 (Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997, 1999; Pyronnet *et al.*, 1999; Scheper *et al.*, 2001). While not essential for translation initiation, eIF4E phosphorylation appears to play a regulatory role, although the details of how this is achieved and which mRNAs are most susceptible are poorly understood (reviewed in Scheper and Proud, 2002). Finally, the cellular polyA binding protein, while itself not an eIF4F core component, physically interacts with eIF4G in the eIF4F initiation complex and stimulates translation initiation (Imataja *et al.*, 1998; Kahvejian *et al.*, 2005). This association between the 5' and 3' ends of the mRNA generates a circular or 'closed loop' topology that is thought to be important for translation (Wells *et al.*, 1998; Kahvejian *et al.*, 2001). It is possible that such interactions act as a checkpoint ensuring that the mRNA is both capped and polyadenylated prior to translation initiation. PABP can also be found associated with two cellular polypeptides, Paip1, an eIF4G-related protein that stimulates translation (Craig *et al.*, 1998), or Paip2, a translational repressor that prevents PABP from interacting with Paip1 and the mRNA polyA tail (Khaleghpour *et al.*, 2001).

All of these components are tethered in place at the 5' end of the mRNA through an interaction with eIF4G. In addition to providing a scaffold on which the eIF4F components eIF4E and eIF4A are assembled, eIF4G also binds to the eIF4E kinase mnk-1 and the polyA binding protein PABP (Imataja *et al.*, 1998; Pyronnet *et al.*, 1999). Aside from our perceived role for eIF4G as a molecular scaffold, little is known regarding its function. This is further highlighted by the existence of at least five eIF4GI isoforms, generated by translation initiation at alternate ATG codons, coupled with differential phosphorylation,

Figure 9 (a) The eIF4F complex and associated factors bound to the mRNA. Upon binding the 7-methyl guanine cap (7m) at the 5' end of the mRNA, eIF4E (4E) recruits eIF4G and eIF4A to assemble the eIF4F complex. The 40S ribosome is recruited through its association with eIF3. eIF4G also interacts with the polyA binding protein bound (PABP) to the 3' end of the mRNA and the eIF4E kinase mnk-1, which phosphorylates eIF4E (depicted as P). (b) The translational repressor 4E-BP1 binds eIF4E and is regulated by phosphorylation. eIF4E bound to the 5'mRNA cap (7m) associates with the translational repressor 4E-BP1. Upon phosphorylation by the cellular kinase mTOR, 4E-BP1 is released from eIF4E allowing the recruitment of eIF4G and eIF4F complex assembly

all of which are of unknown functional significance (Raught *et al.*, 2000; Byrd *et al.*, 2002). In response to apoptotic stimuli, eIF4G is cleaved by cellular caspases (Marissen and Lloyd, 1998; Marissen *et al.*, 2000). Furthermore, mammalian cells contain a homolog of eIF4GI, eIF4GII, which appears to be selectively recruited to capped mRNA upon differentiation (Caron *et al.*, 2004). Finally, recent studies demonstrating that the N-terminus of eIF4G undergoes a conformational change upon engaging eIF4E suggest that the role of eIF4G may indeed go beyond that of an inert scaffold upon which other initiation factors assemble (Gross *et al.*, 2003).

Promoting eIF4F complex assembly in HSV-1-infected cells

Given the importance of eIF4F in the initiation and regulation of cap-dependent translation, recent attention has focused on evaluating the impact of HSV-1

infection on this complex in part because herpesvirus mRNAs are capped (Bartkowski and Roizman, 1976). Indeed, suppression of host polypeptide synthesis in cells infected with human adenovirus or VSV is mediated by dephosphorylation of the cap binding protein eIF4E (Huang and Schneider, 1991; Connor and Lyles, 2002). Since host protein synthesis is profoundly impaired in HSV-1-infected cells, it seemed plausible that a similar mechanism could be operative. In marked contrast to findings in other viral systems, eIF4E is rapidly phosphorylated following infection of primary human cells or a variety of established cell lines with HSV-1. Phosphorylation occurred early in the viral life cycle, was sustained throughout the productive growth cycle, and was sensitive to small molecule inhibitors of p38 and mnk, the latter being the cellular eIF4E kinase. More importantly, inhibiting the eIF4E kinase mnk impaired viral protein synthesis and reduced viral replication by 100-fold in primary human cells (Walsh and Mohr, 2004).

Not only does HSV-1 infection promote eIF4E phosphorylation, it also stimulates phosphorylation of the translational inhibitor 4E-BP1 early in its productive replication cycle. Phosphorylation of 4E-BP1 in cells infected with HSV-1 is sensitive to rapamycin, an inhibitor of the cellular kinase mTOR. Moreover, following its phosphorylation, steady-state levels of 4E-BP1 were observed to decline precipitously in infected cells. Proteasome inhibitors or rapamycin effectively prevented the decrease in 4E-BP1 abundance. Thus, following its phosphorylation by mTOR, the translational repressor 4E-BP1 is degraded by the proteasome in HSV-1-infected cells (Walsh and Mohr, 2004). As eIF4E and 4E-BP1 phosphorylation have been reported to stimulate translation in other biological settings, this raised the possibility that the changes to eIF4F in HSV-1-infected cells might be required to stimulate the cellular translation machinery. In addition, recent work has established that the adenovirus E4 ORF1 and ORF4 proteins can both act, albeit through different mechanisms, to stimulate the cellular kinase mTOR, resulting in 4E-BP1 phosphorylation (O'Shea *et al.*, 2005a,b). Perhaps mutant viral strains unable to stimulate signaling pathways that require mTOR function will be part of future strategies to target oncolytic virus replication to tumor cells with uncontrolled growth factor receptor or mTOR activity.

While the steady-state levels of eIF4F core components eIF4E, eIF4G, and eIF4A remain unchanged in HSV-1-infected cells, phosphorylation of 4E-BP1 relieves translational repression by promoting eIF4F complex assembly. Indeed, in growth arrested primary human cells infected with HSV-1, eIF4E is released from the 4E-BP1 repressor and the amount of eIF4G bound to eIF4E increases dramatically (Walsh and Mohr, 2004). This suggests that HSV-1 can promote the assembly of eIF4F complexes in quiescent resting cells. The ability to stimulate eIF4F complex assembly is likely important for productive viral growth in quiescent cells, as the capacity of resting cells to support high levels of protein synthesis are reduced relative to their

exponentially growing counterparts (Stanners and Becker, 1971; Thomas and Gordon, 1979; Thomas *et al.*, 1981; Thomas and Thomas, 1986). Strikingly, although the amount of eIF4G bound to eIF4E increases substantially upon infection of quiescent cells with HSV-1, a concomitant increase in the amount of PABP bound to eIF4G was not observed (Walsh *et al.*, 2005). While it is not a core component of the eIF4F complex, PABP bound to the polyA tail at the 3' end of the mRNA is known to associate with eIF4G bound at the 5' end of the mRNA; moreover, this association between eIF4G and PABP is capable of stimulating translation. Presently, the significance of the failure to recruit PABP into eIF4F complexes following HSV-1 infection of quiescent fibroblasts is not clear; however, upon infection of quiescent fibroblasts with HCMV, a related member of the herpesvirus family, both eIF4G and PABP are recruited into eIF4E-containing complexes (Walsh *et al.*, 2005). As host polypeptide synthesis is not impaired in HCMV-infected cells, it is tempting to speculate that the differential association of PABP with eIF4F complexes might somehow play a role in host shut-off. Further work will hopefully illuminate the significance of this finding and either challenge or support the aforementioned hypothesis.

All of the changes to the eIF4F complex that occur in HSV-1-infected cells require viral gene expression (Walsh and Mohr, 2004). Consistent with this finding, viruses with mutations in the IE *ICP0* gene were unable to promote phosphorylation of eIF4E along with 4E-BP1 or stimulate the assembly of eIF4F complexes in quiescent cells (Walsh and Mohr, 2004). ICP0 is a master regulator of viral gene expression and one of the first viral polypeptides produced in productively infected cells (Elshiekh *et al.*, 1991; Lium and Silverstein, 1997). It localizes to nuclear ND-10 domains where it induces the redistribution and subsequent destruction of the cellular PML protein. In addition, ICP0 possesses an intrinsic ubiquitin protein ligase activity and functions as a promiscuous transactivator of viral gene expression (reviewed in Everett, 2000; Hagglund and Roizman, 2004). Although ICP0 is not essential for replication in cultured cells, *ICP0* mutant viruses are deficient for growth at low MOIs in cultured cells (Stow and Stow, 1986; Sacks and Schaffer, 1987) and unable to reactivate from latency in a mouse model of infection (Cai *et al.*, 1993; Halford and Schaffer, 2001).

Although genetic analysis has established that the modifications to eIF4F observed in cells productively infected with HSV-1 depend upon the product of the multifunctional regulatory protein encoded by the *ICP0* gene, exactly how ICP0 promotes these changes remains to be determined. Given that ICP0 is one of the first proteins produced in infected cells, it might positively regulate expression of one or more downstream genes whose products act directly on kinases upstream of mnk or mTOR to ultimately result in phosphorylation of eIF4E together with 4E-BP1. Likewise, a downstream effector might directly associate with one or more components of eIF4F to promote complex assembly. Alternatively, it is certainly possible that ICP0 acts

directly and possesses an intrinsic ability to trigger the observed modifications of eIF4F. Future studies aimed at revealing how viral factors promote eIF4F complex assembly may not only contribute to our understanding of how translational is regulated during the herpesvirus life cycle and produce new insights into translational control mechanisms, but also they may expose new strategies to limit selectively replication of oncolytic HSV-1 strains to cancer cells, which have intrinsically greater rates of translation and in some cases, a greater abundance of limiting translation initiation factors. For example, an HSV-1 derivative that is unable to stimulate eIF4F complex assembly in quiescent cells may be unable to replicate effectively in terminally differentiated cells; however, this defect may be suppressed in rapidly dividing tumor cells. While the test of this hypothesis awaits the identification of the viral gene (s) responsible for this phenotype, its inactivation may contribute to selective oncolytic viral replication in malignant cells via a novel mechanism.

Theme and variations: inhibiting translation by sequestering the message

On its simplest level, if the mRNA does not gain access to the cytosol, it is unavailable to ribosomes and will not be translated. Mutant derivatives of two different oncolytic viruses have been isolated that capitalize on this premise to conditionally replicate in cancer cells. The first instance involves sequestering the host interferon β mRNA within nuclei, preventing the subsequent accumulation of host components involved in inactivating eIF2 in neighboring cells, whereas in the second, a viral mRNA critical for proper translational control in infected cells is not exported from nuclei and its protein product is not made.

Taking advantage of the reduced sensitivity of many cancer cells to interferon α/β , VSV has shown promise as an oncolytic virus in mouse models (reviewed by Lichty *et al.* (2004) and in this issue by Barber, 2005). An important pathogen affecting domestic livestock, notably cattle, horses, and swine, VSV is a member of the rhabdoviridae family, possessing a ssRNA genome of negative polarity. Although its natural cycle remains largely unknown, insects in endemic areas transmit VSV. In animals, the infection is seldom fatal and naturally occurring human infections are rare, involving predominately lab workers and livestock workers (reviewed in Rodriguez, 2002). Most are asymptomatic or associated with mild symptoms. However, one case of viral encephalitis was reported in a 3-year-old (Quiroz *et al.*, 1988).

VSV is exquisitely sensitive to type 1 interferons (α/β). Significantly, the ability of normally resistant MEFs to support VSV replication increases with transformation, correlating with a decrease in their interferon responsiveness (Stojdl *et al.*, 2000b; Balachandran and Barber, 2004). Recently, the guanine nucleotide exchange activity of eIF2B was reported to increase in trans-

formed cells, maintaining pools of unphosphorylated eIF2 α sufficient to support VSV mRNA translation and replication (Balachandran and Barber, 2004). This might also play a role in the replication of VSV under hypoxic conditions known to be present in a variety of tumors (Connor *et al.*, 2004).

To constrain VSV replication to neoplastic cells and perhaps develop a suitable oncolytic strain that would be safe in cancer patients, who are often immunocompromised to some degree, natural VSV variants (AV1, 2, 3) known to produce small plaques in interferon-responsive cells have been investigated.

AV strains contain alleles of the M gene, which encodes a structural polypeptide, that are exceptional inducers of antiviral response. The wild-type M protein blocks mRNA export by binding the mRNA nuclear export factor Rae1/mrnp41 (Faria *et al.*, 2005), preventing the cytoplasmic accumulation of host mRNAs, one of which encodes interferon β (Stojdl *et al.*, 2003). The AV mutants are thought to produce mutant M polypeptides unable to prevent efficiently the export of interferon β mRNA to the cytosol. However, many cancer cells are either able to complement the defect in mRNA transport displayed by the M protein or are impaired in their ability to respond to interferon β , and are thus fully permissive for the replication of the AV strains (Stojdl *et al.*, 2003). Although the primary target here clearly is mRNA export, the ultimate effector may be eIF2, as interferon β alerts neighboring cells to the presence of a virus allowing them to accumulate components vital for host defense, one of which is the eIF2 α kinase PKR. Upon activation by dsRNA, PKR phosphorylates eIF2 α inactivating this critical translation initiation factor and thereby conferring resistance to VSV (Stojdl *et al.*, 2000a). Presently, while VSV variants that are deficient in inducing interferon production are useful in destroying certain tumors that do not respond to ifn, it is clear that there are still tumor cells, such as the PC3 prostate cancer cell line, that can mount an interferon response and are unable to support replication of even WT VSV (Ahmed *et al.*, 2004). It is noteworthy, however, that PC3 cell tumors are effectively treated with an oncolytic HSV-1 strain genetically selected to replicate in cancer cells (Taneja *et al.*, 2001).

In a more extreme example of globally altering cellular mRNA metabolism that begins prior to the onset of viral gene expression, infecting HSV-1 particles deposit a virus-encoded ribonuclease into the host cytosol. A FEN-1-related nuclease, vhs enhances the turnover of viral and cellular mRNAs alike (Everly *et al.*, 2002). Vhs is specifically targeted to mRNA through an interaction with cellular translation initiation factors eIF4AII (GS Read, personal communication) together with eIF4H and eIF4B (Feng *et al.*, 2001; Doepker *et al.*, 2004; GS Read, personal communication). Moreover, vhs has recently been found tethered to at least a subset of eIF4F cap-binding complexes (GS Read, personal communication). While not essential for viral replication in cultured cells (Read and Frenkel, 1983), vhs is an important pathogenesis determinant as vhs mutant viruses are attenuated in

mouse models of infection (Strelow and Leib, 1995; Smith *et al.*, 2002). The enhanced turnover of host mRNAs important for innate responses, such as those encoding type 1 interferons, likely contributes to the reduced virulence of these mutants (Leib *et al.*, 1999; Murphy *et al.*, 2003; Duerst and Morrison, 2004). Irrespective of the vhs-promoted destabilization of mRNA that begins upon entry, the ICP27 protein produced at IE times in the HSV-1 life cycle inhibits splicing in infected cells, preventing the proper processing of cellular transcripts, which is linked to their export from nuclei. In turn, ICP27 promotes the export of viral mRNAs the overwhelming majority of which are unspliced (reviewed in Sandri-Goldin, 2004). Thus, HSV-1 uses two functions to destroy and sequester host mRNAs. As ICP27 is absolutely required for viral replication, it is unlikely that ICP27 mutants would be incorporated into oncolytic viral strains unless tumor cells are identified that provide a cellular ICP27-like function in trans. Vhs, on the other hand, is not required for viral replication and vhs mutants might conceivably be used to further limit viral replication to tumors unable to mount an interferon response.

Unlike the scenario involving the VSV M protein, the mRNA culprits retained in the nuclei of cells infected with an oncolytic strain of adenovirus are in fact viral transcripts. A DNA virus that naturally infects humans but is associated with little or mild disease, mutant adenovirus strains that selectively replicate in cancer cells have been described (reviewed in Lichtenstein and Wold, 2004). Perhaps the best characterized of these mutant strains contains a mutation in the E1b region that results in the failure to export late viral mRNA from the nucleus (O'Shea *et al.*, 2005a,b). While the resulting block to replication in normal cells likely results from the combined effects of retaining multiple late mRNAs that specify the various components of the adenovirus capsid within nuclei, it is particularly noteworthy that the late mRNA encoding the 100 K protein is among this class of sequestered transcripts. One of the functions of 100 K is to promote the translation of adenoviral late mRNAs, all of which have a common tripartite leader sequence spliced onto their capped 5' end (Cuesta *et al.*, 2000; Xi *et al.*, 2004). The tripartite leader is in fact a *cis*-acting genetic element that promotes translational initiation by ribosome shunting (Yueh and Schneider, 1996). Central to its ability to promote shunting, the tripartite leader contains a region complementary to a segment of 18S rRNA (Yueh and Schneider, 2000). Following recruitment of the 40S ribosome to the 5' end through an association with eIF4F and eIF3, the search for the AUG codon initially proceeds through a scanning mechanism; however, once the ribosome encounters the tripartite leader, it appears to be transferred to the AUG by a nonlinear translocation process (Yueh and Schneider, 1996). The mechanism through which 100 K promotes shunting involves both its binding to mRNA, which is regulated by tyrosine phosphorylation, and eIF4G (Xi *et al.*, 2004, 2005). The interaction of eIF4G with 100 K displaces the mnk kinase, resulting in the

accumulation of unphosphorylated eIF4E. It is thought that the eviction of mnk from the complex by 100 K plays a role in the inhibition of host protein synthesis in adenovirus-infected cells (Cuesta *et al.*, 2000). A similar shunting strategy is activated following heat shock and promotes the translation of hsp70 mRNAs (Yueh and Schneider, 2000).

Not beginning at the beginning: internal ribosome entry sites and their utility in promoting viral oncolysis

Cis acting translational control elements can also play an important role in viral replication, and as such may contribute to viral pathogenesis. In the case of poliovirus, the internal ribosome entry site or IRES plays a critical role in recruiting the cellular translation machinery to the + sense RNA genome, which serves for all intensive purposes as mRNA. Despite the fact that viral mRNAs are effectively translated, the translation of cellular mRNAs, the vast majority of which contain a 7-methyl guanine cap at their 5' end and require the cap binding protein eIF4E to initiate translation, is rapidly impaired. The inhibition of host polypeptide synthesis is due to the proteolytic cleavage of eIF4G at a specific site such that the eIF4E-binding domain is severed from the remainder of the protein. Thus, although the truncated form of eIF4G cannot be recruited to the 5' ends of cellular mRNAs, it still can be effectively tethered to the poliovirus IRES, selectively allowing the translation of mRNAs that do not require the function of the cap binding protein, eIF4E (reviewed in Hellen and Sarnow, 2001). The poliovirus IRES is also an important determinant that contributes to the neurovirulence of poliovirus (Gromeier *et al.*, 1996). Although the precise mechanisms through which the IRES contributes to neuropathogenesis is not understood, tissue-specific factors that mediate IRES activity, and in turn viral protein synthesis, do not appear to be responsible for poliovirus neurovirulence (Kauder and Racaniello, 2004). Nevertheless, it is possible to attenuate poliovirus by exchanging the polio IRES with a similar genetic element from rhinovirus type 2, a related virus in the same family. Not only is this recombinant virus neuroattenuated, it is rapidly cytolytic in malignant cells that harbor CD155, the poliovirus receptor (Gromeier *et al.*, 2000). This receptor is expressed in a majority of malignant gliomas, a particularly devastating CNS tumor for which new therapies are desperately needed. Significantly, a single intratumoral injection of this virus was effective in eliminating established intracerebral xenografts in a mouse model of malignant glioma.

Concluding remarks

Not only must viruses go to great lengths to commandeer the cellular translational machinery, but it is equally vital that they have the capacity to preserve its activity by countering a potent host response. The

recruitment of 40S ribosomes to the 5' end of capped mRNAs requires successfully engaging the translation initiation factor eIF4F, while simultaneously preserving the activity of eIF2, a target of host innate defenses. Anything short of success in either of these endeavors will undoubtedly compromise the production of viral proteins, the subsequent assembly of viral progeny, and in turn severely curtail viral replication. Since the translation of viral proteins is a fundamental event in

virus replication and pathogenesis, interfering with how viruses manipulate the translational apparatus provides an opportunity to engineer new oncolytic viral strains that selectively replicate in neoplastic cells.

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