A Global View of Translation in Vesicular Stomatitis Virus Infected Cells

A Global View of Translation in Vesicular Stomatitis Virus Infected Cells

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Abstract

Infection of mammalian cells with vesicular stomatitis virus (VSV) results in the inhibition of cellular translation, while viral translation proceeds efficiently. This host "shutoff" is mediated by multiple mechanisms. VSV infection alters the phosphorylation of cellular eIF4E-BP1, thereby sequestering the cap-binding protein eIF4E. The viral matrix protein, M, blocks nuclear export of host mRNPs, and inhibits cellular transcription, suppressing the host mRNA pool. VSV replicates within the cytoplasm, and during transcription the viral polymerase transcribes 5 mRNAs that are structurally identical to cellular mRNAs with respect to their 5′ cap-structure and 3′-polyadenylate tail. We employed the global approach of massively parallel sequencing cytoplasmic, monosome, and polysome-associated mRNA to interrogate the impact of VSV infection of HeLa cells on translation. Analysis of sequencing reads in the different fractions shows > 60% of the cytoplasmic and polysome-associated reads map to the 5 viral mRNAs by 6 hours post-infection (hpi), which corresponds with host shutoff. Consistent with the overwhelming abundance of viral mRNA on polysomes, reads mapping to cellular genes were reduced after infection, although a subset exhibited increased polysome association. Analysis of viral and cellular mRNA distributions within polysome profiles by quantitative PCR supports a redistribution of cellular mRNAs to smaller polysomes and monosomes in infected cells. To test the contribution of blocking nuclear mRNA export to shutoff, we infected cells with a viral mutant in M (M51R), which is

defective in blocking nuclear export. This analysis reveals > 50% of cytoplasmic and polysome-associated reads map to viral mRNAs by 6 hpi, confirming the role of viral mRNA abundance in host shutoff. Furthermore, interferons were differentially expressed 6 hpi with M51R. Interferons were not upregulated at 2 hpi, or during infections with VSV harboring wild-type M, indicating viral replication is required for innate immune sensing, and that blocking nuclear export principally inhibits interferon gene expression. These results provide a global view of host mRNA translation in response to VSV, supporting a model in which viral mRNA abundance is a key determinant of host shutoff, with inhibiting mRNP export and eIF4E sequestration contributing additional effects.

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To my friends: I love you all. You are the most incredible group of human beings, and it has been the greatest joy of my life to grow with you. You're brilliant and loving, funny and insightful, and your support has kept me going when it seems like nothing is going the way I planned. To the six: from basement parties, to misadventures in Vermont, to six and-a-half and beyond. You are my family, and I love you. To my Boston family, thank you for picnics, for the 4th of July, and Sunday night dinners. You all inspire me every day, and I'm so excited to see what the future has in store for us.

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Chapter 1: Introduction

Vesicular stomatitis virus

Replication Cycle

Vesicular stomatitis virus is the prototype of the *Rhabdoviridae* family of non-segmented, negative strand RNA viruses.[1] The single-stranded negative sense genome encodes for the five viral proteins: nucleocapsid (N), which encapsidates the genomic RNA; the phosphoprotein (P) the cofactor of the viral polymerase; the matrix protein (M) which is important for mediating host shutoff as well as virion assembly; the glycoprotein (G) which is responsible for fusing viral membranes with cellular membranes; and the large protein (L) which is the viral RNA-dependent RNA polymerase. The VSV polymerase (L) is responsible for both transcription and replication of the viral genome. The replication cycle of VSV is summarized in Figure 1.1. VSV gains entry to a cell via clatherin mediated endocytosis.[2, 3] The dimensions of the viral particle dictate a requirement for actin to successfully mediate internalization.[4, 5] Endosomal acidification leads to a pH-dependent conformational change in the viral glycoprotein resulting in the fusion of the viral envelope with the host cell membrane of the late endosome, Figure 1.1.[3, 6] The viral core, consisting of the negative sense genomic RNA coated in N, and associated with L and P is competent for all additional steps of replication.[7-9] Indeed, transfection of viral cores alone is sufficient for replication and the production of infectious progeny virus.[10] Upon escape from the endosomal compartment, the viral polymerase initiates a primary round of transcription to produce the five viral messenger RNAs (mRNAs), Figure 1.1.[11-13]

The VSV mRNAs are transcribed by L via a start-stop mechanism that results in the production of 5 individual mRNAs, Figure 1.2A.[14-18] These transcripts are

Figure 1.1. Vesicular stomatitis virus replication cycle. Schematic of principle steps during VSV replication in a mammalian cell. After attachment (1) and endocytosis (2), pH-dependent fusion in the late endosome deposits the viral core into the cytoplasm (3). Viral N-RNA cores associated with L and P undergo an initial round of primary transcription, producing the five individual viral mRNAs (4). Viral mRNAs are translated in the cytoplasm, and translation by membrane-bound ribosomes leads to the G protein being cotranslationally inserted into the endoplasmic reticulum (5). G then traffics through the Golgi and secretory pathway and is ultimately deposited in microdomains on the plasma membrane. The other four viral mRNAs are translated by soluble ribosomes in the cytoplasm, and the production of new proteins leads to replication of the viral genome through a positive sense antigenome intermediate (6). Newly replicated and encapsidated genomic RNAs can then be transcribed to amplify the viral mRNAs, so-called “secondary transcription” (7), and/or trafficked to the plasma membrane where they bud out through G-rich microdomains from the infected cell (8-9).

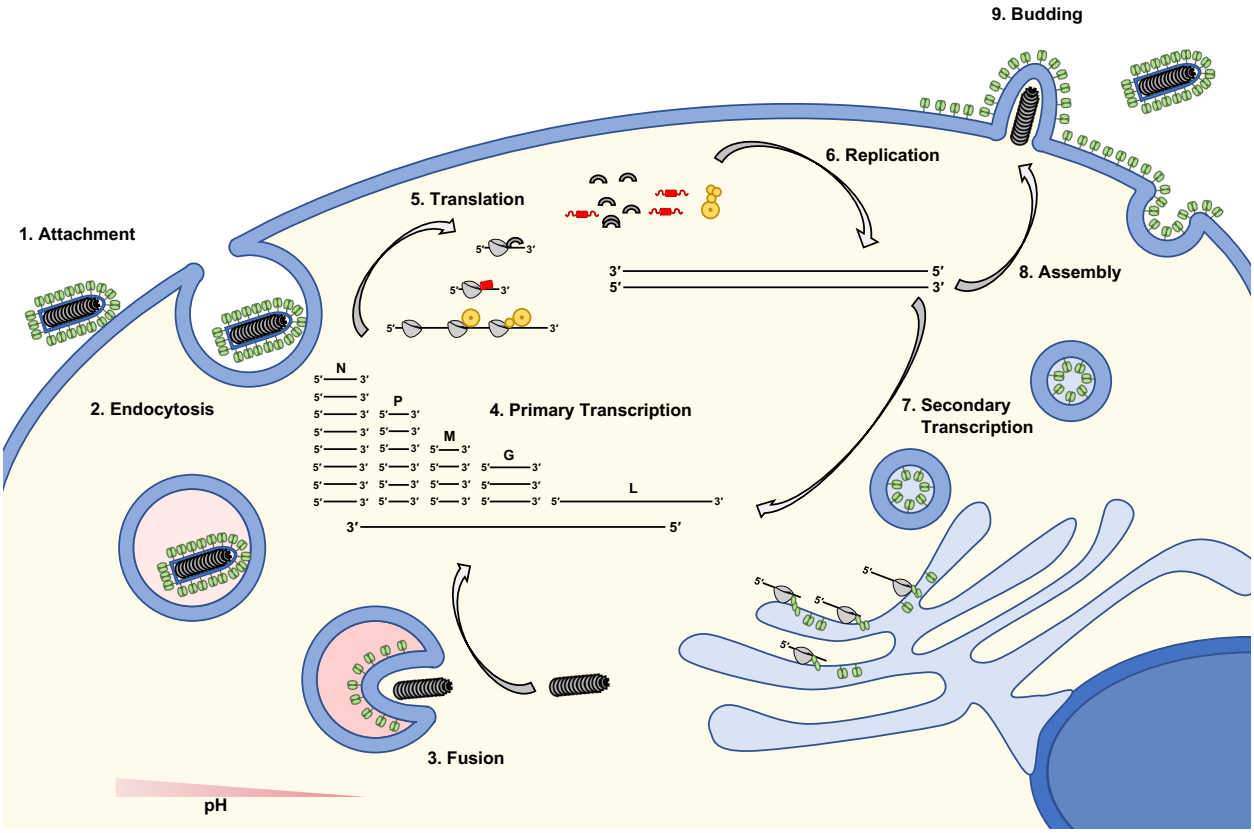

Figure 1.1 (Continued). Vesicular stomatitis virus replication cycle.

Figure 1.2. Structure and function of the large protein (L), the RNA-dependent RNA polymerase of vesicular stomatitis virus. (A) Organization of the VSV genomic RNA. The five viral genes, shown to scale, are transcribed in a cascade of abundance related to their position on the viral genome such that N>P>M>G>L. (B) Cryo-EM structure of VSV L with the RdRp domain colored in blue, the capping domain in pink, and the methyltransferase domain in green. The C-terminal domain and the linker region are both colored in gray. The figure is based on the structure 5a22 deposited in the Protein Data Bank (PDB). (C) Cap structure of VSV mRNAs from infected cells. N-7 cap methylation (green) and 2′-O methylation (cyan) of the first adenosine residue A_1 are performed by the methyltransferase domain of L. This activity can be reconstituted *in vitro*. Additional N6 methylations at A_1 and A_2 (red), as well as 2′-O at A_2 (cyan) have been observed only in mRNA isolated from infected cells. At the 3′ end of VSV mRNA is a polyadenylate tail added by the polymerase through reiterative copying of a 7 residue polyU tract. (D) Host shutoff during VSV infection. HeLa cells infected with VSV at MOI 3 were incubated with ^{35}S methionine and cysteine to label newly synthesized proteins at the indicated times post infection. Between 2 and 6 hpi cellular protein synthesis is inhibited while initiation continues on viral transcripts.

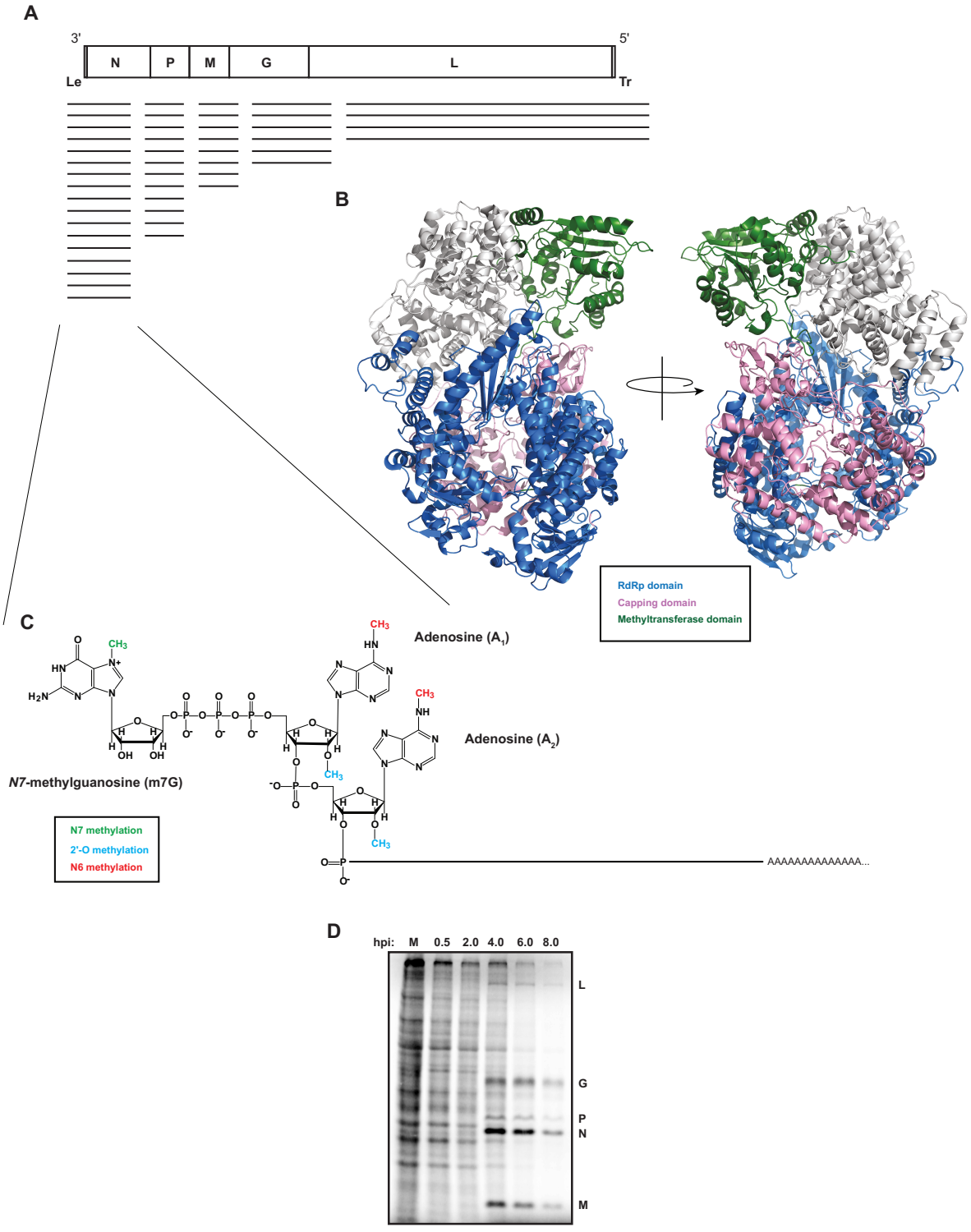

Figure 1.2 (Continued). Structure and function of the large protein (L), the RNA-dependent RNA polymerase of vesicular stomatitis virus.

present in a cascade of abundance corresponding to their position on the viral genome, Figure 1.2A.[19-21] Attenuation of transcription at the gene junctions results in an ~30% decrease in abundance between each transcript, with N being the most abundant transcript, followed by P, M, G, and L.[18, 22]

Translation of viral mRNAs occurs in the cytoplasm, and for all viral mRNAs with the exception of G, translation occurs on soluble cytosolic ribosomes, Figure 1.1.[23-25] G is translated predominantly by membrane-bound ribosomes.[24-26] Co-translational insertion of G into the endoplasmic reticulum leads to its trafficking through the secretory pathway, and ultimate deposition in microdomains on the plasma membrane.[27, 28] Viral cores of N-RNA are transported with P and L to the plasma membrane where they are condensed by M and bud from the cell through G-containing microdomains.[29] Budding of viral particles is facilitated by the late-domain motifs of M, which recruit the cellular endosomal sorting complex required for transport (ESCRT).[30] Viruses with mutations in the M late-domain motifs assemble viral particles, but the mature virions fail to pinch off from the plasma membrane.[30-32]

Structure and function of L

The viral polymerase (L) is a multifunctional protein responsible for all steps of viral transcription and genome replication.[8, 11] Core enzymatic activities of the polymerase, as well as the architecture of the protein are summarized in Figure 1.2. The polymerization domain contains a classical finger and palm structure characteristic of many RNA and DNA polymerases, Figure 2B.[33] L cannot directly engage the template N-RNA, but relies on its co-factor, the phosphoprotein P, to bridge the interaction

between L and the N-RNA.[34-37] Association of L with the template RNA requires the transient dissociation of 2-3 molecules of N from the N-RNA complex.[33, 38] L initiates transcription from a single entry point on the 3′ end of the viral RNA, producing a short leader RNA with a 5′ terminal triphosphate before sequentially transcribing the 5 viral mRNAs, which all begin with the conserved transcriptional start sequence "AACAGnnAUC".[19, 21, 39, 40] This sequence is required for proper mRNA processing and gene expression.[41, 42] Evidence exists for a second entry site at the first gene start sequence of N *in vivo*, but this observation has not yet been fully reconciled with *in vitro* results supporting a single entry site.[39, 43, 44]

Once nascent transcripts have reached 31 nucleotides in length, they are capped by the viral capping domain, Figure 1.2B, via an unusual covalent enzyme-RNA intermediate.[45, 46] This classifies the VSV capping enzyme as a polyribonucleotidyl transferase (PRNTase), rather than the typical guanylyl transferases used during the capping of cellular mRNAs.[45, 47] The caps are then sequentially methylated at the 2′-O and N-7 positions by the L methyltransferase domain, with 2′-O cap methylation preceding and facilitating N-7 methylation.[48-56] The structure of VSV mRNA caps is depicted in Figure 1.2C. L terminates transcription at the end of each mRNA, and through reiterative copying of a 7 residue polyU tract adds a polyA tail of variable length.[41, 57-64] The capped, methylated, and polyadenylated viral mRNAs are structurally identical to cellular mRNAs, and can be further modified by cellular methyltransferases at the first two adenosine residues of the transcription start sequence A_1A_2CAG contained in all viral 5′ untranslated regions (UTRs) to form a 5′ end with the structure $m^7Gppp(m^6)A_1mp(m^6)A_2(m)pCpApG$-, Figure 1.2C.[49, 65, 66]

During genome replication, L enters at the 3′ end of the viral RNA and ignores all signals for gene start-stop junctions to synthesize a full-length complementary, positive sense antigenome.[67-69] This antigenome then serves as a template for genome replication. Genome replication is dependent upon N protein synthesis for encapsidation of genomic RNA.[70, 71] Genome replication produces additional negative strand genomes which are transcribed during secondary transcription, thereby amplifying viral mRNA levels, Figure 1.1. New genomes can also be packaged into budding virions.

Translation of VSV mRNAs

Consistent with the structural identity between fully processed eukaryotic mRNA and VSV mRNAs, translation of viral messages is thought occur through the canonical initiation pathway. In support of this hypothesis, β-elimination of the methylated caps of viral mRNAs *in vitro* led to decreased association of the de-capped mRNAs with ribosomes following incubation in wheat germ translation extracts.[72-75] Furthermore, VSV mRNAs do not contain an IRES. In fact, the viral mRNAs all have 5′ UTRs significantly shorter than the average cellular 5′ UTR, which ranges from ~100-200 nucleotides across species.[76] The viral N, P and L mRNAs have 5′ UTRs of 14 nucleotides or less, and all the viral mRNAs are AU-rich, suggesting complex secondary RNA structures do not drive translation initiation.[40, 57, 77] While VSV translation is thought to be cap-dependent, it exhibits an unusual hypersensitivity to depletion of the large ribosomal subunit protein, rpl40.[78]

Rpl40 is synthesized as an C-terminal fusion peptide to ubiquitin. The ubiquitin is cleaved off, contributing to the pool of free ubiquitin in the cell, and rpl40 is incorporated

into the 60S ribosomal subunit.[79, 80] Incorporation of rpl40 into the ribosome occurs late during assembly, after the subunits are localized to the cytoplasm.[81] Structural data indicates rpl40 is surface exposed, and sits in close proximity to the GTPase stimulating eIF5B factor, suggesting it may play a role in regulating subunit joining.[82] It has also been postulated that rpl40 may prevent translation initiation by immature ribosomes, indicating rpl40 may function as a quality control checkpoint during ribosome processing and maturation.[82] Consistent with this, deletion of rpl40 is lethal in yeast, but both yeast and mammalian cells can be depleted of rpl40 with minimal effects on polysome formation and rRNA processing.[78, 81, 83]

The precise mechanism by which rpl40 contributes to VSV translation is unknown, but depletion of rpl40 in HeLa cells using siRNA prevents GFP expression from an eGFP containing reporter virus.[78] This block to infection is downstream of entry as transfected viral cores are also defective in gene expression, but occurs after primary transcription, which is unaffected by rpl40 depletion.[78] *In vitro* translation extracts prepared from yeast cells depleted of rpl40 are unable to translate exogenous reporter mRNA containing the VSV UTRs, indicating the phenotype is evolutionarily conserved, and dependent on a *cis*-acting element in the viral UTRs.[78] Deep sequencing of polysome-associated mRNAs in wild-type yeast cells, and cells depleted of rpl40 identified cellular mRNAs that also exhibited sensitivity to rpl40, but did not identify any sequence elements or structural similarities with viral mRNA.[78]

Host Shutoff in VSV-infected Cells

Infection of mammalian cells with VSV results in the rapid inhibition of host cell gene expression, Figure 1.2D.[84] In HeLa cells labeled with ^{35}S-methionine, which is incorporated into newly synthesized proteins, synthesis of host cell proteins is rapidly inhibited between 2 and 6 hours post infection, Figure 1.2D. Translation of cytoplasmic viral transcripts, which are structurally indistinct from cellular messages, continues unabated, Figure 1.2D. The decrease in ^{35}S labeling of cellular proteins during infection is concomitant with the collapse of large polysomes, and a corresponding increase in 80S monosomes, indicative of a defect in translation initiation.[14, 85] Consistent with this, it was demonstrated that rates of elongation were unchanged during VSV infection.[86]

Despite the profound inhibition of cellular mRNA translation, and global suppression of polysome formation, the rate of total ^{35}S-methionine incorporation in VSV-infected cells decreases only ~35% by 4.5 hours post infection.[87] This is due to the robust synthesis of viral proteins. The discrepancy between continued initiation on capped and methylated viral mRNAs during a profound inhibition of cellular cap-dependent translation has been the focus of intense study for decades. Cumulatively, these studies have demonstrated that the inhibition of host cell gene expression is mediated by 1) the pleiotropic effects of the viral matrix protein (M), which inhibits transcription from all cellular polymerases and blocks nuclear export of cellular mRNPs, and 2) decreased translation initiation through activation of eIF4E-BP1 and subsequent sequestering of the cap-binding protein, eIF4E.

Eukaryotic Translation Initiation

Overview

Canonical translation in eukaryotic cells initiates with recognition of the 5′ ends of mRNAs by the eIF4F complex, summarized in Figure 1.3. The eIF4F complex consists of the scaffolding protein eIF4G, the cap-binding protein eIF4E, and the helicase eIF4A.[88-90] The cap-binding protein eIF4E recognizes the 5′ methylguanosine cap of eukaryotic mRNAs and has long been considered the rate-limiting factor for initiation, although recent work suggests physiological levels are in excess of the amount required for development and tumorigenesis.[91-93] Recruitment of the eIF4F complex to the 5′ end of mRNAs allows for interactions between eIF4G and polyA-binding protein (PABP) bound to the polyA tails of cellular messages.[89, 90, 94] These interactions lead to the circularization of mRNAs forming a “closed-loop” structure, which is thought to enhance initiation and may facilitate translational control by 3′ mRNA elements.[88, 89, 95]

Activation of an mRNA by eIF4F binding leads to recruitment of the 40S subunit of the ribosome in association with eIFs 1, 1A, 3, and 5 as well as the ternary complex composed of eIF2 and the initiating methionine tRNA.[88-90] This pre-initiation complex (43S PIC) then scans the 5′ UTR of the mRNA until a start codon within a suitable context is identified. Scanning and start codon recognition are facilitated by the eIF1 and eIF1A proteins.[96] Upon start codon recognition, eIF1 dissociates from the PIC, and the GTPase-activating factor eIF5 stimulates eIF2 to hydrolyze GTP and release P_i.[88-90] 60S ribosome subunit joining is then catalyzed by eIF5B, forming an elongation competent 80S ribosome.[97]

Figure 1.3. Eukaryotic translation initiation and viral subversion. Overview of the recruitment of ribosomes to a canonical mammalian mRNA, beginning with cap recognition by eIF4F. Interactions between the 5′ and 3′ ends of the mRNA lead to circularization and loading of the small 40S subunit of the ribosome in complex with initiation factors, called the “43S PIC”. The 43S PIC scans the 5′ UTR until a suitable start codon is recognized, at which point the energy from GTP hydrolysis leads to 60S joining and the formation of an elongation competent 80S ribosome. Steps in the initiation pathway that are targeted by viruses with diverse replication strategies are highlighted in blue boxes.

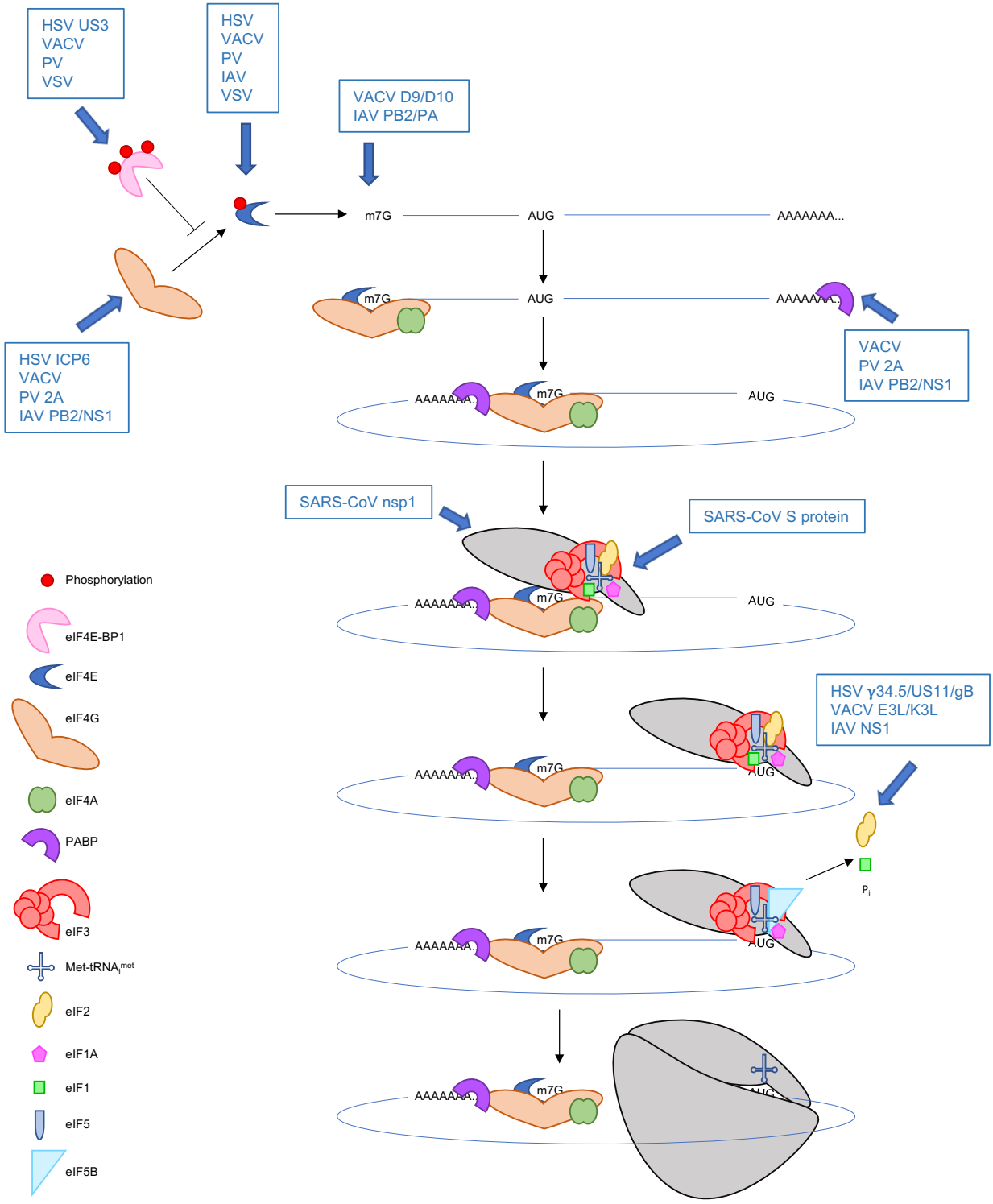

Figure 1.3 (Continued). Eukaryotic translation initiation and viral subversion.

For cellular mRNAs on which initiation is efficient, multiple ribosomes may bind and translate the message simultaneously, leading to polyribosome formation. Ribosome density on an mRNA is often used as a surrogate for the translation efficiency of a given message. RNAs with three or more ribosomes bound are generally considered well translated, while messages bound by a single ribosome, or monosome, are considered poorly translated.[98]

Because eukaryotic translation initiation requires the coordinated functions of over a dozen initiation factors, it is a prime target for the regulation of cellular protein synthesis. Indeed, initiation is the primary step of protein synthesis targeted for regulation both by the cell, and by viruses that infect eukaryotic organisms.[99] Cap binding by eIF4E is regulated by phosphorylation.[100, 101] An additional level of regulation is conferred by a family of eIF4E-binding proteins (eIF4E-BPs), which are also regulated by phosphorylation, Figure 1.3.[90, 100, 101] Hypophosphorylation of eIF4E-BPs leads to their activation and binding to eIF4E, thereby decreasing the rate of cellular cap-dependent translation initiation. Phosphorylation levels of eIF4E-BPs are controlled by the mammalian target of rapamycin (mTOR) signaling pathway, which coordinates multiple homeostatic input signals such as amino acid availability and growth factor signaling.[102] Activation of the mTOR pathway results in sequential eIF4E-BP hyperphosphorylation and the release of eIF4E, allowing the cap-binding complex to initiate protein synthesis and leading to an increase in cellular cap-dependent translation.[90, 100, 101]

In particular, eIF4E is thought to be important for the translation of mRNAs with long, structured 5′ UTRs. This effect is thought to be mediated through eIF4E's ability to

recruit and stimulate the activity of the eIF4A helicase.[89, 90] Additionally, the translation of mRNAs with a 5′ terminal oligopyrimidine (TOP) motif has been shown to be hypersensitive to the inhibition of the mTOR. Genetic studies have indicated this effect is dependent on the eIF4E-BPs, but the precise mechanism leading to decreased translation efficiency and polysome association of TOP mRNAs following mTOR inhibition has not been defined.[103, 104]

Downstream of cap recognition, additional levels of translation initiation regulation are conferred by eIF3 and eIF2.[89, 90] Regulation of eIF2 occurs through phosphorylation of its alpha subunit. Phosphorylation of eIF2α in response to stresses such as endoplasmic reticulum stress or the detection of viral nucleic acids, results in eIF2α tightly binding the guanine nucleotide exchange factor eIF2b, inhibiting its activity.[88, 94] This prevents ternary complex recycling and leads to a global inhibition of canonical translation initiation.[88, 94] However, the translation of eIF2α stress response mRNAs such at ATF4 and ATF5 is enhanced, allowing the cells to respond and recover from stress.[88, 89]

The initiation factor eIF3 is itself composed of over a dozen independent subunits that associate along the mRNA entrance channel of the 40S ribosome, and make extensive contacts with the 5′ UTRs of cellular mRNAs.[89, 90] This allows eIF3 to serve as a platform for regulating the translation of subsets of cellular mRNAs. Recent work has identified mRNAs regulated by an eIF3-specific mechanism during transformation and tumorigenesis.[105] Additionally, eIF3d has been shown to exhibit cap-binding activity, suggesting that eIF3d could potentially substitute for eIF4E during non-canonical translation initiation.[106] During the cellular response to heat shock,

heat shock protein mRNAs are co-transcriptionally modified with *N6*-methyladenosine in their 5′ UTRs. This methylation results in non-canonical translation initiation through eIF3 recognition of the epigenetic mark, and direct recruitment of the ribosome by eIF3, independent of eIF4E.[107-109]

Structure and Function of eIF4E

The cap-binding protein eIF4E, and its preference for binding m7G, were first identified through crosslinking studies of 5′ radiolabeled reovirus mRNA.[110] Subsequent crystallographic studies demonstrated the protein forms a cup around the m7G moiety via eight anti-parallel beta sheets.[111, 112] The complex is stabilized by the aromatic stacking of two tryptophan residues with the guanosine cap, and additional stability is conferred by a third tryptophan interacting with the positive charge of the N-7 methyl group.[111, 112] Consistent with these structural data, rate constants of purified eIF4E for variously modified cap analogues determined that eIF4E exhibits the highest affinity for m7GTP.[113]

Measurements of eIF4E abundance suggest that there are 0.2-0.3 molecules per ribosome in mammalian cells, leading to the generally accepted hypothesis that eIF4E is the rate-limiting factor for cellular translation initiation.[90-92, 114, 115] Consistent with this model, increased eIF4E activity has been linked to oncogenesis, and overexpression of eIF4E alone is sufficient to transform NIH-3T3 cells.[100, 101, 116] That eIF4E is limiting has been contradicted by work claiming that estimates of free eIF4E levels in rabbit reticulocyte lysates vastly underestimate the amount of total eIF4E.[117] Furthermore, overexpression of eIF4E does not increase global protein

synthesis rates but instead increases the translational efficiency of specific mRNAs involved in oncogenesis.[100] More recently, a mouse model of eIF4E haploinsufficient mice suggested that physiological levels of eIF4E in mammals are in excess of the levels needed for normal development or tumorigenesis.[93]

In addition to cap binding, eIF4E contains a binding site for eIF4G, the scaffolding protein of the canonical eIF4F initiation complex. A recurring theme from the study of eukaryotic initiation factors is that cooperative binding between factors leads to synergy and enhanced mRNA recognition. Binding of *Saccharomyces cerevisiae* eIF4G to eIF4E increases the association rate of the complex with RNA.[118] Within the eIF4F complex, eIF4E stimulates the helicase activity of the DEAD-box helicase eIF4A, and the interaction of eIF4A with eIF4G and eIF4B increases the helicase's processivity to allow unwinding of cap-proximal RNA duplexes.[119, 120] This activity is thought to be particularly important for translation initiation on mRNAs with long, highly structured 5′ UTRs. Ectopic overexpression of eIF4E enhanced translation of reporter mRNAs with increased 5′ UTR structures, likely through its ability to recruit and stimulate eIF4A.[121, 122] In yeast, ribosome profiling experiments indicated that the functions of eIF4A and its cofactor eIF4B were important for the translation efficiency of cellular RNAs with a propensity for more structured 5′ UTRs.[123, 124] In mammalian cells, chemical inhibition of eIF4A helicase activity confirmed a role for the initiation factor in stimulating the translation of RNAs with structured 5′ UTRs.[125-128] However, diminished eIF4E levels in haploinsufficient mice had no measurable effect on the polysome association of mRNAs with structured 5′ UTRs.[93] Beyond enhancing eIF4A activity, the interaction

between eIF4E and eIF4G may stimulate translation by contributing to the circularization of cellular mRNAs.[129, 130]

Eukaryotic eIF4G contains a binding domain for PABP, and thereby acts as a bridge between 5′ end recognition through eIF4E and 3′ end recognition by PABP, Figure 1.3.[129, 131, 132] This “closed-loop” model of translation has been studied primarily in the yeast system. Consistent with the model, deletion of the PABP-binding motifs in eIF4G leads to decreased rates of protein synthesis *in vitro* and slower cell growth.[131, 133, 134] Furthermore, inhibition of translation initiation by cap analogs appears to be more difficult once translation has commenced on an RNA.[135, 136] In HeLa cells, cleavage of eIF4G appears to have less of an impact on mRNAs already engaged in translation and minimally affects the translation of ribosomal protein mRNAs.[137, 138] Notably, housekeeping genes such as ribosomal proteins exhibit high ribosome density and tend to have short open reading frames, suggesting that they have a high potential to form closed-loop structures.[95, 139-143]

Circularization of mRNA may enhance translation efficiency by increasing the probability that a terminating ribosome could re-initiate translation on the same molecule due to the proximity of the 3′ and 5′ ends. Indeed, re-initiation appears to occur more efficiently than *de novo* initiation.[144] This may be in part due to a higher local concentration of initiation factors around a closed-loop mRNA.[95] However, the nature of the secondary structures adopted by mammalian RNAs within a cell remain controversial.

Regulation of eIF4E

eIF4E function is regulated by phosphorylation, both by direct phosphorylation of eIF4E at serine 209, and by the phosphorylation of a family of eIF4E-binding proteins (eIF4E-BPs).[100, 101] Direct phosphorylation of eIF4E is performed by the MAPK-interacting serine/threonine kinases (MNKs), which associate with the C-terminal HEAT domains of eIF4G.[145-150] Studies into the functional consequence of this posttranslational modification have produced conflicting results. While there are reports that bulk translation increases as a result of serine 209 phosphorylation, biochemical assays have demonstrated that serine 209 phosphorylation leads to a decreased affinity of eIF4E for the cap.[151-154] However, the magnitude of this effect was measured to be less than five-fold, suggesting it may have only a modest impact in a setting of redundant, synergistic interactions such as those that occur during eIF4F mRNA activation and 43S PIC recruitment. While it is known that the phosphorylation of eIF4E at serine 209 is required for transformation in mouse models, the precise mechanism by which this occurs remains unknown.[152, 155, 156] The regulation of eIF4E by the activation of eIF4E-binding proteins has been more clearly elucidated.

The eIF4E-BP family consists of three members in mammals, eIF4E-BP1, BP2, and BP3 that bind eIF4E via a conserved YXXXXLΦ motif also present on eIF4G.[157-160] As this would suggest, the binding sites for eIF4G and eIF4E-BPs on eIF4E are overlapping. Inactivation of cap-dependent translation by eIF4E-BPs therefore occurs through competitive inhibition with eIF4G.[161, 162] Regulation of eIF4E-BP function is coordinated by mTOR, which phosphorylates eIF4E-BPs in response to changes in cellular homeostasis.[163-168] Hypophosphorylated eIF4E-BPs are active and can bind

eIF4E thereby preventing eIF4G association and inhibiting cap-dependent translation initiation.[157] The active forms of eIF4E-BPs can dissociate eIF4E-eIF4G complexes *in vitro*, a property that is abrogated by their sequential phosphorylation.[169] Phosphorylation of eIF4E-BPs by mTOR at threonines 37 and 46 is thought to be a pre-requisite for their inhibition, and modifications at these sites are followed by phosphorylations at serine 65 and threonine 70 that completely abrogate eIF4E-BP binding to eIF4E.[167, 168] Once released from eIF4E-BPs, eIF4E can associate with eIF4G and stimulate cap-dependent translation.

Host Shutoff During Viral Infection

Viruses with Diverse Replication Cycles Induce Host Shutoff

Viruses are entirely dependent on cellular ribosomes for the translation of viral proteins.[99] To direct synthesis of viral proteins, viruses frequently manipulate the canonical translation initiation pathway, often at the expense of host cell protein synthesis, Figure 1.3. This shut down in cellular cap-dependent translation is termed "host shutoff".[99] VSV is not the only virus that inhibits cellular protein synthesis, and viruses with widely divergent replication cycles induce host shutoff in infected cells.

Herpesviruses are DNA viruses that replicate in the nucleus of infected cells.[1] Comprehensive studies on the manipulation of cellular processes by virus-encoded factors have been published for the prototype alphaherpesvirus, herpes simplex virus 1 (HSV-1). HSV-1 does not encode an RNA polymerase, therefore it depends on cellular polymerases for the transcription of viral mRNAs. Consistent with RNA processing by cellular factors, viral messages are capped, methylated, and polyadenylated by cellular

machinery.[170] Following export to the cytoplasm, the viral mRNAs initiate translation through the canonical cap-dependent pathway and bind host ribosomes. Paradoxically, infection with HSV-1 results in the collapse of polysomes and a profound inhibition of cellular cap-dependent translation.[171, 172]

In contrast to the nuclear life-cycle of herpesviral replication, large DNA viruses of the poxvirus family, such as vaccinia virus (VACV), replicate in the cytoplasm of infected cells.[173] Because VACV mRNAs do not have access to nuclear localized factors, they are capped, methylated, and polyadenylated by virus-encoded enzymes in the cytoplasm.[174-183] Consistent with the structural identity between viral and cellular transcripts, translation of viral mRNAs is believed to be cap-dependent. As with herpesviruses, VACV infection also results in a profound inhibition of cellular gene expression, and the collapse of polysomes.[184-187]

In addition to DNA viruses, RNA viruses of both positive and negative sense, and that replicate in either the nucleus or the cytoplasm of infected cells are known to induce host shutoff. Coronaviruses (CoV) are positive-strand RNA viruses that contain the largest known RNA genomes.[188] The genomic and subgenomic viral RNAs are capped, methylated, and polyadenylated.[189-196] It is thought that the capping reaction is performed by virus-encoded enzymes because severe acute respiratory syndrome-CoV (SARS-CoV) nsp13 has a RNA 5′-NTPase activity. However, a virus-encoded RNA guanylyltransferase has not yet been identified.[188, 197, 198] N-7 cap methylation of viral transcripts is catalyzed by CoV nsp14, which uses *S*-adenosyl methionine (SAM) as the methyl donor.[199-201] The 2′-O methyltransferase nsp16 then modifies the first adenine of the genomic and subgenomic mRNAs producing a

fully methylated cap structure.[202-207] The enzyme(s) catalyzing the polyA tail reaction are currently unknown, but it has been observed that tail length on viral transcripts changes during infection, implying that tail length may be regulated.[208, 209]

Consistent with the capped, methylated, and polyadenylated structure of viral genomic and subgenomic RNAs, the translation of coronavirus transcripts is thought to occur by a cap-dependent mechanism. Constitutively active eIF4E-BP1 that harbors alanine mutations at all four possible phosphorylation sites restricts coronavirus gene expression when overexpressed in infected cells.[210] Furthermore, small molecule inhibition of the eIF4E-eIF4G interaction completely inhibits the replication of HCoV-229E, at a concentration that only inhibits cellular translation by ~40%, suggesting that coronaviruses may have a greater dependency on the cellular eIF4F complex than cellular mRNAs.[211] Despite the sensitivity of coronaviruses to manipulation of the cap-binding complex, infection with coronaviruses is usually associated with a profound inhibition of cellular cap-dependent translation.[212-215] Large polysomes collapse and the number of 80S monosomes increases during infection, suggesting that translation initiation is inhibited.[216]

In contrast, during infection with influenza virus, host cell gene expression is profoundly inhibited, despite polysome integrity being maintained.[217] Influenza is a single-stranded RNA virus with a negative sense genome, that replicates in the nucleus of infected cells.[218] Infection with influenza results in host shutoff that is driven in part by virus-mediated inhibition of splicing, accumulation of cellular mRNA in the nucleus, and the degradation of cellular mRNA.[219-228] Although the mRNAs of influenza have

cellular m7G caps, the translation of viral proteins in infected cells is insensitive to depletion of eIF4E by siRNA, overexpression of hypophosphorylated and therefore active eIF4E-BP1, or inactivation of mTOR by rapamycin.[210] While the function of the cap in recruiting ribosomes to influenza virus mRNAs is unclear, picornavirus mRNAs are able to recruit the ribosome in the absence of an m7G cap.

Poliovirus is the prototype enterovirus of the *Picornaviridae* family.[1] The positive sense viral genome is uncapped, and yet it directs robust viral protein synthesis during host shutoff.[229, 230] Consistent with the inhibition of cellular translation, polyribosomes rapidly collapse in infected cells.[231, 232] Poliovirus mRNAs are actively translated during cellular cap-dependent shutoff through the use of an internal ribosome entry site (IRES).[233] The poliovirus IRES is a complex mRNA structural element that directs ribosome loading with a reduced dependence on cellular initiation factors and a 5′ m7G RNA cap.[229, 230, 234] Such structures have since been identified in *Pestivirus* and *Hepacivirus*, of which hepatitis C virus is the prototype. In their most extreme form, the IRES of *Dicistroviridae*, a family of arthropod pathogens, is able to directly bind eukaryotic ribosomes and assemble 80S ribosomes to initiate protein synthesis in the absence of any cellular initiation factors.[235-239]

Despite the profound inhibition of cellular translation following poliovirus infection, microarray studies of polysome-associated mRNAs identified a subset of cellular mRNAs that remained ribosome-associated during infection.[240] These mRNAs included proto-oncogenes and members of the MAPK pathway, and it was proposed that many may contain cellular IRES elements.[240] Despite the identification and

characterization of multiple elements within cellular mRNAs resembling IRESes, their ability to function as *bona fide* IRES elements has remained controversial.[240-244]

Although diverse virus families induce host shutoff, there are two common themes that emerge with regards to the mechanisms used by viruses to suppress cellular gene expression. In general, viruses suppress host cell gene expression by, 1) altering the pool of cytoplasmic mRNA, and 2) regulating the assembly of the cap-binding complex, eIF4F. In addition to promoting the translation of viral mRNAs, host shutoff enhances viral replication by preventing the expression of interferon (IFN) and interferon-stimulated genes (ISGs) in infected cells.

Targeting the Cap

The essentiality of the m7G cap of cellular mRNAs for efficient recruitment of ribosomes suggests that it would be an ideal target for viruses that inhibit host cell gene expression. Indeed, both the DNA virus vaccinia virus, and the RNA virus influenza virus, directly target the caps of cellular mRNAs, Figure 1.3.

VACV encodes two decapping enzymes, D9 and D10, that remove the m7G cap of cellular mRNAs, targeting them for degradation by cellular exonucleases.[245-250] The depletion of cellular messages coincides with robust viral transcription, allowing viral messages to dominate the mRNA pool.[251]

The influenza virus polymerase associates with the C-terminal domain of cellular RNA polymerase II (Pol II), where it has access to newly synthesized cellular pre-mRNAs.[252-254] Influenza polymerase is a heterotrimeric complex composed of the PA, PB1, and PB2 subunits. The PB2 subunit recognizes the m7G cap of cellular pre-

mRNAs, binds the cap, and positions the cellular pre-mRNAs for cleavage by the PA subunit endonuclease.[255-261] PA cleaves the cellular pre-mRNAs ~10-13 nucleotides downstream from the cap.[259, 260, 262-264] This host cell-derived capped and methylated oligo then serves as a template for viral mRNA transcription, producing viral mRNAs with cellular m7G caps and heterogeneous 5′ terminal sequences.[255] The viral polymerase further modifies the transcripts by adding a polyadenylate tail, making them structurally indistinguishable from cellular transcripts.[265, 266]

Targeting eIF4F

The importance of eIF4F assembly to translation initiation on cellular mRNAs is highlighted by the diverse mechanisms by which viruses target and manipulate the assembly of this complex, Figure 1.3. HSV-1 targets the eIF4F complex for dual functions in infected cells; 1) to target cellular mRNAs for degradation, and 2) to stimulate translation of viral messages. Herpesvirus host shutoff is achieved primarily through degradation of capped, cytoplasmic mRNAs.[267-270] This degradation is nonspecific, and capped viral mRNAs are degraded as well.[271, 272] The virus host shutoff (vhs) protein, which is deposited in cells as a component of the infecting virion, contains ribonuclease activity responsible for degrading mRNA.[273-279] Vhs has been shown to bind eIF4H and eIF4A, and it associates with the eIF4F cap-binding complex, providing it with access to fully processed mRNAs.[280-283] Although it has been shown that the cleavage of mRNAs may occur in polysomes, ribosome association does not seem to be required for degradation by vhs.[284, 285] It is believed that high

rates of transcription of viral genes prevent depletion of viral mRNA levels, allowing for their continued translation during host shutoff.

HSV-1 also modifies and regulates components of eIF4F to stimulate translation of viral proteins. In quiescent HSV-1 infected cells, eIF4E becomes phosphorylated at serine 209.[286] Although the precise mechanism by which eIF4E phosphorylation functions to stimulate translation remains poorly defined, it appears to be important for the formation of eIF4F complexes in HSV-1 infected cells, because preventing the phosphorylation of eIF4E through Mnk1 inhibits viral replication.[286] To further aide in assembly of functional eIF4F complexes, the viral ICP6 protein physically interacts with the eIF4G scaffolding protein and enhances the association of eIF4G with eIF4E.[287]

Vaccinia virus infection also leads to eIF4E phosphorylation at serine 209, suggesting that larger pools of activated eIF4E may be available during infection.[288] Furthermore, eIF4E, PABP, and the scaffolding protein eIF4G are re-localized during vaccinia infection to sites of active viral replication called viral factories.[288, 289] It is thought that this redistribution of initiation factors contributes to host shutoff by limiting the availability of initiation factors to cellular transcripts, while increasing the local concentration of factors around viral mRNAs. This appears to be an active re-localization by the virus, as the virus-encoded ssDNA-binding phosphoprotein I3 has been shown to bind eIF4G and recruit it to the viral factories.[290]

While eIF4GI and eIF4A are thought to be essential for influenza protein expression, eIF4E is actually dephosphorylated during infection, suggesting that the cellular cap-binding complex may be remodeled.[291, 292] Similar observations that eIF4E is dephosphorylated have been made during VSV infection.[293] However, the

contribution of eIF4E phosphorylation to translation during RNA virus infection remains unclear, as dephosphorylation may reflect the dissociation of the eIF4F complex, and not be a driving mechanism in host shutoff. Consistent with the importance of eIF4G during influenza virus infection, it has been observed that the viral polymerase PB2 domain and NS1 protein can interact with eIF4GI and PABP and recruit them to viral mRNAs.[210, 294-296] However, the precise contributions of these interactions to translation initiation is unclear, given that NS1 is not absolutely required for viral protein synthesis or host shutoff.[297]

Picornaviruses such as poliovirus encode a 2A protease that cleaves the eIF4G scaffolding protein, thereby preventing interactions between the 5′ and 3′ end of cellular mRNAs.[298-300] Proteolysis of PABP has also been observed during poliovirus infection, and the cleavage of these cellular initiation factors coincides with a profound inhibition of cellular cap-dependent translation.[298, 301, 302] There are also reports that eIF4E is re-distributed to the nucleus following eIF4G cleavage, and that eIF4E-BP1 becomes hypophosphorylated and activated during poliovirus infection.[303, 304] However, the contribution of these effects to host shutoff beyond eIF4G cleavage are unclear. Consistent with host shutoff in poliovirus infected cells being driven by eIF4F alterations, cellular mRNAs are depleted from polysomes but are not degraded during infection.[305, 306]

Targeting eIF4E-Binding Proteins

The regulation of eIF4E function by eIF4E-binding proteins represents a powerful node of translational regulation. Consistent with the importance of this regulatory

pathway, diverse virus families manipulate the phosphorylation status of eIF4E-BP1, Figure 1.3. HSV-1 infection in quiescent cells leads to increased eIF4E-BP1 phosphorylation, suggesting eIF4E is liberated and able to bind viral transcripts.[286] Furthermore, the HSV-1 US3 kinase inactivates a negative regulator of mTOR, leading to proteasomal degradation of hyperphosphorylated eIF4E-BP1.[307] A similar situation occurs during vaccinia virus infection, where eIF4E-BP1 becomes hyperphosphorylated and is subsequently degraded by the proteasome.[288]

Not all viral infections are associated with decreased eIF4E-BP1 activity. Indeed, infection with the DNA virus, SV40, as well as the RNA viruses poliovirus, EMCV, and VSV all lead to dephosphorylation of eIF4E-BP1, coincident with the appearance of host shutoff.[293, 303, 308] Although the precise mechanism by which VSV activates eIF4E-BP1 is unknown, it appears to involve the Akt pathway.[309] It is thought that dephosphorylation of eIF4E-BP1 leads to binding and sequestration of eIF4E during infection, leading to a global decrease in eIF4F activation of cellular mRNAs and a decrease in cap-dependent translation. Poliovirus and EMCV translation is unaffected by eIF4E-BP1 activation because both viruses initiate translation via an IRES, however, how VSV mRNAs continue to direct translation initiation under conditions of eIF4E-BP1 dephosphorylation remains to be resolved.

Targeting eIF2

As previously mentioned, eIF2 function can be regulated by phosphorylation of its alpha subunit in response to a variety of cellular stresses, including viral infection. Detection of double-stranded RNA produced during infection activates RNA-dependent

protein kinase (PKR), which phosphorylates eIF2α and leads to a global inhibition of translation initiation.[310] Although PKR is activated during HSV-1 infection, the virus counteracts eIF2α phosphorylation through the viral γ34.5 phosphatase, which binds cellular protein phosphatase 1α (PP1) and maintains eIF2α in an unphosphorylated, functional state.[311-313] Late during infection, the viral gene US11 interacts with PKR and inhibits its activation.[314-318] HSV further maintains eIF2α functionality by preventing the activation of PKR-like ER kinase (PERK), which is an effector of the unfolded protein response that also phosphorylates eIF2α, through associations between PERK and the viral glycoprotein gB.[319]

Vaccinia virus also counteracts PKR and PERK activation. The viral E3L gene product binds double-stranded RNA to prevent activation of PKR.[320-326] An additional gene product, K3L, serves as a pseudosubstrate for both PKR and PERK, thereby preventing phosphorylation of eIF2α and permitting translation initiation to continue.[327-329]

Among RNA viruses, influenza virus utilizes multiple mechanisms to antagonize PKR activation. Influenza recruits the cellular inhibitor of PKR, P58(IPK) to dampen the response to infection.[330-332] Additionally, the virally-encoded NS1 protein interacts with PKR and inhibits the phosphorylation of eIF2α.[333-335] Infection of cells with poliovirus and VSV results in eIF2α phosphorylation at later times post-infection, with differential impacts on viral translation.[336-338] In the case of poliovirus, at late times post-infection the virus exhibits a reduced requirement for eIF2 to initiate translation, potentially as a result of a viral protease cleaving eIF5B.[339-342] This allows poliovirus to continue to direct IRES-mediated viral protein synthesis.[340-342] In contrast, eIF2α

phosphorylation late during infection with VSV is thought to suppress translation of viral proteins.[338] However, this occurs after alterations to eIF4E and eIF4E-BP1 that are thought to be responsible for suppressing cellular translation.[293, 338]

Targeting mRNA Export and Stability

Targeting assembly of the eIF4F complex allows viruses to subvert the cellular translation initiation pathway at the expense of host cell translation. In addition to manipulating translation initiation, many viruses also modulate the pool of available mRNA in infected cells. While this clearly contributes to host cell shutoff in some cases, it also serves to abrogate the expression of interferons (IFNs) and interferon-stimulated genes (ISGs).

As previously described, infection with the DNA virus HSV-1 results in the degradation of capped, cytosolic mRNA.[267-270] De-capping by VACV enzymes likely leads to degradation of cellular mRNA by the host cell Xrn1 pathway, although this has not yet been definitively shown.[343, 344] Additionally, transcription from cellular polymerases is inhibited during VACV infection, also potentially contributing to host cell shutoff and the inhibition of IFN gene expression.[185, 345, 346] Beyond DNA viruses, multiple RNA viruses encode endonuclease domain-containing proteins that target mRNA for cleavage and degradation.

One of these is the multifunctional nsp1 protein of coronaviruses. SARS-CoV nsp1 binds directly to 40S ribosomal subunits and inhibits 80S ribosome formation, while the virus S protein has been shown to bind eIF3f and inhibit translation later in infection.[347, 348] The viral 7a protein has also been suggested to inhibit cellular

translation, but the precise mechanism remains unknown.[349] In addition to inhibiting translation directly, coronaviruses degrade cellular mRNAs.[216, 350-352] The SARS-CoV nsp1 protein induces endonucleolytic cleavage of cytoplasmic cellular mRNAs that results in their degradation by the cellular Xrn1 5′-3′ exonuclease pathway.[285, 347, 352] Viral mRNAs escape cleavage, allowing for their efficient association with cellular ribosomes.[353, 354] The Middle East respiratory syndrome-CoV (MERS-CoV) nsp1 protein induces degradation of actively transcribed nuclear mRNAs during infection, while the cytoplasmic pool of cellular RNAs remains unaltered.[355] Additionally, infection with mouse hepatitis virus (MHV) has been shown to lead to cleavage of ribosomal 28S rRNA in the cytoplasm starting ~5 hours post infection.[356] While this slightly precedes the timing of host shutoff, viral protein synthesis continues unabated.[356]

In addition to “cap-snatching” the m7G caps of cellular pre-mRNAs, influenza specifically targets cellular mRNAs for degradation. Ribosome frameshifting during translation of the PA mRNA leads to production of a protein containing the PA endonuclease domain, with a C-terminal X-ORF domain.[357] This PA-X gene product suppresses cellular gene expression by targeting and degrading Pol II-transcribed cellular mRNAs.[357, 358] Viral mRNAs escape degradation by PA-X.[358]

Although it has been observed that cytoplasmic cellular mRNAs are not degraded during picornavirus infection, transcription from all three cellular DNA-dependent RNA polymerases is inhibited.[359-366] Mechanistically, the viral 3C protease cleaves the cellular TATA-binding protein (TBP) inhibiting cellular transcription, although some RNAs activated by NF-κB appear to be resistant to this inhibition.[367-

370] It has also been reported that the poliovirus 2A protein degrades components of the nuclear pore, such as Nup98.[371, 372] The effect of this degradation on the nuclear export of cellular mRNAs has produced conflicting data, but regardless, inhibition of nuclear mRNA export and cellular transcription would serve redundant functions.[371, 372] The matrix protein of VSV has also been reported to inhibit transcription from cellular polymerases through interactions with TFIID, as well as block the directed export of mRNPs out of the nucleus by interacting with Rae1•Nup98.[373-375]

Inhibition of Cellular Gene Expression during VSV Infection

As outlined above, viruses have evolved multiple mechanisms to shut off host cell gene expression, and this shut off is often achieved through the simultaneous action of multiple effectors. During VSV infection, the activation of eIF4E-BP1 and subsequent sequestration of eIF4E is thought to suppress cellular translation. Additional contributions to host shutoff are mediated by the viral matrix protein, M, which inhibits transcription by cellular polymerases, and blocks the nuclear export of cellular mRNPs.

The first evidence that VSV inhibited transcription by cellular polymerases was from experiments demonstrating that the incorporation of tritiated uridine into cellular mRNA was blocked during infection.[376, 377] Although transcription was found to be inhibited from all three cellular RNA polymerases, the greatest effect was observed on Pol II.[378, 379] Subsequent work identified M as the viral component responsible for inhibiting cellular transcription.[380-385] This inhibition seems to depend on an interaction between M and TFIID, the cellular transcription factor containing, among

other factors, the TATA-binding protein.[373] Although M is distributed throughout the nucleus as well as the cytoplasm, a large fraction of the protein is localized to the nuclear periphery.[375, 386, 387] The current model for how M mediates inhibition of endogenous transcription is that through interactions with components of the nuclear pore, M is able to associate with sites of active transcription and repress the activity of cellular polymerases.[388] These interactions include the well-documented ability of M to associate with the Rae1•Nup98 nuclear pore complex and block the nuclear to cytoplasmic export of mRNP complexes.

Crystallographic data indicates M interacts with the Rae1•Nup98 nuclear export complex at a 1:1:1 stoichiometry.[389] The Rae1 binding site on M, termed the finger region, interacts with a groove on Rae1 that is thought to make contacts with the phosphate backbone of mRNA on its export out of the nucleus.[389, 390] This suggests that the interaction between Rae1 and M sterically occludes the binding of cellular mRNAs to Rae1, inhibiting their export. Consistent with this, M can outcompete ssRNA for Rae1 binding *in vitro*, and a naturally occurring viral mutant containing a methionine to arginine mutation at residue 51 (M51R) in the finger region is defective in host shutoff.[389, 391] In Xenopus oocytes injected with recombinant wild-type M protein, but not M51R, M was observed to localize to the nuclear periphery, and the directed transport of snRNAs and mRNAs was blocked.[386] The transport of tRNAs was unaffected by M, suggesting not all nuclear export is inhibited by M.[392] Consistent with this finding, the Rae1•Nup98 axis is not the only mechanism for RNA export from the nucleus to the cytoplasm.[393, 394]

If a primary function of M were to inhibit nuclear export of mRNPs, and not abolish transcription by cellular polymerases, it would follow that a nuclear accumulation of cellular polyA mRNA should be observed following infection with VSV. Indeed, there have been several reports that transfection of M alone, or infection with VSV leads to the nuclear accumulation of polyA mRNA in HeLa cells, BHK-T7 cells, and mouse embryonic fibroblasts.[374, 375, 387, 395, 396] However, attempts to replicate this result in other labs have not always been successful. This may reflect the presence of alternative export pathways, nuclear degradation of RNA triggered by export stress, or cell-type dependent variations in the contributions of M to host shutoff. It is possible that in certain cell types, the inhibition of mRNA transcription by M plays a more significant role than the inhibition of nuclear mRNP export.

Underscoring the importance of M-mediated inhibition of transcription and nuclear export to infection, is the observation that these functions are conserved among vesiculoviruses. The matrix proteins of the closely related vesiculoviruses Chandipura and spring viremia carp virus, have also been shown to localize to the nuclear periphery in transfected HeLa cells, and block the cytoplasmic transport of mRNA when injected into Xenopus oocytes.[397] Additionally, the matrix protein of Chandipura virus can inhibit expression of a chloramphenicol acetyl transferase (CAT) reporter transfected into 293 cells, providing indirect evidence that it may also have inhibitory effects on transcription by cellular polymerases.[398] Notably, although the sequence identity between VSV and Chandipura or spring carp viremia virus M protein is only ~50%, a methionine, at position 51 in VSV, is conserved in a comparable position between all

three, and is essential for the transport blocking ability of VSV, Chandipura, and spring carp viremia virus M.[397]

Although the functions of M in blocking cellular transcription and nuclear mRNP export contribute to inhibiting host cell gene expression, they do not fully explain the suppression of cellular translation observed during VSV infection. It is well established that VSV does not target cellular mRNAs for degradation, and that the inhibition of cellular protein synthesis occurs before significant turnover of cellular mRNA.[87, 399] Indeed, host cell mRNA can be extracted from infected cells and translated *in vitro*, demonstrating the mRNA is intact and competent to direct protein synthesis.[87, 400] Rather, VSV infection is thought to inhibit cap-dependent translation initiation on cellular mRNAs through modulating the availability of the cap-binding complex eIF4F. During VSV infection both dephosphorylation of eIF4E at serine 209 and dephosphorylation of eIF4E-BP1 at serine 65 have been observed.[293] Pulldown experiments using m7G sepharose demonstrated that as eIF4E-BP1 became hypophosphorylated during infection, the levels of eIF4G associated with the cap-binding complex diminished by 6 hours post-infection to ~50% of the levels pulled down in uninfected cells.[293] Simultaneously, the level of eIF4E-BP1 pulled down with m7G sepharose increased, suggesting eIF4E-BP1 was disrupting eIF4F complexes and sequestering eIF4E.[293] Consistent with this, addition of ribosomal salt washes containing eIF4F, among other factors, stimulated translation in extracts prepared from infected cells.[401]

Although the precise mechanism leading to eIF4E-BP1 activation is unknown, it appears that M can affect the activation of the Akt pathway.[309] However, expression of M alone actually stimulates translation in HeLa cells, suggesting either another viral

component is necessary to affect eIF4E-BP1 activation, or that the effects on eIF4E-BP1 are the result of a cellular response to infection.[402] Regardless of the mechanism leading to modulation of the eIF4F complex, translation of viral mRNAs continues during eIF4E-BP1 hypophosphorylation.

Although the translation of VSV mRNAs is thought to be cap-dependent, multiple lines of evidence suggest it may not be eIF4E-dependent. VSV can replicate during conditions when canonical cap-dependent translation is suppressed, such as during rapamycin treatment, hypoxia, eIF4E depletion by siRNA, and eIF4G cleavage by the poliovirus 2A protease.[293, 395, 403] Furthermore, infection proceeds despite activation of eIF4E-BP1.[293] These discrepancies could be reconciled if eIF4F complex formation is not actually rate-limiting in cells, as has been suggested. Alternatively, it is possible that initiation on VSV mRNAs may be more efficient than canonical cellular initiation due to the short 5′ UTRs of viral messages. The continued transcription of viral mRNAs throughout the course of infection has also been proposed to play a role.[404, 405] However, a unifying model for how VSV induces host shutoff while capped and methylated viral mRNAs are translated has not emerged.

Models to explain viral mRNA translation during host shutoff

The first model to reconcile the robust translation of VSV mRNAs with cap-dependent translation shut down, was proposed by Harvey Lodish and Mary Porter in 1980. They hypothesized that an overabundance of viral mRNA led to competition for a limiting pool of cellular ribosomes.[87] Due to abundance, viral mRNAs were better able to compete for ribosome binding, leading to a redistribution of ribosomes from cellular

messages onto viral transcripts.[87] This model elegantly explained why polysomes collapsed and monosomes increased during infection, despite only modest suppression of the total rate of translation as measured by ^{35}S incorporation.

They presented evidence that the total amount of translatable mRNA in infected BHK, Vero, and L cells increased 2-3 fold during infection.[87] By performing polysome profiling and then quantifying the amount of specific cellular proteins translated *in vitro* from polysome fractions, they demonstrated that the majority of cellular mRNA remained polysome-associated in infected cells, but was shifted towards smaller polysomes.[87] Crucially, they used this method to show that viral and cellular messages of similar sizes were translated on polysomes containing similar numbers of ribosomes.[87] This demonstrated that viral mRNAs were not preferentially translated, but rather that ribosomes were equally likely to bind cellular or viral transcripts. Therefore, they concluded that robust viral protein synthesis and decreased cellular protein synthesis was due to viral mRNA overabundance. This result validated earlier studies of the molar ratios of viral proteins and mRNAs that indicated VSV protein levels were regulated at the level of transcription.[22] A follow-up study by Lodish and Porter utilizing VSV temperature sensitive mutants defective in mRNA transcription, supported their initial conclusions by demonstrating a correlation between the level of viral mRNA transcription and the level of host shutoff.[400]

Notably, Lodish and Porter also described the presence of sub-ribosomal mRNPs during VSV infection, and postulated that these could be explained by a cellular factor required for initiation that becomes limiting after infection.[87] However, in general, they found that no more than ~25% of cellular and viral mRNAs were

associated with non-translating mRNPs during infection.[87] This is in contrast to a subsequent study reporting the presence of a puromycin-resistant mRNP particle in VSV infected CHO cells.[406] The authors reported that the particle co-sedimented with polysomes on a sucrose gradient, and contained the five viral mRNAs complexed with the N protein.[406] By dissociating polysomes with puromycin and then isolating the mRNP, they showed that by 4.5 hours post infection, ~97% of the total VSV mRNA was associated with the particle, and that the particle was translationally inactive in wheat germ extracts.[406] Because only 3% of the viral mRNA remained bound by polysomes, the authors concluded that competition for ribosome binding cannot explain host shutoff.[406] Subsequent work by a different group using puromycin treatment followed by northern blotting of polysome fractions from HeLa cells concluded that only ~18% of the viral mRNA is ribosome-associated, and that it is associated with large polysomes.[404] The results from these studies, interpreted with results obtained using defective-interfering particles (DI particles) to modulate viral mRNA levels, led to the competition model falling out of favor. However, the presence of an N-viral mRNA mRNP is incompatible with experiments that immunoprecipitated N from infected cells and found only genomic viral RNA associated with immune complexes.[407]

It is also worth mentioning that the interpretation of the experiments using defective-interfering particles to modulate viral mRNA transcription are confounded by the use of high multiplicities of infection (MOI). In this study, the infectious VSV MOI used was 5, and DI particles were added up to a MOI of 56.[408] Considering that the particle to pfu ratio for the infectious stock is unknown, and even more particles were added in conditions with high DI particle MOIs, the target cells were likely exposed to a

high dose of M protein. The incoming M from both infectious and defective particles will immediately act to block nuclear export and inhibit cellular transcription. Additionally, there will likely be higher levels of defective nucleic acids and abortive transcription products that could serve as potent activators of innate immunity, further complicating the interpretation of the experiments. However, in response to the challenges to the competition model, the consensus in the field has been that the effects of infection on eIF4F complex formation drives the inhibition of host cell translation.

While this model is not necessarily mutually exclusive to the competition model, it also does not provide a sufficient explanation for all of the observations regarding VSV host shutoff and viral gene expression.

Innate immunity and the discrimination of self vs. non-self RNAs

RNA viruses that replicate in the cytoplasm of eukaryotic hosts must evade detection by host cell sensors of foreign nucleic acids. This pressure becomes particularly acute during replication, when there is the possibility that uncapped RNA, or double-stranded RNA intermediates may be formed.[409] These substrates are potent activators of the cytosolic innate immune sensors of foreign RNA, RIG-I and MDA5. RIG-I recognizes short, triphosphate RNAs, and MDA5 recognizes longer, double-stranded RNA molecules.[410-414] Detection of foreign nucleic acids by RIG-I or MDA5 leads to activation and association of the molecules with their adaptor protein MAVS, which is localized to the outer mitochondrial membrane.[409, 415, 416] Activation of MAVs by RIG-I or MDA5 initiates a downstream signaling cascade resulting in the expression of interferons (IFNs) and interferon-stimulated genes (ISGs) that establish a

cellular antiviral state.[409, 415, 416] To evade detection by RIG-I and MDA5, positive sense RNA viruses induce alterations to host cell membranes forming compartments that physically separate viral replication from the cytoplasm.[417] Negative strand RNA viruses do not sequester their replication in membrane-bound compartments but have evolved other mechanisms to counter host defenses. Many of these strategies involve disassembly of signaling cascades downstream of detection by RIG-I and MDA5.[409, 415, 416]

RIG-I is the cytosolic RNA sensor believed to be responsible for recognizing VSV RNA, as IFN is not induced when RIG-I knockout MEFs are infected with VSV, and VSV replicates to higher titers in RIG-I knockout mice.[411, 418] The viral M protein is believed to antagonize the innate immune response and ablate the expression of IFN and ISGs by blocking transcription from cellular polymerases, and inhibiting nuclear mRNP export. Studies comparing the effects of wild-type VSV infection with infection by the M51R mutant in M, support that blocking nuclear export may be the primary mechanism by which M antagonizes the innate immune system.

The M51R mutant in M

A mutation in M at methionine 51, M51R, was identified in two separate strains of temperature sensitive VSV mutants defective in host shutoff, *ts*O82 and *ts*1026RI.[382, 384, 419-423] The *ts*O82 virus is temperature sensitive in chicken cells, but neither the *ts*O82 virus nor a recombinant virus containing only the M51R mutation exhibit temperature sensitivity in most cell lines used for the study of VSV, such as BHK and HeLa cells.[391, 422] Crystallographic studies of M in complex with the Rae1•Nup98

nuclear export complex localize methionine 51 to a region of M making extensive contacts with Rae1. Therefore, even a conservative amino acid substitution such as arginine, may be sufficient to disrupt the interaction between M and Rae1.[389] Consistent with this, recombinant M51R protein does not localize to the nuclear periphery when microinjected into Xenopus oocytes, and does not inhibit directed transport within those same cells.[386] Similarly, wild-type M localizes to the nuclear rim in HeLa or MEF cells transfected with M alone, or infected with a GFP containing reporter virus, and leads to the accumulation of nuclear polyA mRNA in these cells.[374, 375, 387] A mutant in residues 52-54 of M, where each residue has been substituted for alanine, M(D), exhibits a more diffuse cellular localization compared with wild-type M, and is unable to mediate nuclear accumulation of polyA mRNA when transfected into HeLa cells.[375]

Because M blocks cellular transcription and nuclear mRNP export, one potential consequence of M-mediated functions is that the expression of interferons and interferon-stimulated genes is inhibited in VSV-infected cells. Consistent with this hypothesis, cells transfected with a luciferase reporter gene drive by the IFN-β promoter and either co-transfected, or infected, with wild-type M were unable to activate luciferase expression.[384, 391] However, cells transfected with M containing the M51R mutation, or infected with VSV_{M51R}, were able to activate luciferase expression, demonstrating that the inhibition of host cell gene expression by wild-type M abrogates interferon signaling in infected cells.[384, 391] Indeed, wild-type M appears to be sufficient to inhibit interferon signaling in these cells. This is likely through M's ability to block transcription and the nuclear export of mRNPs. Notably, infection of MEFs with

VSV led to an accumulation of IFN-β RNA in the nucleus of wild-type infected cells as assessed by microarray and RT-qPCR, but equal distributions of IFN-β RNA between the nucleus and cytoplasm in cells infected with a mutant virus harboring the M51R mutation.[424] Additionally, Nup98 is an interferon-stimulated gene, and overexpression of Nup98/Nup96 or addition of IFN-γ in M transfected cells can overcome the nuclear retention of cellular polyA mRNA.[387]

The papers reporting IFN induction during infection with VSV_{M51R} did not identify the stimulatory molecule recognized by the innate immune system, and a convincing demonstration of the physiological ligand detected during VSV infection has not been put forth.[425] Initial reports suggested that viral genomic RNA could activate the innate immune response, but the relevance of purified and transfected RNAs to the physiological ligand is unclear.[412] Although the viral genomic and antigenome RNA have a 5′-terminal triphosphate, as do the short, uncapped leader and trailer products produced during transcription and replication, these RNAs are coated in the viral nucleocapsid protein (N) and are therefore shielded from detection.[38, 67, 426-430]

Viral mRNA is likely not a significant source of immune activation, because it is capped and methylated at the 2′-O position.[49, 66] While methylation at the N-7 position is thought to facilitate cap recognition and translation efficiency, methylation at the 2′-O position has been shown to be a determinant of self vs. non-self discrimination by IFIT1 (ISG56) and IFIT2 (ISG54).[431-434] The precise function of the methylations added by L to VSV mRNA remains enigmatic, as a viral mutant defective in all methyltransferase activity *in vitro* retains the ability to induce host shutoff, directs viral protein synthesis and is pathogenic in mice.[435] While these studies do not definitively

rule out compensatory methylations by a cellular methyltransferase, they suggest that the inhibitory effects of M on cellular gene expression are sufficient to counteract host defense strategies.

However, VSV replication produces large numbers of defective-interfering particles (DI particles). Copy-back DI particles, which have complementary 3′ and 5′ ends are potent activators of the interferon response.[436] Indeed, infection with a single copy-back DI particle is capable of inducing a quantum yield of interferon, suggesting that the release of such particles may account for the robust induction of interferon in a VSV infected animal.[436]

Addressing lingering questions in VSV biology

In the studies presented here, we have interrogated the mechanism of VSV-induced host cell shutoff by massively parallel sequencing cytoplasmic and polysome-associated mRNA. We found that viral mRNA abundance is a key determinant of host shutoff, but cannot fully explain the movement of cellular mRNAs out of large polysomes towards smaller polysomes and monosomes. This reconciles the viral mRNA abundance model proposed by Lodish and Porter with observations that the cellular cap-binding complex, eIF4F, is remodeled during VSV infection. We specifically tested the contribution of blocking cellular nuclear mRNP export to host shutoff by sequencing cytoplasmic and polysome-associated mRNA from cells infected with VSV harboring the M51R mutation in M. Our results indicate that blocking nuclear export of cellular mRNAs does not contribute significantly to the translational suppression of the host. Rather, we identify the function of M in blocking nuclear export as a key factor in suppressing the

cellular antiviral response. These results expand our understanding of host-pathogen interactions and underscore the robustness of the activity of the VSV polymerase.

Chapter 2: Vesicular stomatitis virus mRNA abundance is a major determinant of efficient viral protein synthesis.

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Abstract

Infection of mammalian cells with vesicular stomatitis virus (VSV) results in the inhibition of cellular translation while viral translation proceeds efficiently. The VSV 241 kDa large protein (L) which contains an RNA-dependent RNA polymerase (RdRp) transcribes 5 mRNAs in the cytoplasm. The mRNAs are structurally indistinct from those of the cell as two additional enzymatic activities of L – a polyribonucleotidyl transferase and a dual specificity cap methyltransferase – add and methylate the 5′ cap-structure. A 3′ polyadenylate tail is added through a pseudotemplated mechanism of RdRp stuttering on a U7 tract. Here we employed the global approach of massively parallel sequencing cytoplasmic, monosome- and polysome-associated mRNA to interrogate the impact of VSV infection of HeLa cells on translation. Analysis of relative sequence reads in the different fractions shows > 60% of cytoplasmic and polysome-associated reads map to the 5 viral mRNAs by 6 hours post-infection, which corresponds to the time point at which robust host shutoff is observed. Consistent with this overwhelming abundance of viral mRNA in the polysome fraction, the reads mapping to cellular genes were reduced. Analysis of a subset of viral and cellular mRNA distributions within polysomes using quantitative PCR shows a redistribution of cellular mRNAs to smaller polysomes and monosomes in infected cells. These results underscore the importance of viral mRNA abundance in VSV infected cells as a key determinant of host cell shutoff. Viral induced suppression of cellular pools of the rate-limiting factor for translation initiation – the cap-binding protein eIF4E – likely enhances this effect by shifting cellular mRNAs to smaller polysomes and monosomes.

Introduction

Nonsegmented negative-strand (NNS) RNA viruses share a common strategy of mRNA synthesis, our understanding of which has largely come from studies of vesicular stomatitis virus (VSV). The template for mRNA synthesis comprises the viral genomic RNA completely encased within a viral encoded nucleocapsid protein (N) sheath. The N-RNA template is transcribed by the viral encoded RNA-dependent RNA polymerase (RdRp) which resides within the 241 kDa L protein.[8, 11] The L protein contains two additional enzymatic activities that catalyze 5′ mRNA cap-structure formation through unconventional mechanisms.[45, 54, 56] The viral mRNAs contain a 3′ polyadenylate tail that is added in a pseudotemplated manner of reiterative copying of a polyU-tract by the viral RdRp.[57, 61]

The L protein contains all of the enzymatic activities required for formation of a mature mRNA, but cannot engage the N-RNA template directly. The viral encoded phosphoprotein (P) bridges interactions between N-RNA and L allowing the polymerase access to the RNA template.[9, 34, 35, 38] Two additional genes in the viral genome encode the matrix protein (M) that is essential for budding of viral particles, and a single transmembrane glycoprotein (G) that mediates attachment of virus to cells, and catalyzes fusion of viral and cellular membranes during entry. The arrangement of those 5 genes on the template is 3′-N-P-M-G-L-5′, and during L-catalyzed mRNA synthesis a localized transcriptional attenuation occurs at each gene-junction resulting in a gradient of mRNA where N>P>M>G>L.[18, 19, 21, 22]

The 5 viral mRNAs are transcribed in the cytoplasm and are structurally identical to those generated by the host. Infection of mammalian cells by VSV results in a

profound shut off of host gene-expression as evidenced by metabolic labeling of protein synthesis.[84] The viral and cellular mRNAs are, however, structurally indistinct and translated in the cytoplasm. Consequently, the mechanism responsible for this host shutoff must account for ongoing viral translation. Eukaryotic mRNA translation typically involves binding of the 43S pre-initiation complex (PIC) comprising the small ribosomal subunit and eukaryotic initiation factors (eIF) near the m7G cap of mRNA.[88, 90, 94] The recruitment of the small subunit requires the recognition of the mRNA cap structure by the eIF4F complex comprising a scaffolding protein eIF4G, the eIF4A helicase, and the cap-binding protein eIF4E, which is the rate-limiting factor for initiation.[88, 90, 94] Following binding to the mRNA, the small ribosomal subunit then undergoes scanning to the initiating methionine where the large 60S subunit joins to form the elongation competent 80S complex, which is accompanied by the release of the initiation factors.[88, 90, 94]

For VSV, multiple mechanisms have been proposed to account for the observed shut off of host protein synthesis. Early work led to the model that viral mRNA abundance in the cytoplasm resulted in competition for ribosome binding between cellular and viral transcripts.[87, 400] The viral M protein also inhibits host gene-expression through multiple mechanisms. Those mechanisms include the hypophosphorylation of the eIF4E-binding protein eIF4E-BP1, which sequesters eIF4E thereby reducing the pool of the rate-limiting factor for translation initiation.[293, 309] The M protein also suppresses host gene-transcription by inhibition of RNA polymerase II.[381, 382, 385] A further onslaught on host gene expression is accomplished by the M-mediated block of mature mRNP export from the nucleus to the cytoplasm by direct

binding to the Rae1•Nup98 nuclear export complex.[374, 375, 389] In addition to those mechanisms, evidence indicates that ongoing transcription of viral mRNAs plays a role in efficient viral protein synthesis.[404, 405] In a genome wide RNAi screen, we previously reported that viral translation was hypersensitive to the loss of the large ribosomal protein L40, providing further evidence of differential recognition of the viral mRNAs from those of the host.[78, 437]

We now interrogate global mRNA translation in VSV infected cells using RNA sequencing of the cytoplasmic mRNA transcriptome, and parallel sequencing of polysome-associated mRNAs. The results provide strong support of the importance of the abundance of viral mRNA in contributing to host shutoff by leading to a redistribution of cellular ribosomes onto viral messages. By combining this RNAseq analysis with examining the distribution of specific viral and cellular mRNA within polysomes, we also demonstrate that cellular mRNAs shift to smaller polysomes and monosomes consistent with a reduction in the pool of eIF4E. We conclude that the overwhelming abundance of viral mRNA plays a key role in the shut off of host protein synthesis, a strategy that has recently been recognized for other highly cytopathic RNA viruses.

Results

Viral RNA comprises 60% of the cytoplasmic mRNA 6 hours post-infection.

To interrogate the impact of VSV infection on global translation we isolated cytoplasmic, monosome- and polysome-associated mRNAs from HeLa cells at 2 and 6 hpi and compared the relative sequence reads obtained by deep-sequencing (Figure 2.1A). As evident from the polysome profiles, VSV infection results in a small but reproducible increase in monosomes and large polysomes by 2 hpi, followed by a collapse in the pool of large polysomes and an increase in monosomes by 6 hpi (Figure 2.1B). Analysis of the reproducibility of sequencing reads between biological replicates of each fraction yields Pearson correlations >0.97 for cytoplasmic, monosome- and polysome-associated mRNA pools, validating reproducibility between the replicates.

Mapping of the sequence reads to the viral and cellular genomes highlights that by 6 hpi > 60% of reads in the cytoplasmic and polysome fractions are viral (Figure 2.1C). This increase from the <1% observed at 2 hpi (Figure 2.1C) emphasizes the impact of the exponential phase of viral RNA replication and secondary transcription of the viral genome on viral mRNA production. The viral sequence reads cover all 5 mRNAs, with clear dips in coverage that map to gene-junctions (Figure 2.2A). Consistent with the order of transcription of the viral genome, and the localized transcriptional attenuation at gene-junctions, the relative reads that map to each viral gene typically diminish with distance from the single 3′ promoter (Figure 2.1D and Figure 2.2B & 2.2C).

Similar analysis of the sequencing reads that map to cellular genes supports that like the viral mRNAs, the level of reads in the polysome fraction mirrors that in the

Figure 2.1. Viral RNA comprises 60% of the cytoplasmic mRNA at 6 hours post-infection. (A) Schematic representation of the experimental design. Where indicated, HeLa cells were infected at MOI 10 with VSV for 2 or 6 hours. At 2 or 6 hpi cytoplasmic extracts were prepared and used directly for RNA isolation, or were subject to polysome profiling. RNA was isolated from fractions corresponding to 80S monosomes, or polysomes containing 3 or more ribosomes, and mRNA was used to make deep sequencing libraries. (B) Polysome profiles from uninfected (black) or VSV (red) infected HeLa cells. Cytoplasmic extracts were sedimented through a 10-50% sucrose gradient, and 0.5 ml fractions were collected while continuously monitoring absorbance at UV254nm. (C) Results of fragment mapping to the concatenated hg38 (human) and VSV genomes for cytoplasmic, monosome, and polysome samples at 2 and 6 hpi. Trimming and mapping was performed in CLC Genomics Workbench. (D) Cytoplasmic gene cascade for the 5 viral mRNAs at 2 and 6 hpi, respectively. Expression level is presented as Transcripts per Kilobase Million (TPM) to normalize for gene length and library size, error bars denote standard deviation.

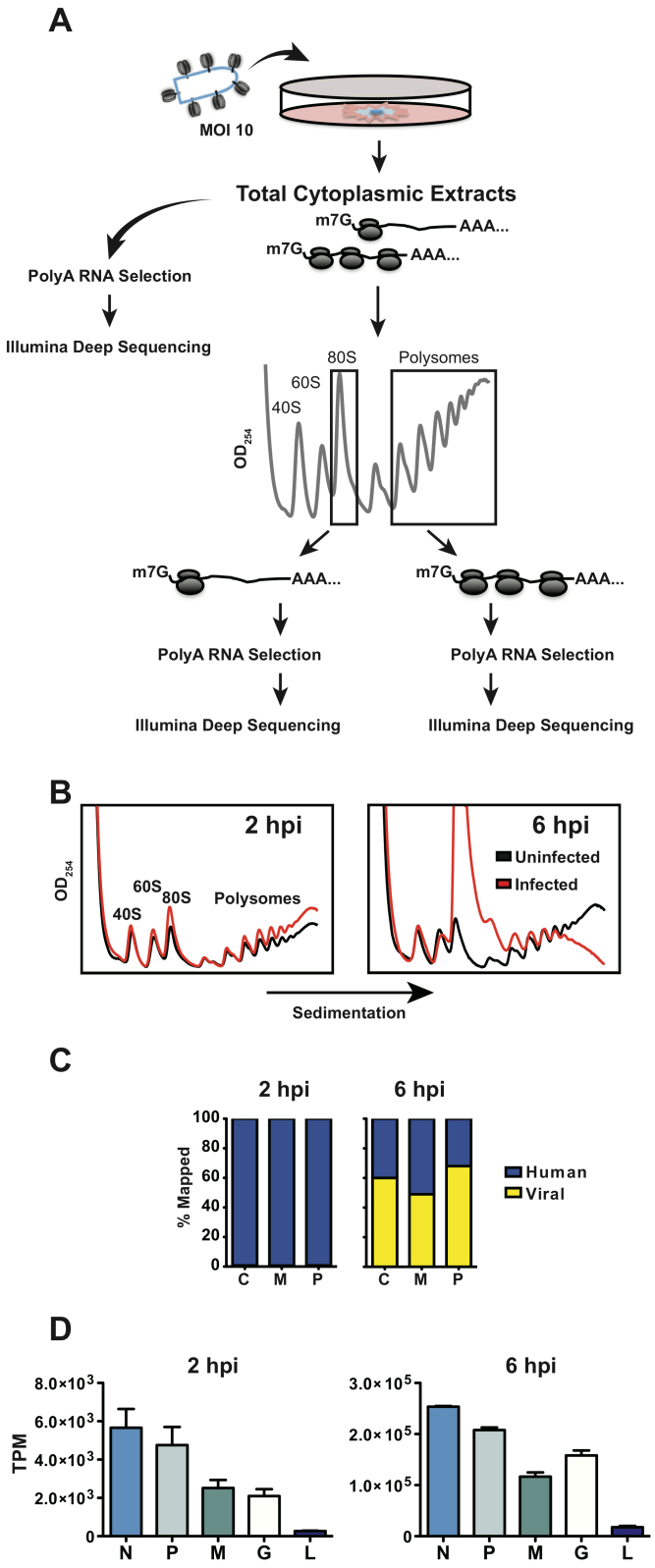


Figure 2.1 (Continued). Viral RNA comprises 60% of the cytoplasmic mRNA at 6 hours post-infection.

Figure 2.2. The viral gene cascade at 2 and 6 hpi. (A) Mapping coverage to the viral genome in the cytoplasm or on polysomes at 6 hpi. Mapping and coverage analysis was performed in CLC Genomics Workbench. (B) Gene cascade on monosomes and polysomes for the 5 viral mRNAs at 2 hpi. Expression level is presented as Transcripts per Kilobase Million (TPM) to normalize for gene length and library size, error bars denote standard deviation. (C) Gene cascade on monosomes and polysomes for the 5 viral mRNAs at 6 hpi, presented as in B.

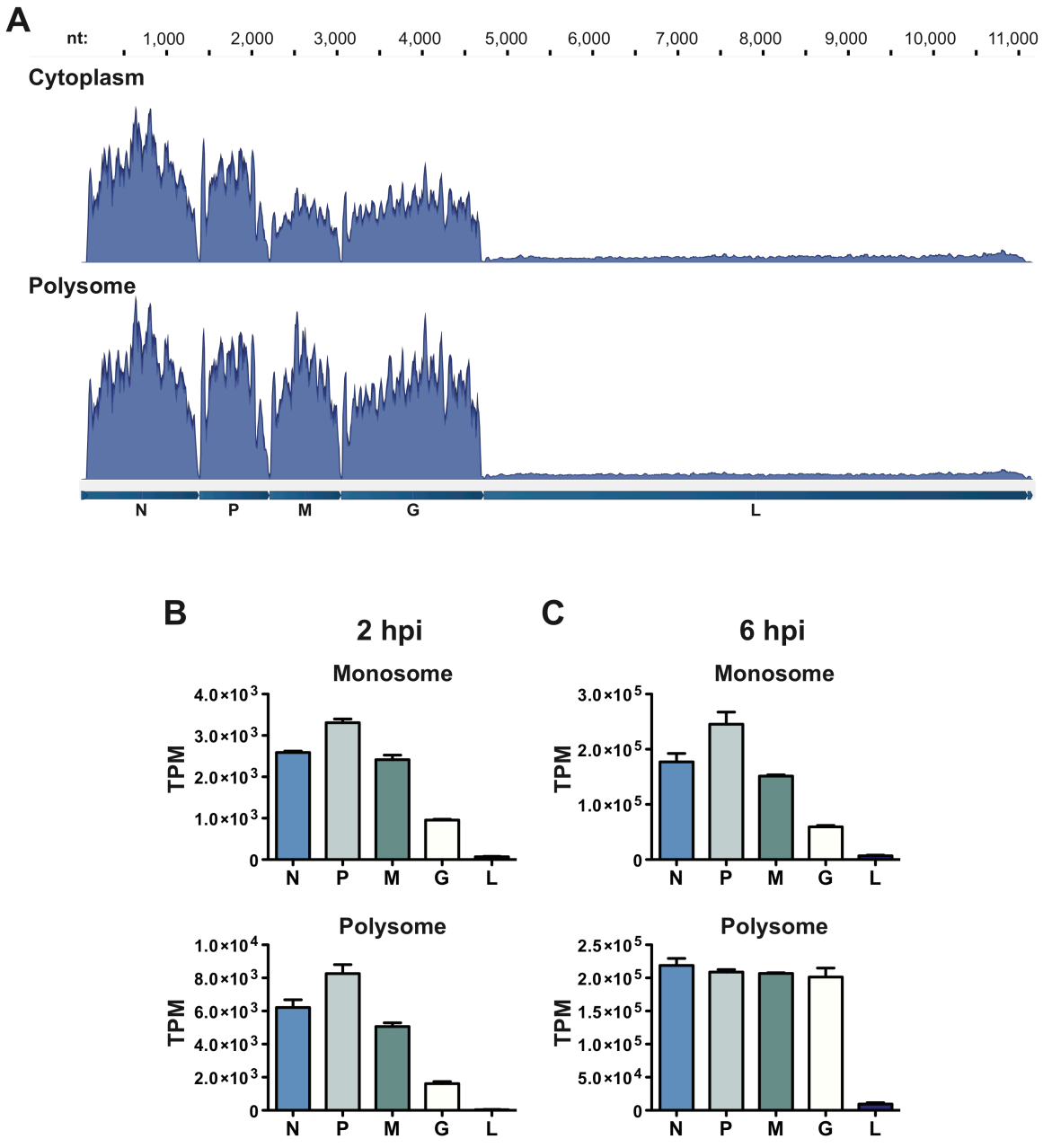


Figure 2.2 (Continued). The viral gene cascade at 2 and 6 hpi.

cytoplasmic fraction in uninfected cells and at 2 and 6 hpi (Figure 2.1C). An overwhelming abundance of viral mRNA produced by the viral polymerase thus competes with the pool of cellular mRNA for polysome association, supporting the model first proposed by Lodish and Porter. We note that viral mRNAs are, however, underrepresented (49%) and cellular mRNAs overrepresented (51%) in the monosome fraction at 6 hpi compared to their total abundance (Figure 2.1C). This finding is consistent with a differential effect on viral versus host mRNA due to the suppression of the cellular pool of eIF4E. Such suppression should result in reduced ribosome loading on mRNA and could account for the shift of some cellular mRNAs out of the polysome pool. Thus, multiple mechanisms including viral mRNA abundance and eIF4E inactivation contribute to translational inhibition of the host.

The relative abundance of individual cellular mRNAs in the cytoplasm and on polysomes decreases between 2 and 6 hpi.

To determine how VSV infection affects the distribution of each individual mRNA within the respective pools, we plotted the TPM for each mRNA mapped to the human and viral genome in uninfected and infected cells in all 3 fractions (Figure 2.3). At 2 hpi, the relative reads of viral mRNAs are similar to that of highly expressed cellular mRNAs in each fraction (Figure 2.3A). The reads that map to each individual cellular mRNA were largely unaffected early during infection (Figure 2.3B). This analysis shows that at 2 hpi the polysome association of cellular mRNA reflects cytoplasmic abundance.

By 6 hpi, reads that map to 4 of the 5 viral genes, N, P, M and G exceed those of any cellular mRNA, and reads mapping to the L gene were exceeded by only a few

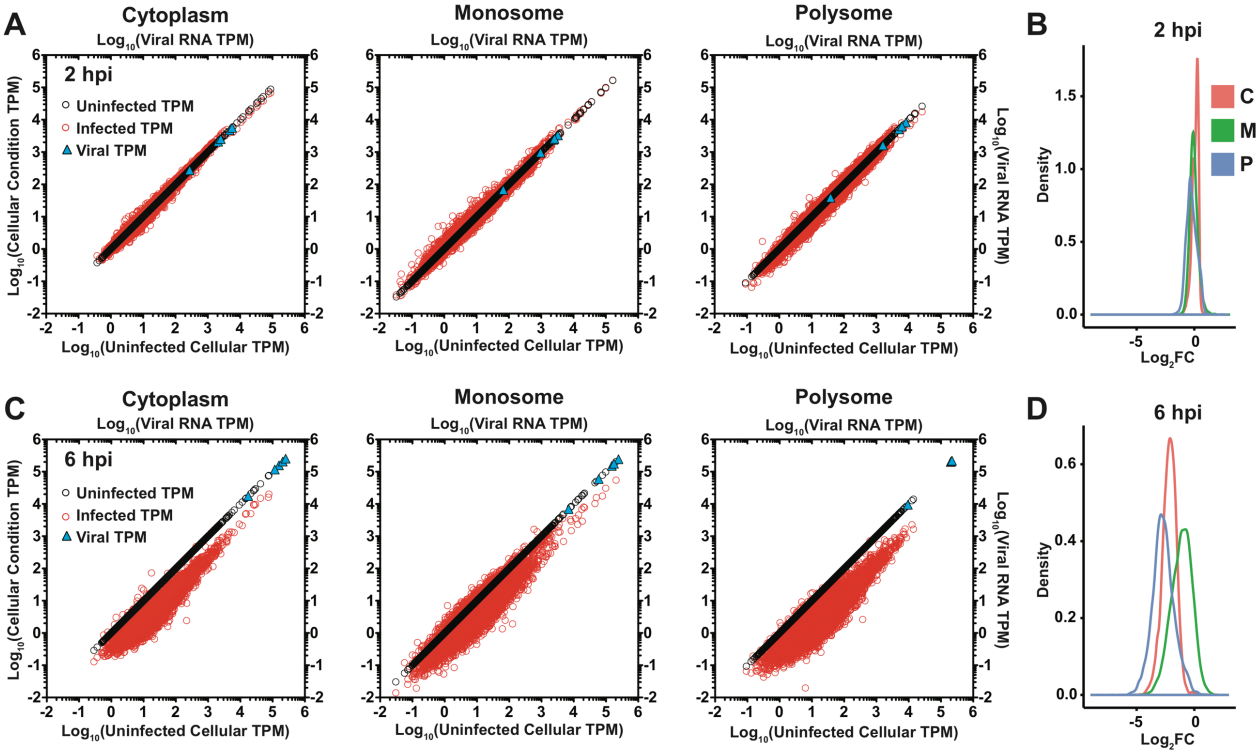


Figure 2.3. The relative abundance of cellular mRNAs in the cytoplasm and on polysomes decreases between 2 and 6 hpi. (A) Scatter plots of Transcripts per Kilobase Million (TPM) for exonic regions at 2 hpi. The TPM in uninfected cells for a given cellular mRNA is graphed on the bottom x-axis, and the TPM for a given cellular mRNA in either uninfected (black circles), or VSV infected cells (red circles) is graphed on the left y-axis. The relative abundance of viral mRNAs in TPM is plotted on the top x-axis and right y-axis. (B) Density plots of the $\log_2$ fold change in TPM for cellular mRNAs between uninfected or VSV infected cells at 2 hpi. C, M, and P denote “cytoplasm”, “monosome”, and “polysome”, respectively. (C) Scatter plots of TPM for individual mRNAs at 6 hpi, presented as in A. (D) Density plots of the $\log_2$ fold change in TPM at 6 hpi, presented as in B.

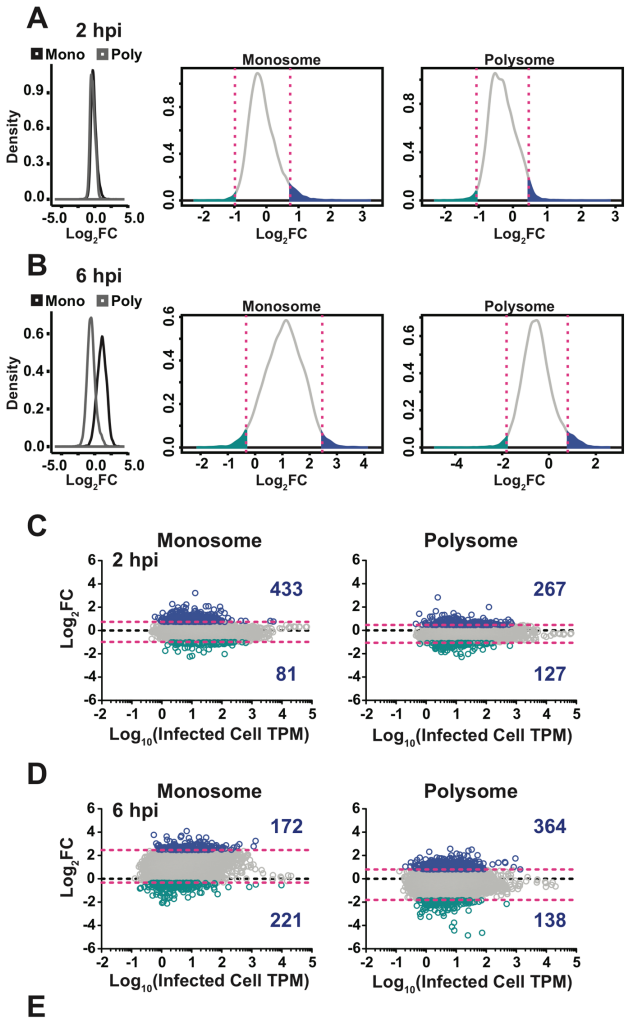
cellular genes (Figure 2.3C). This is concurrent with a 2-fold or greater decrease in the cytoplasmic and polysome abundance of the majority of cellular mRNAs (Figure 2.3C & 2.3D). Some cellular transcripts show increased monosome association at 6 hpi, indicating a non-uniform response to viral infection (Figure 2.3D). This may reflect a shift of cellular mRNAs out of the large polysomes toward smaller polysomes and monosomes.

The translation efficiency of cellular mRNAs is differentially impacted by VSV infection.

We next mined our sequence data to address the question; Are cellular mRNAs subject to differential translation regulation following VSV infection? For each individual mRNA, we divided the polysome TPM by the cytoplasmic TPM as a measure of potential translation efficiency (TE). We also performed a similar analysis for the monosome TPM divided by the cytoplasmic TPM to identify whether specific cellular mRNAs are differentially present in the monosome pool. We are cognizant of the fact that this measure cannot account for shifts of any given mRNA from large to small polysomes and thus likely represents an underestimate of the extent of regulation.

The fold change in TPM was plotted at 2 and 6 hpi with the subset of mRNAs that change >2 standard deviations of the mean (highlighted by blue and green dots) as mRNAs undergoing the most translational regulation (Figure 2.4A-D). Using this approach, we observe evidence of translational regulation at 6 hpi consistent with the timing of host cell shutoff (compare Figure 2.4C & 2.4D). This analysis demonstrates that the majority of monosome- or polysome-associated reads for cellular mRNAs are not significantly changed at 2 hpi, although we note that 433 cellular mRNAs exhibit an

Figure 2.4. The translation efficiency of cellular mRNAs is differentially impacted by VSV infection. (A) Density plots of the $\log_2$ fold change in translation efficiency (TE) between uninfected cells and cells infected for 2 hours for both monosome and polysome fractions. Individual density plots are shown to the right. The region of the density within the 95% confidence interval of the mean is shaded gray. Magenta lines denote $\pm$ 2 standard deviations of the mean. Regions with increased TE relative to the mean are shaded blue, and regions with decreased TE are shaded green. (B) Analysis at 6 hpi, presented as in A. Downstream analyses were performed on genes in the green or blue regions. (C) Plots of $\log_2$ fold changes in TE at 2 hpi plotted against cytoplasmic abundance in infected cells. Magenta lines denote $\pm$ 2 standard deviations of the mean $\log_2$ fold change. Genes outside the 95% confidence interval are denoted by blue (increased) or green (decreased) dots. (D) The $\log_2$ fold changes in TE at 6 hpi as presented in C. (E) Gene ontology analysis on mRNAs with decreased translation efficiency, analysis was performed using GOseq in R. (F) Gene ontology analysis for mRNAs under positive translational control, as determined using GOseq in R.



E

GO Term	p value	BH p value
response to biotic stimulus	1.60E-06	0.0147
protein phosphorylation	2.56E-06	0.0147
regulation of phosphorylation	2.85E-06	0.0147
regulation of signal transduction	4.35E-06	0.0164
regulation of protein phosphorylation	6.97E-06	0.0164
positive reg. of phosphorus metabolic process	9.11E-06	0.0164
positive regulation of phosphate metabolic process	9.11E-06	0.0164
negative regulation of cell proliferation	979E-06	0.0164
positive regulation of phosphorylation	1.10E-05	0.0164
MAPK cascade	1.13E-05	0.0164
regulation of MAPK cascade	1.34E-05	0.0164

F

GO Term	p value	BH p value
RNA binding	2.83E-18	4.39E-14
poly(A) RNA binding	8.76E-18	6.80E-14
ATPase activity	1.96E-13	1.02E-09
helicase activity	2.86E-13	1.11E-09
nucleoside-triphosphatase activity	1.22E-12	3.77E-09
organelle lumen	1.50E-12	3.89E-09
membrane-enclosed lumen	2.14E-12	4.74E-09
macromolecular complex	2.79E-12	5.42E-09
pyrophosphatase activity	5.04E-12	7.34E-09
hydrolase activity	5.12E-12	7.34E-09

Figure 2.4 (Continued). The translation efficiency of cellular mRNAs is differentially impacted by VSV infection.

increase in monosome association (Figure 2.4A & 2.4C). The number of cellular mRNAs exhibiting translational regulation at 2 hpi is likely overestimated, given that most mRNAs with increased translation efficiency increase less than 2-fold (Figure 2.4A & 2.4C). At 6 hpi this analysis reveals evidence of increased reads of 172 cellular mRNAs in the monosome fraction, 364 in the polysome fraction and 138 with a larger reduction in polysome association (Figure 2.4B & 2.4D). It is important to interpret these results in light of the fact that globally at 6 hpi 49% of the monosome-associated reads are viral; thus, the 172 cellular mRNA represent those that are most shifted into the monosome pool. Overall, 5,297 cellular mRNAs are enriched >2-fold in monosomes at 6hpi (Figure 2.4B & 2.4D). Similarly, at 6 hpi almost 70% of the polysome-associated reads are viral; thus, the enhanced polysome association of the 364 cellular mRNAs is likely an underestimate (Figure 2.4B & 2.4D).

Gene ontology analysis indicates that the 364 mRNAs with increased polysome association are enriched for proteins involved in RNA binding, and the 138 mRNAs with decreased polysome association are enriched for proteins involved in cellular response to stimuli and signaling cascades (Figure 2.4E & 2.4F). In the graph presented, the majority (95%) of cellular mRNAs show an alteration within 2 standard deviations of the average change in polysome association (Figure 2.4B & 2.4D), but this analysis is based on TPM and therefore does not take into account shifts of any given mRNAs position within polysomes. Consequently, the graphs shown are presented to draw attention to the transcripts that are most altered as a starting point to identify whether specific transcript features correlate with translation efficiency.

Targets of positive translational regulation are longer and more AU-rich.

We next mined our sequence data to determine whether cellular mRNAs with enhanced or suppressed polysome-associated reads share any common features. The prime determinant of polysome-associated reads related to the cytoplasmic reads for any given transcript, consistent with mRNA abundance being a major determinant of translatability (Figure 2.5A & 2.5B). We extracted mRNA sequences and annotations from the UCSC Genome browser for the subset of cellular mRNAs that exhibit the largest increase or decrease in polysome-associated reads and examined whether their size, GC content, polyA tail length or half-life correlated with increased or decreased sequence reads (Figure 2.5C-F, Figure 2.6).[438, 439] This analysis revealed that cellular mRNAs with reduced reads in the polysome fraction in general have higher GC content (Figure 2.5D, Figure 2.6B-D). By contrast those cellular mRNAs associated with increased reads in the polysome fraction were typically longer and have a lower GC content (Figure 2.5C-F, Figure 2.6A-D).

Further examination of the correlation between transcript length and enhanced polysome-associated reads indicated that the correlation corresponds to longer coding regions in these mRNAs, as opposed to 5′ or 3′ UTR (Figure 2.5C, E-F, Figure 2.6A). The correlation between lower GC content and increased polysome-associated reads was not related to localized GC content of the UTRs or coding region (Figure 2.5D, Figure 2.6B-D). We did not observe a correlation between poly(A) tail length and polysome-associated reads (Figure 2.6F). Enhanced polysome-associated reads trended towards longer mRNA half-life which would relate directly to mRNA abundance (Figure 2.6E).

Figure 2.5. Targets of positive translational regulation are longer and more AU-rich. (A) Analysis of translation efficiency in uninfected cells for mRNAs with high cytoplasmic abundance (purple) or low cytoplasmic abundance (orange) as compared to mRNAs with cytoplasmic abundance within 2 standard deviations of the mean abundance (gray). Cytoplasmic abundance by TPM is from the data set published with this paper. ***$p < 2.2 \times 10^{-16}$; **$p < 5.0 \times 10^{-5}$; *$p < 0.05$; all others $p > 0.05$ as determined by the Wilcoxon rank sum test compared to mRNAs with relative abundance levels within the 95% confidence interval of the mean. Hinges correspond to the 25^{th}-75^{th} percentiles, and whiskers denote 1.5 times the inter-quartile range. (B) Analysis as in A for cytoplasmic abundance in infected cells. (C-F) mRNA characteristics for mRNAs with increased translation efficiency (blue) or decreased translation efficiency (green) at 6 hpi as compared to mRNAs with translation efficiency measurements within 2 standard deviations of the mean. CDS is defined as the coding region of the gene sequence, and size is the number of nucleotides. Analysis was performed as in A.

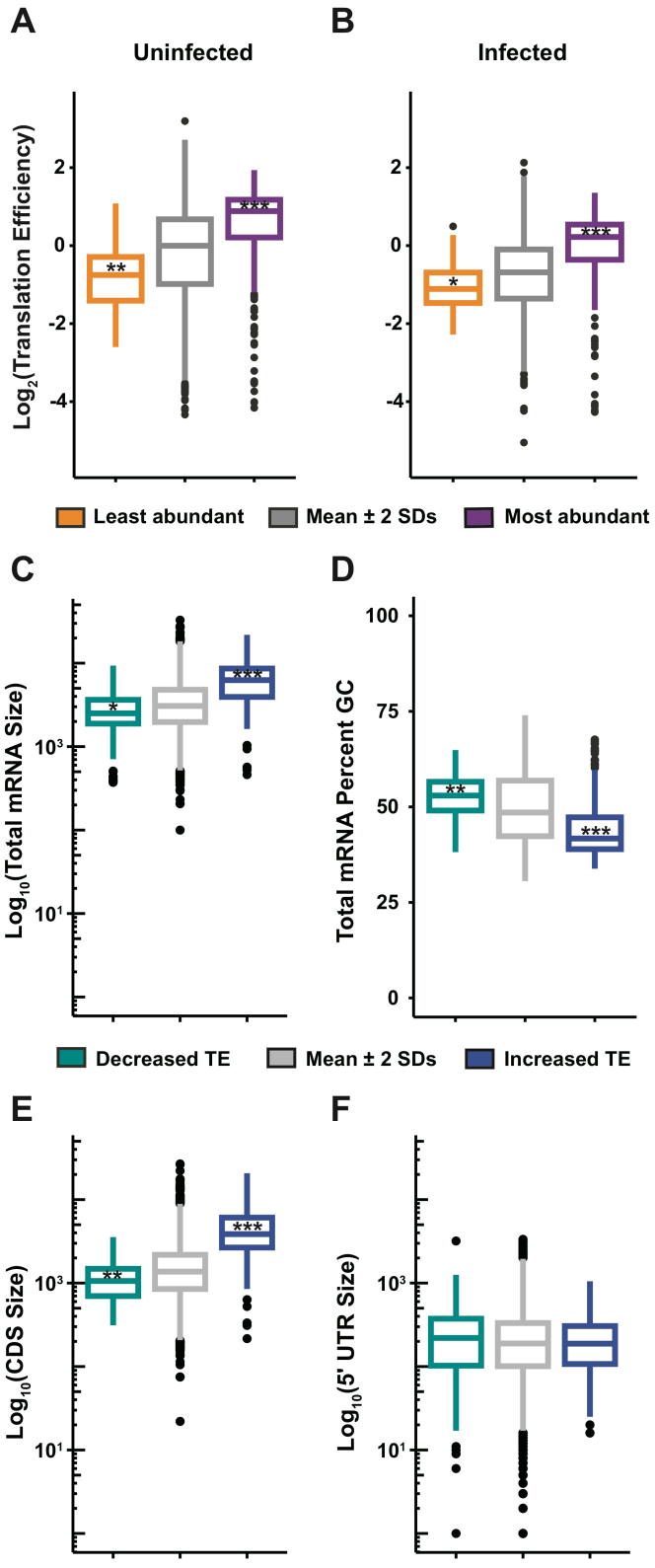


Figure 2.5 (Continued). Targets of positive translational regulation are longer and more AU-rich.

Figure 2.6. Lower GC content is a general, and not localized feature, of mRNAs under positive translational regulation. (A-F) Analysis of mRNA characteristics for RNAs with increased (blue) or decreased (green) translation efficiency as compared to mRNAs with translation efficiency measurements at 6 hpi within 2 standard deviations of the mean. ***$p<2.2 \times 10^{-16}$, **$p<5.05 \times 10^{-5}$, *$p<0.05$; all other $p>0.05$ as determined by the Wilcoxon rank sum test compared to the translation efficiency of mRNAs within the 95% confidence interval. Hinges correspond to the 25^{th}-75^{th} percentiles, and whiskers denote 1.5 times the inter-quartile range. CDS is defined as the coding region of the gene sequence, and size is the number of nucleotides. RNA half-life data are from Tani et al., 2012; and poly(A) tail length was determined by Chang et al., 2014.[438, 439]

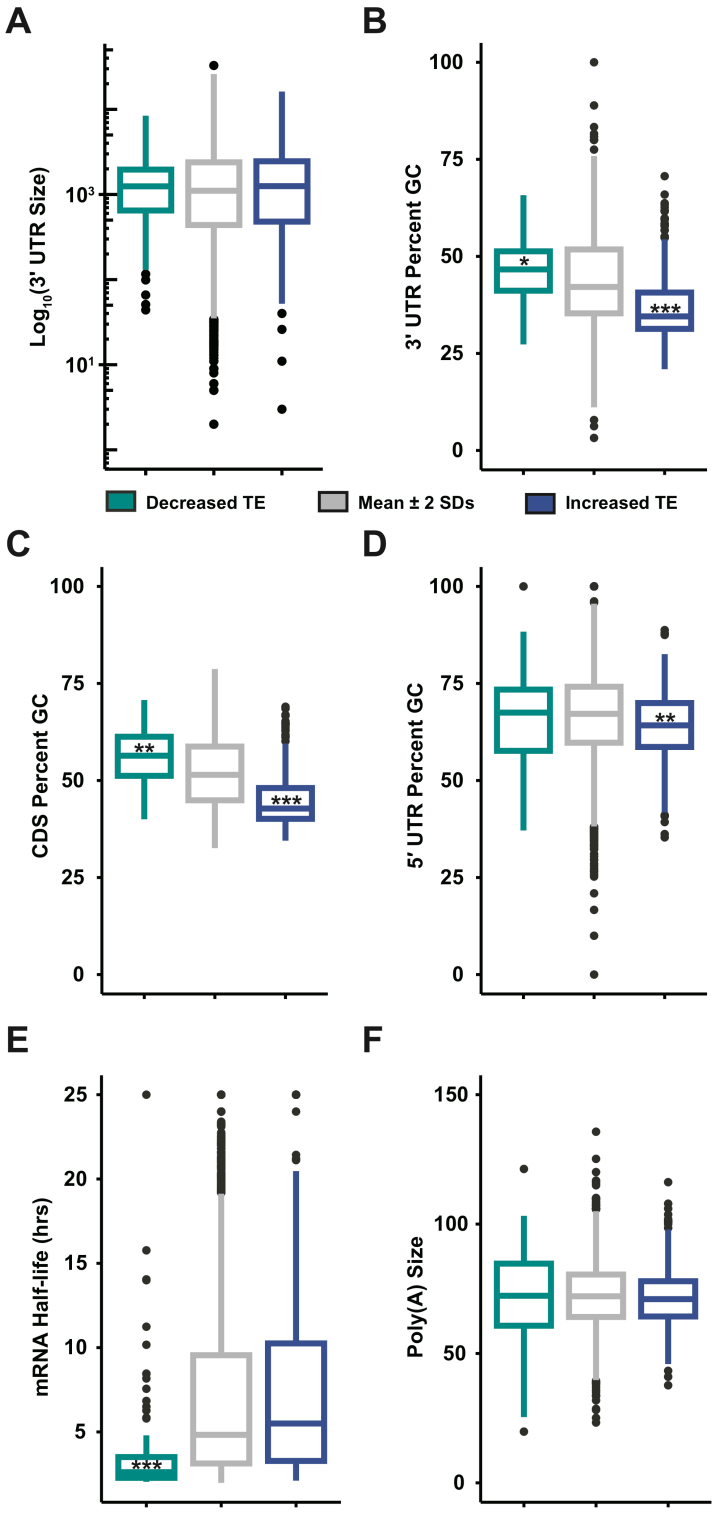


Figure 2.6 (Continued). Lower GC content is a general, and not localized feature, of mRNAs under positive translational regulation.

Altered distribution of cellular mRNA within polysomes following infection.

Our analysis of translation efficiency of cellular mRNAs at 6 hpi identified 502 cellular mRNAs with altered polysome association. However, our sequencing data cannot discriminate between transcripts associated with large or small polysomes and viral infection reduces large polysomes. We selected a subset of viral and cellular mRNAs and interrogated the distribution of mRNA across polysome profiles by RT-qPCR (Figure 2.7). We selected the viral N and G mRNAs as representative transcripts translated in the cytoplasm by soluble or endoplasmic reticulum-associated ribosomes, respectively. We examined the cellular transcripts COL4A2 and TGIF with reads that show a fold change >2 standard deviations of the mean fold change, and ACTN4 and UBE2B which exhibit altered polysome-associated reads >2-fold, but were within the 95% confidence interval. GAPDH and ACTB were selected because their polysome-associated reads did not change significantly at 6 hpi.

Consistent with the robust production of viral proteins at 6 hpi, the VSV N and G mRNAs were localized in fractions corresponding to 3 or more ribosomes (Figure 2.7A). The distribution of ACTN4 and COL4A2 shifted towards polysome fractions containing 3 or more ribosomes compared with their distribution in uninfected cells (Figure 2.7B). The distribution of ACTB and GAPDH mRNA shifted towards smaller polysomes by 6 hpi when compared to uninfected cells, and we observe an increase in the fraction of mRNA associated with free subunits (Figure 2.7C). This shift was not captured by the translation efficiency measurements due to pooling polysome fractions for sequencing. UBE2B and TGIF mRNAs were redistributed out of polysome fractions by 6 hpi (Figure 2.7D). These qPCR data are therefore consistent with our RNAseq results. Dissociation

Figure 2.7. Altered distribution of cellular mRNA within polysomes following infection. (A-D) The distribution of mRNA in polysome profiles from uninfected or infected HeLa cells at 6 hpi. A representative polysome trace from infected cells at 6 hpi is shown in light gray, and the mRNA polysome distribution in uninfected cells is shown in black, and infected cells in red. The RNA distribution is presented as the fraction of the total amount of a given RNA recovered. Error bars denote the standard deviation from three independent replicates. (A) Polysome distributions of VSV N and G. (B) RNAs with increased translation efficiency by RNAseq. (C) RNAs with unchanged translation efficiency following infection. (D) mRNAs with decreased translation efficiency.

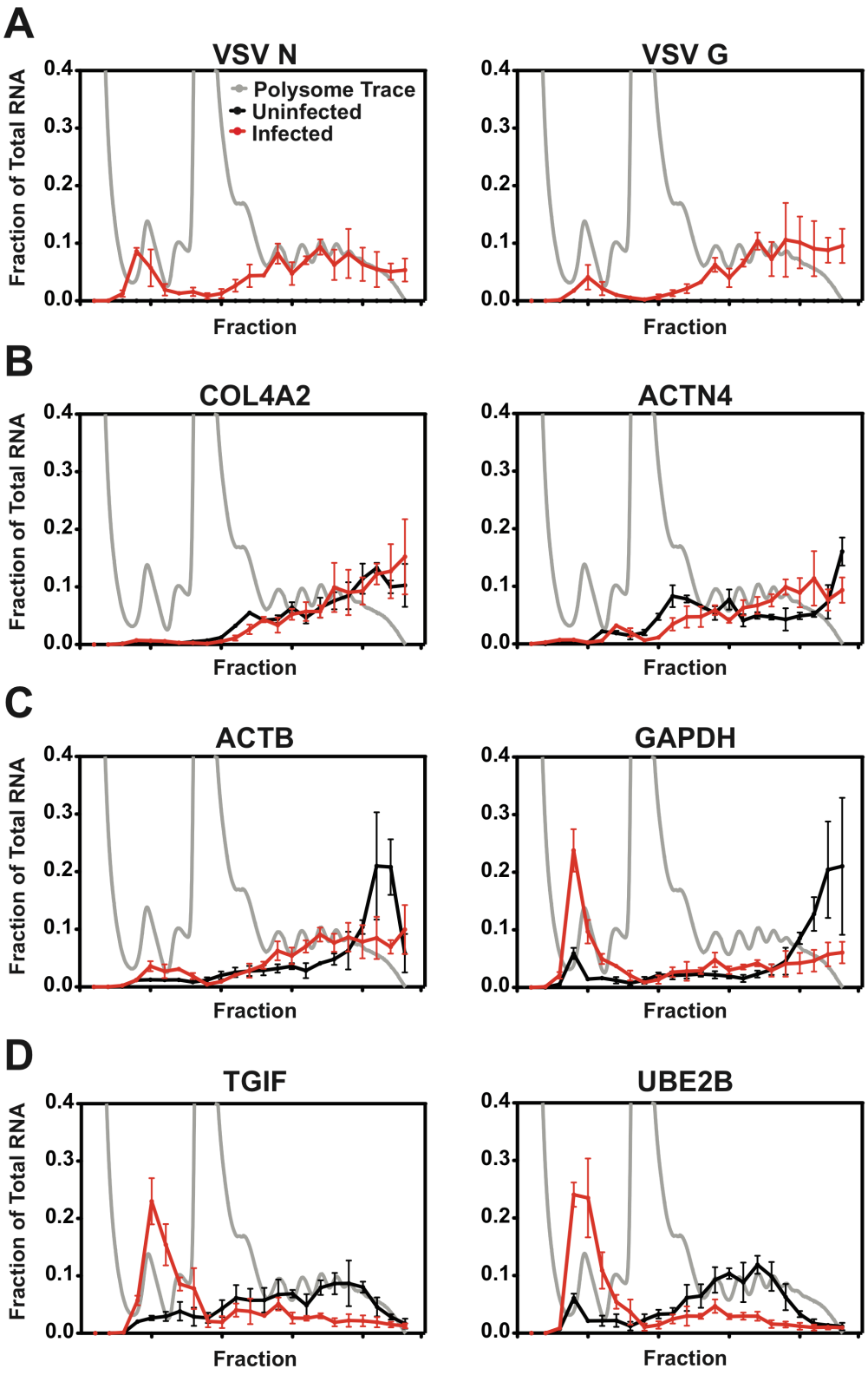


Figure 2.7 (Continued). Altered distribution of cellular mRNA within polysomes following infection.

of ribosomal complexes by treating lysates with EDTA prior to polysome profiling, released mRNA from polysomes and shifted the distribution towards fractions containing free subunits, indicating that RNAs found in the polysome fractions are associated with ribosomal complexes (Figure 2.8). In VSV infected cells, the shift towards smaller polysomes identified for several cellular mRNAs, highlights that in addition to viral mRNA abundance, the redistribution of cellular mRNAs to smaller polysomes will correlate with a reduction in translation and thus also contribute to host shutoff (Figure 2.7C & 2.7D).

Cap methylation on viral mRNA is not required for polysome association at 6 hours post-infection.

Our results indicate that viral mRNAs are associated with large polysomes at a time post-infection when many cellular mRNAs are shifted from large to small polysomes (Figure 2.7). To interrogate the role of cap methylation in mediating the polysome association of viral RNA, we infected HeLa cells with VSV mutants defective in methylating the caps of viral mRNAs *in vitro* and performed polysome profiling and qPCR (Figure 2.9). The VSV mutant G1670A is defective in performing N-7 cap methylation, but retains the ability to methylate viral mRNA caps at the 2′-O position.[54] Analysis of the distribution of VSV N and G mRNA within polysomes at 6 hpi revealed that the majority of viral mRNA was bound by large polysomes in G1670A infected cells, similar to what was observed during wild-type infection (Figure 2.9A & 2.9B). This is consistent with the robust production of viral proteins that has previously been observed in G1670A infected cells, and suggests that N-7 cap methylation is not required for

Figure 2.8. RNAs are ribosome-associated at 6 hours post-infection. (A-E) the distribution of mRNA in polysome profiles from uninfected or infected HeLa cells at 6 hpi in the presence or absence of EDTA. EDTA was added to a final concentration of 50mM and lysates were incubated for 5 minutes on ice. Lysates were spun through gradients containing EDTA. A representative polysome trace from infected cells at 6 hpi following EDTA treatment is shown in light gray. The mRNA polysome distribution from untreated, uninfected lysates is shown in black; and untreated, infected cells in red. RNA distributions from EDTA treated, uninfected lysates are shown in dark gray; and EDTA treated, infected lysates in pink. The RNA distributions are presented as the fraction of the total amount of a give RNA recovered. Error bars denote the standard deviation from at least two independent replicates. Polysome distributions of (A) ACTN4, (B) ACTB, (C) UBE2B, (D) VSV N, and (E) VSV G.

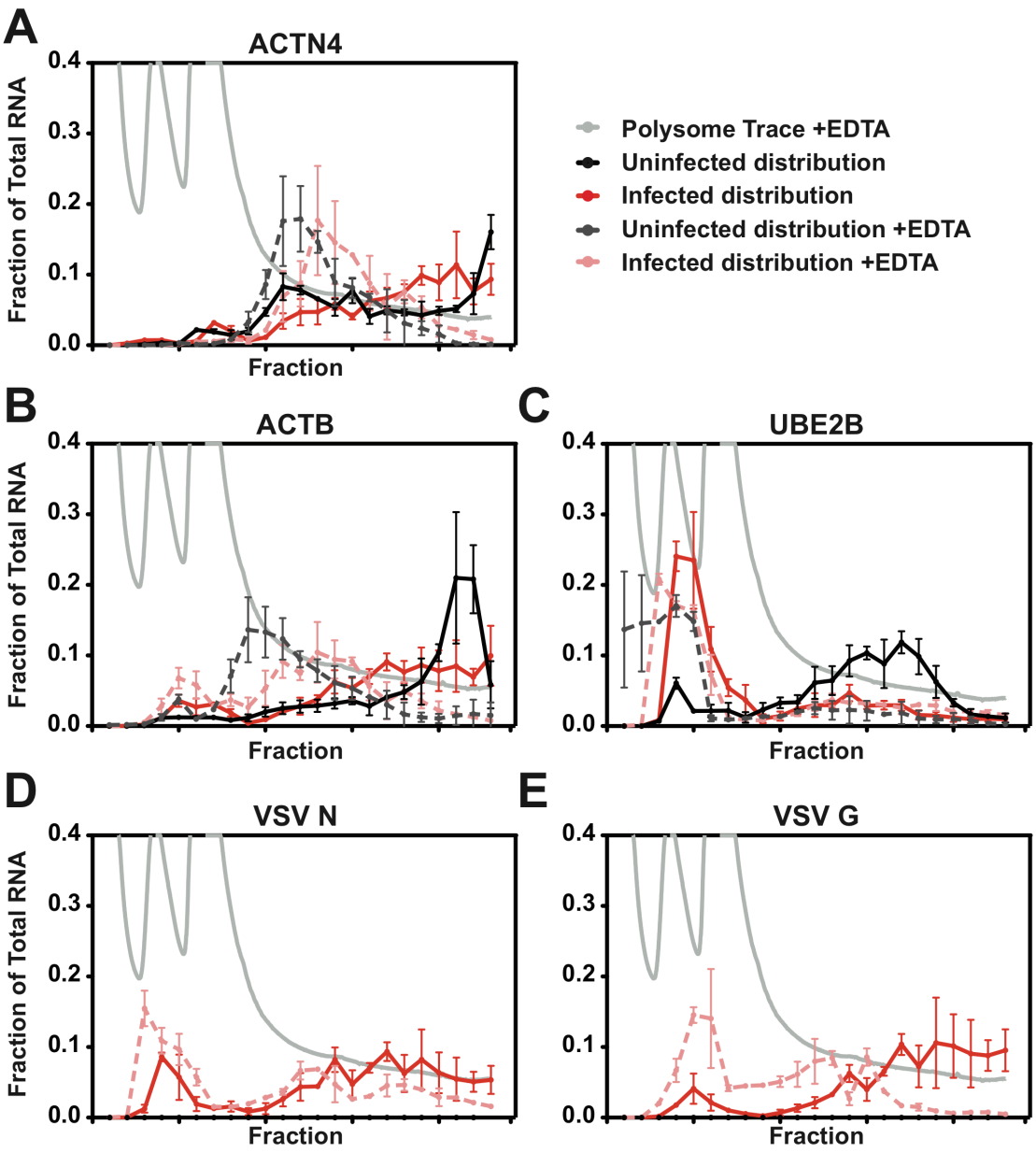


Figure 2.8 (Continued). RNAs are ribosome-associated at 6 hours post-infection.

Figure 2.9. RNA produced by viral mutants defective in cap methylation is polysome-associated in infected cells. (A) Polysome distribution of VSV N and G mRNA at 6 hpi with wild-type VSV. Results of qPCR for each individual polysome fraction are presented as a fraction of the total VSV N or VSV G mRNA recovered, in red. Error bars denote standard deviation from two independent replicates, and a representative polysome trace from infected cells is shown in gray. (B) Polysome distribution of VSV N and G mRNAs at 6 hpi with a VSV mutant deficient in performing N-7 cap methylation, G1670A, presented as in A. (C) Polysome distribution of VSV N and G mRNAs at 6 hpi with a VSV mutant, G4A, defective in both N-7 and 2′-O cap methylation, presented as in A.

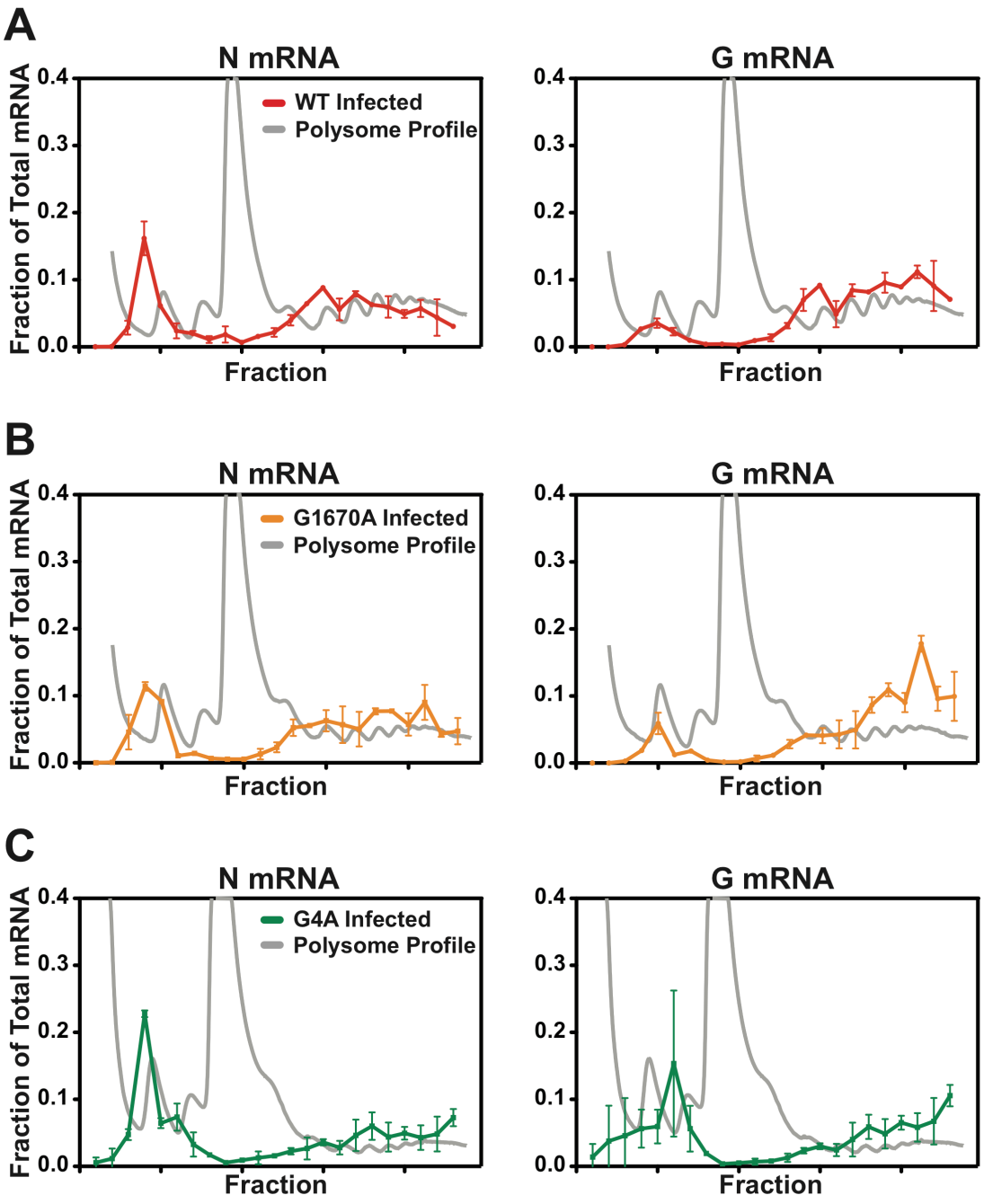


Figure 2.9 (Continued). RNA produced by viral mutants defective in cap methylation is polysome-associated in infected cells.

translation initiation on viral transcripts.[54] The VSV mutant G4A is entirely deficient for cap methylation *in vitro*, performing neither N-7 nor 2′-O methylation on viral mRNA caps.[54] At 6 hpi, polysome profiling of G4A infected cells revealed and increased ratio of sub-polysome to polysome mRNA, although a large fraction of viral RNA is associated with large polysomes (Figure 2.9C). Both viruses were able to drive host shutoff as evidenced by large polysome collapse and increased monosomes (Figure 2.9). These results indicate that cap methylation on viral transcripts is not absolutely required for efficient polysome formation, consistent with the observation that VSV RNAs are translated when eIF4E is depleted.[395]

Discussion

Viral RNA abundance as a general host shutoff mechanism.

We applied a global, unbiased RNAseq approach to address the question of how VSV inhibits host cell translation, while maintaining robust synthesis of viral proteins? Our results support a model first proposed by Lodish and Porter in 1980, that an overwhelming abundance of viral mRNA leads to a redistribution of ribosomes onto viral messages.[87, 400] At a global level, we found that >60% of the cytoplasmic mRNA maps to viral sequences by 6 hpi, and that this corresponds to ~70% of the total polysome-associated mRNA being of viral origin (Figure 2.1C). This suggests that a major mechanism by which VSV inhibits host cell translation relates to the power of the 241kDa viral polymerase.

Consistent with the abundance model, we also find a correlation between abundance and translation efficiency for cellular mRNAs at 6 hpi (Figure 2.5A & 2.5B). We also found a strong correlation between increased translation efficiency and long open reading frame length (Figure 2.5E). In a situation of RNA competition, we would expect that abundant, long mRNAs would exhibit increased likelihood for ribosome association. We note, however, that although such longer mRNAs remain polysome-associated, the number of ribosomes on the mRNAs would likely decrease owing to the sequestration of eIF4E.

Recent work on a mouse coronavirus, a positive-strand RNA virus, influenza virus, a negative strand RNA virus with a segmented genome, and vaccinia virus, a DNA virus, have also observed that >50% of the bulk mRNA in infected cells maps to the viral genome.[217, 440, 441] This suggests that overwhelming viral RNA abundance

may be a common mechanism used by highly cytopathic viruses that shut off host cell gene expression. In contrast to VSV, influenza, coronaviruses, and vaccinia all target cellular mRNAs for cleavage and degradation.[245, 249, 250, 260, 285, 347, 352, 357, 358] Thus, VSV represents the first case in which viral dominance of the cytoplasmic mRNA pool is achieved without degrading cellular mRNA. This highlights the robustness of the viral polymerase, and the exponential amplification of viral mRNA as a result of genome replication and secondary transcription.

Inhibitory effects of eIF4E-BP1 on eIF4E during VSV infection.

The profound inhibition of cellular translation that occurs during VSV infection is also impacted by the activation of eIF4E-BP1 and sequestration of eIF4E.[293] Although our sequencing experiment was not designed to specifically test the contribution of eIF4E sequestration to host shutoff, mining our dataset did not identify specific features associated with eIF4E dependence on cellular mRNAs with decreased translation efficiency after infection. It remains unclear why VSV translation is relatively insensitive to eIF4E sequestration, despite the presence of a 5′ cap structure on the viral mRNAs, but our results demonstrate that a fully methylated cap structure is not required for loading ribosomes onto viral mRNAs (Figure 2.9). Additional mechanisms beyond viral RNA abundance may account for the reduced requirement of VSV mRNA on the presence of cap methylation or eIF4E to direct efficient protein synthesis.

The underrepresentation of viral mRNA and overrepresentation of cellular mRNA in monosomes at 6 hpi (Figure 1), is consistent with the timing and consequences of eIF4E sequestration. This helps move cellular mRNAs out of polysomes into

monosomes, and likely explains the shift of cellular mRNAs from large to small polysomes (Figure 2.7). The identification of classes of cellular mRNAs that are differentially shifted within polysomes, further suggests that although sequestration of eIF4E impacts translation, this likely augments the competition provided by viral mRNA abundance on translation.

Characteristics of cellular mRNAs exhibiting increased translational efficiency.

VSV mRNAs are AU-rich, have short UTRs, and are generally thought to be relatively unstructured in cells.[40, 57, 77] These characteristics would predict that viral mRNAs should be less sensitive to eIF4E inhibition, thereby giving the virus an advantage when eIF4E-BP1 is activated. Indeed, viral RNAs are less dependent on N-7 cap methylation for directing efficient polysome association (Figure 2.9). We note that the cellular mRNAs with increased translation efficiency at 6 hpi also had lower GC content (Figure 2.5 & 2.6). The length of cellular UTRs did not correlate with translation efficiency and thus does not help explain how the short UTR viral transcripts are translated (Figure 2.5 & 2.6).

The mRNAs identified as having increased translation efficiency after VSV infection were enriched in RNA binding and poly(A) RNA binding annotations by gene ontology analysis (Figure 2.4). This may reflect a cellular response to the disruption of mRNA homeostasis due to robust viral transcription and an increased mRNA burden. How the cell would sense this stimulus, and the mechanisms coordinating the response, remain unexplored. We also note that the terms enriched in the transcripts whose reads were reduced on viral infection are associated with signaling.

Based on our global analysis of polysome-associated mRNAs in a VSV infected cell, we propose that an overwhelming abundance of viral mRNA leads to competition for cellular ribosomes in agreement with a model first proposed by Lodish and Porter. We have extended this model by reconciling it with the observation that eIF4E-BP1 is activated following VSV infection, through our finding that cellular mRNAs are overrepresented in monosome fractions at 6 hpi. We propose that viral mRNA abundance is the key determinant driving host shutoff during efficient viral protein synthesis, but that additional contributions are provided by eIF4E sequestration which differentially shifts cellular mRNAs into smaller polysomes and monosomes.

Materials and Methods

Cells and Viruses

HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Tissue Culture Biologicals, Tulare, CA). Viral stocks were grown on Syrian golden hamster kidney BSRT7 cells (a gift from K. Conzelmann), and purified on linear 15-45% sucrose gradients prepared in NTE (10mM Tris pH 7.4, 100mM NaCl, 1mM EDTA). Viral titers were determined by plaque assay on African green monkey kidney Vero cells (ATCC, CCL-81), as previously described.[32] For infections, HeLa cells were first washed with Hanks' Balanced Salt Solution (HBSS) and then infected at a MOI of 10 for 1 hour at 37°C in serum free DMEM. At 1 hour post-infection, the inoculum was removed, the cells were washed again with HBSS, and the infection was allowed to continue for the indicated time in DMEM containing 10% FBS.

Polysome Profiling

For polysome profiling, HeLa cells were treated with DMEM containing 100ug/ml cycloheximide at 37°C for 3 minutes. Cells were washed twice with 1X ice-cold phosphate buffered saline (PBS) containing 100ug/ml cycloheximide, and kept on ice or at 4°C for the remainder of the protocol. Cells were scraped into a 1.5 ml microcentrifuge tube in 1X PBS with 100ug/ml cycloheximide, and pelleted at 300 × *g* for 10 minutes. Cells were resuspended in a hypotonic buffer of 5mM Tris (pH 7.4), 2.5mM $MgCl_2$, 1.5mM KCl, and RNAsin (Promega, Madison, WI). Cycloheximide and DL-Dithiothreitol (DTT) were added sequentially to 100ug/ml and 3uM, respectively.

Triton X-100 and sodium deoxycholate were then added sequentially to 0.5% (volume/volume), cells were briefly vortexed, and incubated for 15 minutes on ice. Polysome extracts were clarified by spinning for 2 minutes at 12,000 × *g*. Sucrose gradients were prepared with a Gradient Master Station (Biocomp, Fredericton, Canada) using 10% and 50% w/v sucrose dissolved in 15mM Tris (pH 7.4), 15mM $MgCl_2$, and 150mM NaCl. RNAsin and 100ug/ml cycloheximide were added immediately before gradient preparation. Gradients were subjected to centrifugation for 2 hours at 40,000 × *g* in a Beckman Coulter ultracentrifuge using an SW40Ti rotor, and 500ul fractions were collected while monitoring UV254 on a Gradient Master Station.

RNA Extraction, Library Preparation, and RNAseq

RNA was extracted from total cytoplasmic RNA fractions, polysome fractions, or monosome fractions using LS Trizol (Invitrogen) following the manufacturer's protocol. Equal amounts of RNA as determined by spectrophotometry using a Nanodrop 2000 (Thermo Fisher, Waltham, MA) were used for library preparation using the Illumina TruSeq vII RNA Library Preparation Kit (Illumina, San Diego, CA), and sequenced at the Whitehead Institute (Cambridge, MA) on an Illumina HiSeq2500 system. Reads were trimmed and mapped to the concatenated hg38 and VSV genomes using CLC Genomics Workbench (Qiagen, Redwood City, CA).

Translation efficiency and differential gene expression analysis

Translation efficiency measurements used Transcripts per Kilobase Million (TPM) as determined by co-mapping to the human and viral genomes in the following formula:

$$\text{Translation Efficiency (TE)} = \frac{\text{TPM from Polysome Library}}{\text{TPM from Cytoplasmic Library}}$$

This ratio was determined for uninfected and infected cells, and presented as the $\log_2$ fold change.

Differential gene expression analysis was performed using DESeq2, and gene ontology analysis was performed in R using GOseq.

mRNA Characteristics and Statistical Analysis

UTR and CDS sequences were downloaded from the UCSC table browser using "KnownCanonical" mRNA identifiers. Non-protein coding RNAs were excluded from the analysis. Poly(A) tail length and mRNA half-lives were pulled from published data sets.[438, 439] Graphs and statistical analyses were performed in R using the built-in "wilcox_test" statistical test, and "geom_boxplot" or "geom_density" functions in ggplot and cowplot.

RT-qPCR

Total RNA was recovered from polysome fractions using LS Trizol (Invitrogen) using the manufacturer's protocol, and 500ng total RNA as determined by spectrophotometry from each polysome fraction was reverse-transcribed using oligod$(T)_{20}$ priming and Superscript III Reverse Transcriptase (Thermo Fisher) at 50°C for 1 hour. cDNA was treated with RNAseA and RNAseH for 15 min at 37°C, and diluted

1:5 for cellular gene-specific qPCR or 1:125 for viral gene-specific qPCR using Power Sybr Green (Thermo Fisher). Relative quantitation was determined by $\Delta\Delta C_t$ using the following primer pairs:

ACTB	Forward: 5′ ACCCAGCACAATGAAGATCA 3′
	Reverse: 5′ CTCGTCATACTCCTGCTTGC 3′
ACTN4	Forward: 5′ ACATCTCCGTGGAAGAGACC 3′
	Reverse: 5′ GGAAGTTCTGCACATTGACG 3′
COL4A2	Forward: 5′ AACGGGATTCCATCAGACAC 3′
	Reverse: 5′ ATGCCTCTTATTCCTGGTTCC 3′
GAPDH	Forward: 5′ AGCCTCAAGATCATCAGCAATG 3′
	Reverse: 5′ ATGGACTGTGGTCATGAGTCCTT 3′
TGIF	Forward: 5′ CACCGTTACAATGCCTATCC 3′
	Reverse: 5′ GATTTGGATCTTTGCCATCC 3′
UBE2B	Forward: 5′ CAATTCAGTCTCTGCTGGATG 3′
	Reverse: 5′ AACAATGGCCGAAACTCTTT 3′
VSV G	Forward: 5′ GTGGGATGACTGGGCTCCAT 3′
	Reverse: 5′ CTGCGAAGCAGCGTCTTGAA 3′
VSV N	Forward: 5′ GAGTGGGCAGAACACAAATG 3′
	Reverse: 5′ CTTCTGGCACAAGAGGTTCA 3′

Polysome distribution is presented as the fraction of the total recovered RNA for each individual polysome fraction.

Chapter 3: The matrix protein of vesicular stomatitis virus blocks expression of interferon in cells infected with wild-type VSV.

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Abstract

Infection of mammalian cells with vesicular stomatitis virus (VSV) results in the inhibition of cellular protein synthesis. An overwhelming abundance of viral mRNA, and sequestration of the rate-limiting factor for translation initiation (eIF4E) accomplish this. The viral matrix protein (M) also contributes through inhibition of host RNA polymerases, and separately by blocking nuclear export of cellular mRNPs. This combined assault on the host translation, transcription and mRNP export machinery shuts off the host cell. VSV is exquisitely sensitive to interferon, and this multi-pronged assault on the cell helps evade the innate immune system. A virus containing a mutation in the matrix protein gene, VSV_{M51R}, is defective in blocking mRNP export and fails to inhibit interferon-β gene expression. To determine how blocking mRNP export influences mRNA translation we performed a global analysis of cytoplasmic, monosome- and polysome-associated mRNAs using massively parallel sequencing. Following infection of HeLa cells with VSV_{M51R} >50% of the cytosolic and polysome-associated mRNA reads map to the 5 viral genes by 6 hpi, compared to >60% in cells infected with wild-type virus. This result demonstrates that the inhibition of cellular transcription and mRNP export by M play relatively modest roles in global inhibition of host protein synthesis. We detect a significant upregulation in transcripts corresponding to interferons and interferon-stimulated genes (ISGs) by 6 hpi in cells infected with VSV_{M51R} but not VSV_{WT}, and demonstrate that they are associated with polysomes and are actively translated. These results indicate that viral replication is necessary for activation of the innate immune system and demonstrate that M protein suppresses

cytosolic interferon gene expression in the primary infected cell by blocking host gene expression.

Introduction

RNA viruses that replicate in the cytoplasm have evolved to contend with surveillance by cytosolic innate immune sensors of foreign nucleic acids. The cytosolic sensor RIG-I recognizes short, triphosphate RNA, and MDA5 recognizes longer dsRNA molecules.[409, 415, 416] Those molecules likely arise through errors of viral replication, thus providing the ligands for RIG-I and MDA5 leading to interferon gene expression and the establishment of an antiviral state.[409, 415, 416] To evade detection by these sensors, positive sense RNA viruses induce alterations in cellular membranes, thereby providing physically distinct compartments in which replication occurs.[416, 417] Negative sense RNA viruses sequester their replication machinery in non-membrane bound liquid compartments that form through phase separation.[442-445] Like many positive sense RNA viruses, they also encode accessory proteins that antagonize innate immune signaling.[409, 415, 416]

Transcription and replication of negative strand viruses has been studied extensively for vesicular stomatitis virus (VSV), the prototype of the *Rhabdoviridae* family. In contrast to positive sense viruses, where the infecting genome can immediately be translated by host ribosomes, negative-strand viruses must initiate a primary round of transcription to produce viral mRNAs.[12, 13, 417] The viral mRNAs are then translated by host ribosomes, producing proteins that replicate the negative-sense genome through a positive sense antigenome intermediate.[67-69] New genomes can serve as templates for transcription, thereby amplifying the viral mRNA pool and enhancing protein production, or are assembled into new virions.[446] VSV

replicates exclusively the cytoplasm of infected cells, and must shield its RNAs from detection by RIG-I and MDA5.[23]

VSV mRNAs are capped and methylated by the viral large protein (L) which contains both a capping domain, and a methyltransferase domain responsible for sequentially modifying the 5′ guanosine cap through addition of methyl groups at the 2′-O and N-7 positions.[45, 48-50, 54, 56] Evidence from stalling transcription reactions *in vitro* demonstrates that those reactions can occur at a nascent chain length of 31-nt, a strategy that may help avoid the production of RNA ligands for RIG-I and MDA5.[46] The viral genome and antigenome RNAs, however, are not capped and have a 5′ terminal triphosphate moiety.[447, 448] Both genome and antigenome RNA are completely encased in a sheath of viral nucleocapsid protein (N), the most abundant protein produced in infected cells, and are never exposed to the cytosolic milieu.[38, 67, 70, 429, 430, 449] Two additional RNA products are synthesized in infected cells, a 47 and 45-nt triphosphate leader RNA copied from the 3′ end of the genome and antigenome, respectively.[426] In addition to modifying viral mRNAs and sequestering genomic RNA in a protein coat, VSV subverts cellular immunity by inducing a profound inhibition of cellular gene expression upon infection.

Viral mRNA abundance is a key determinant of host shutoff, leading to competition for cellular ribosomes and the redistribution of the translational apparatus onto predominately viral messages.[87, 400] The viral matrix protein, M, does not have any known enzymatic activity, but inhibits host cell transcription by interacting with TFIID, and nuclear export of cellular mRNPs by interacting with the Rae1•Nup98 nuclear export complex.[373-375, 381, 382, 385, 386, 389, 392] A viral mutant in M

harboring a single amino acid change at position 51, M51R, is defective in inhibiting nuclear mRNA export.[386, 397] Furthermore, M51R is unable to inhibit expression from a transfected reporter driven by the interferon-beta (IFN-β) promoter.[384, 391] Infection of mouse embryonic fibroblasts with VSV_{M51R}, results in the cytoplasmic appearance of IFN-β, suggesting that inhibiting nuclear export blunts the innate immune response to VSV.[424]

It is thought that RIG-I detects VSV infection, because RIG-I knockout MEFs support higher viral replication, and knockout mice are more susceptible to VSV.[411, 418] However, the viral nucleic acid sensed by infected cells and how M blockade of nuclear export directly contributes to host shutoff and immune evasion remain unclear.[415, 425] To interrogate the role of blocking nuclear mRNA export on host shutoff and immune evasion, we infected HeLa cells with wild-type VSV and VSV_{M51R} and performed a global analysis by massively parallel sequencing cytoplasmic, monosome- and polysome-associated mRNA. These results extend our previous work demonstrating that viral mRNA abundance is a key determinant driving host shutoff. By combining our RNAseq analysis with quantitative PCR, we demonstrate that blockade of nuclear mRNA export prevents the cytoplasmic expression of interferons and interferon-stimulated genes (ISGs). We conclude that viral replication is necessary for innate detection of VSV in an infected cell, but that during wild-type infection M nuclear blockade ablates cellular activation of the interferon pathway.

Results

Viral RNA comprises 50% of the cytoplasmic mRNA at 6 hours post-infection.

To interrogate the role of nuclear mRNP export blockade on translation, we infected HeLa cells with VSV_{M51R} and deep-sequenced the cytoplasmic, monosome-, and polysome-associated mRNA (Figure 3.1A). As evident by polysome profiling, M51R infection does not affect bulk polysomes at 2 hpi, but by 6 hpi results in increased monosomes and polysomes (Figure 3.1B). This is consistent with the inability of M51R to completely inhibit host cell gene expression (Figure 3.1B). Analysis of the reproducibility of sequencing reads mapped to a concatenated hg38 and M51R genome, yields Pearson correlation values >0.97, demonstrating reproducibility between cytoplasmic, monosome, and polysome replicates.

Mapping sequencing reads to the human and viral genomes demonstrates that >50% of the reads are viral at 6hpi (Figure 3.1C). The increase from <1% at 2 hpi is consistent with our previous observations from wild-type VSV infected HeLa cells, and indicates that defective nuclear blockade plays only a minor role in viral dominance of the cellular mRNA pool. The sequence reads mapping to the viral genome cover all 5 mRNAs, with clear drops in coverage at gene-junctions (Figure 3.2A). Relative reads mapping to each viral gene diminish with increasing distance from the 3′ leader, consistent with the sequential polar transcription of the viral genes (Figure 3.1D).

Analysis of sequencing reads that map to cellular genes at 2 and 6 hpi supports that the level of cellular genes in the polysome fraction mirrors that in the cytoplasm (Figure 3.1C). For wild-type VSV, we observed that 60-70% of the cytoplasmic and polysome reads were viral, supporting that an overwhelming abundance of viral mRNA

Figure 3.1. Viral RNA comprises 50% of the cytoplasmic mRNA at 6 hours post-infection. (A) Schematic representation of the experimental design. HeLa cells were uninfected, or infected at MOI 10 with M51R for 2 or 6 hours at 37°C. At 2 or 6 hpi cytoplasmic extracts were prepared and used directly for RNA isolation, or were subjected to polysome profiling. RNA was isolated from fractions corresponding to monosomes, or polysomes containing 3 or more ribosomes, and mRNA was used to make deep sequencing libraries. (B) Polysome profiles from uninfected (black) or M51R (blue) infected HeLa cells. Cytoplasmic extracts were spun through a 10-50% sucrose gradient and fractionated into 0.5 ml fractions while continuously monitoring absorbance at UV254nm. (C) Results of fragment mapping to concatenated hg38 (human) and M51R genomes for cytoplasmic, monosome, and polysome samples at 2 and 6 hpi. Trimming and mapping were performed in CLC Genomics Workbench. (D) Cytoplasmic gene cascade for the 5 viral mRNAs at 2 and 6 hpi, respectively. Expression level is presented as Transcripts per Kilobase Million (TPM) to normalize for gene length and library size, error bars denote standard deviation.

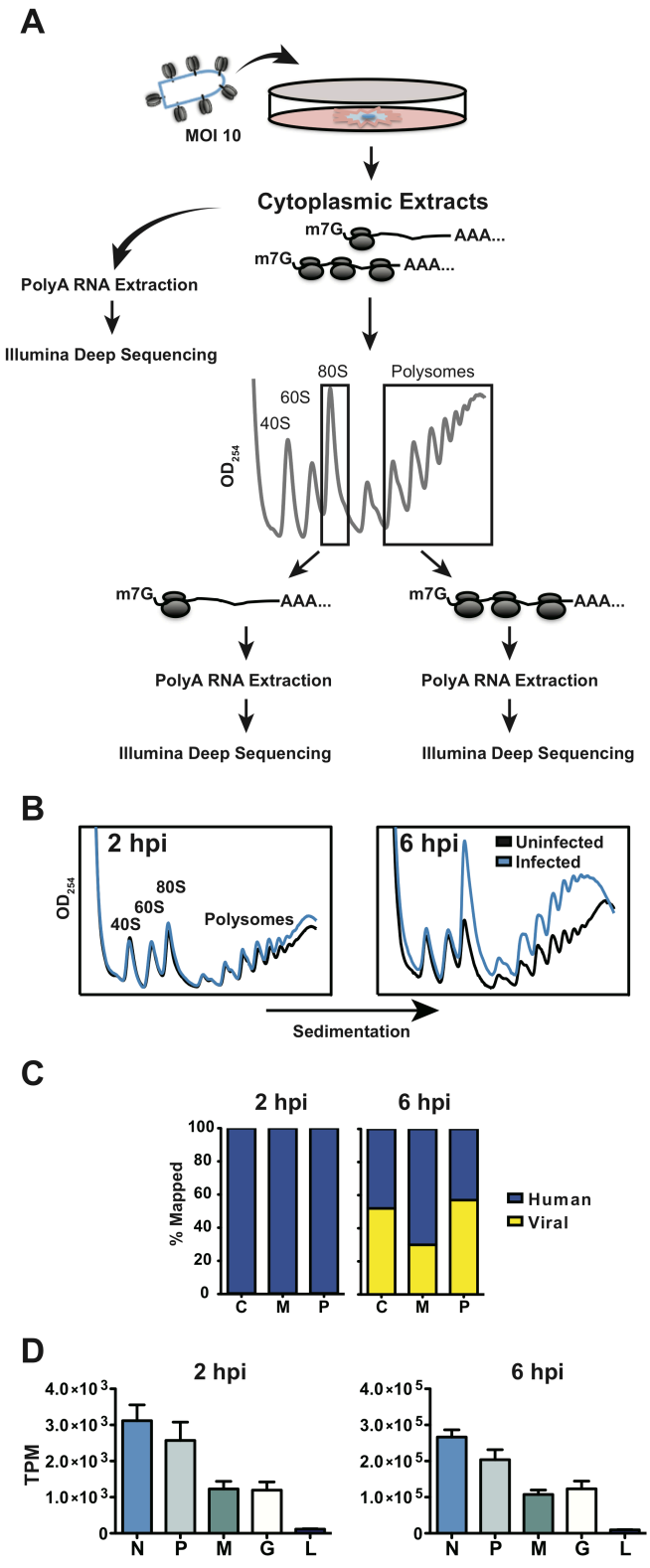

Figure 3.1 (Continued). Viral RNA comprises 50% of the cytoplasmic mRNA at 6 hours post-infection.

Figure 3.2. The M51R gene cascade at 2 and 6 hpi. (A) Mapping coverage to the M51R genome from a representative replicate at 6 hpi. Coverage analysis and visualization was performed in CLC Genomics Workbench. (B) Gene cascade in monosome and polysome fractions for the 5 viral mRNAs at 2 hpi. Expression level is presented as Transcripts per Kilobase Million (TPM) to normalize for gene length and library size, error bars denote standard deviation. (C) Gene cascade in monosome and polysome fraction at 6 hpi, presented as in *B*.

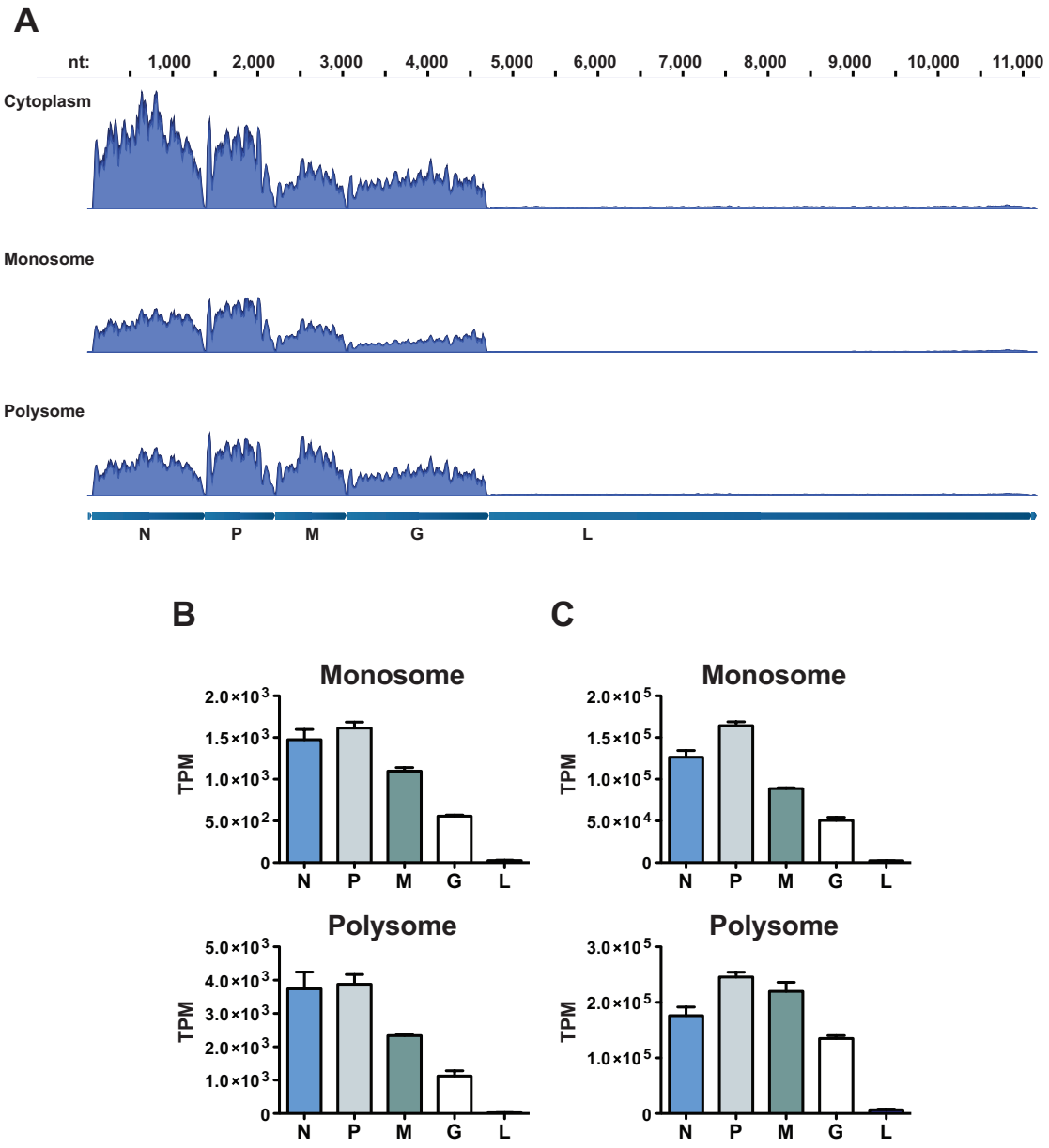

Figure 3.2 (Continued). The M51R gene cascade at 2 and 6 hpi.

leads to inhibition of host translation. In VSV_{M51R} infected cells, we note an underrepresentation of viral mRNAs (30%) and an overrepresentation of cellular mRNAs (70%) in the monosome fraction at 6 hpi compared to their cytoplasmic abundance (Figure 3.1C). This indicates that additional mechanisms contributing to host shutoff, such as eIF4E inactivation, may affect the association of some cellular mRNAs with monosomes.

Cytoplasmic abundance determines polysome association during M51R infection.

To determine how M51R infection affects the distribution of individual mRNAs within the cytoplasmic, monosome, and polysome pools, we plotted the TPM for each mRNA in uninfected and infected cells for all 3 fractions (Figure 3.3A & Figure 3.4A). At 2 hpi the relative abundance of viral mRNAs is similar to that of highly expressed cellular mRNAs (Figure 3.3A). The relative reads mapping to individual cellular mRNAs were largely unaffected at 2 hpi (Figure 3.3B). However, by 6 hpi the reads mapping to each of the 5 viral genes, with the exception of L, was greater than any cellular mRNA in the cytoplasm and polysome fractions (Figure 3.4*A*). Concurrently, we observed a 2-fold or greater decrease in the cytoplasmic and polysome abundance of the majority of cellular mRNAs (Figure 3.4A & 3.4B). We note that some cellular mRNAs show increased monosome association at 6 hpi, consistent with the non-uniform response to viral infection we previously observed in wild-type infected cells (Figure 3.4B).

We then mined our data set to address whether cellular mRNAs are subject to translation regulation during M51R infection. For each individual mRNA, we divided the polysome TPM by the cytoplasmic TPM as a measure of potential translation efficiency.

Figure 3.3. Cytoplasmic abundance determines polysome association at 2 hpi. (A) Scatter plots of Transcripts per Kilobase Million (TPM) for exonic regions of uninfected cells (black) or cells infected with VSV_{M51R} for 2 hours (blue). TPM of M51R viral mRNAs are denoted in magenta triangles. The TPM for each cellular mRNA in uninfected cells is graphed on the bottom x-axis, and the TPM for each cellular mRNA under the different conditions is graphed on the left y-axis. The relative abundance of viral mRNAs is plotted on the top x-axis and the right y-axis. (B) Density plots of the $\log_2$ fold change in TPM between uninfected or M51R infected cells at 2 hpi. C, M, and P denote “cytoplasm”, “monosome”, and “polysomes”, respectively. (C) The $\log_2$ fold change in the polysome to cytoplasmic or monosome to cytoplasmic TPM ratio is plotted against cytoplasmic abundance at 2 hpi with VSV_{M51R} for individual mRNAs. Magenta lines denote $\pm$ two standard deviations from the mean $\log_2$ fold change, and mRNAs outside the 95% confidence interval are highlighted in blue.

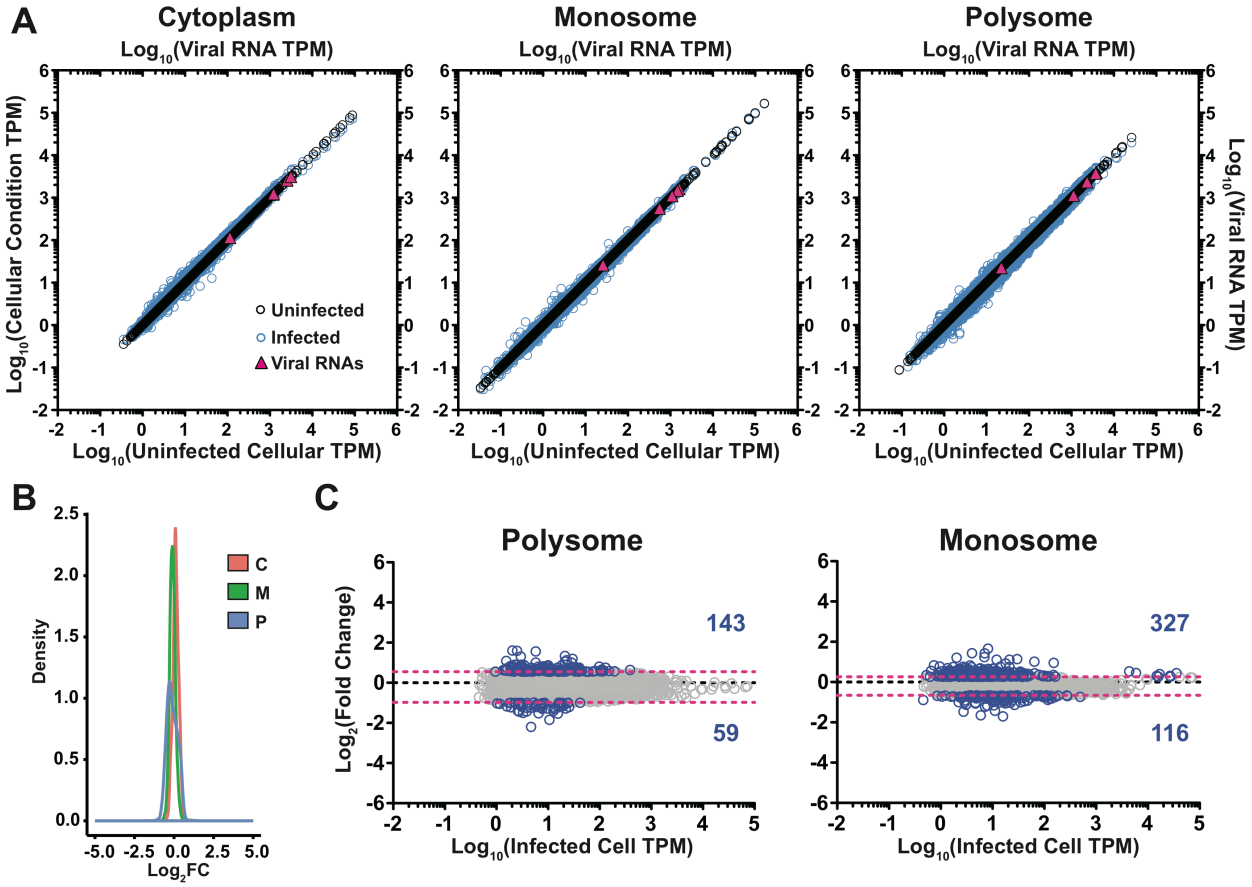

Figure 3.3 (Continued). Cytoplasmic abundance determines polysome association at 2 hpi.

Figure 3.4. Cytoplasmic abundance, and not translation regulation, is a key determinant of cellular gene expression during M51R infection. (A) Scatter plots of Transcripts per Kilobase Million (TPM) for exonic regions of uninfected cells (black) or cells infected with VSV_{M51R} for 6 hours (blue). TPMs of M51R viral mRNAs are denoted in magenta triangles. The TPM for each cellular mRNA in uninfected cells is graphed on the bottom x-axis, and the TPM for each cellular mRNA under the different conditions is graphed on the left y-axis. (B) Density plots of the $\log_2$ fold change in TPM for cellular mRNAs between uninfected or M51R infected cells at 6 hpi. C, M, and P denote "cytoplasm", "monosome", and "polysome", respectively. (C) Analysis of translation efficiency (TE) for the least abundant and most abundant cytoplasmic mRNAs in M51R infected cells at 6 hpi. The most abundant mRNAs (purple) or least abundant mRNAs (orange) were compared to mRNAs with abundance measurements within two standard deviations of the mean abundance (gray). Translation efficiency is defined as the polysome expression level divided by the cytoplasmic expression level. Cytoplasmic TPM is from the data set published along with this paper. ***$p < 2.2 \times 10^{-16}$; **$p < 5.0 \times 10^{10-5}$ as determined by the Wilcoxon rank sum test compared to mRNAs within the 95% confidence interval. Hinges correspond to the 25^{th}-75^{th} percentiles, and whiskers denote 1.5 times the inter-quartile range. (D) The $\log_2$ fold change in the polysome to cytoplasmic or monosome to cytoplasmic TPM ratio is plotted at 6 hpi with VSV_{M51R} for individual cellular mRNAs. Magenta lines denote ± two standard deviations from the mean $\log_2$ fold change, and mRNAs outside the 95% confidence interval are shown in blue.

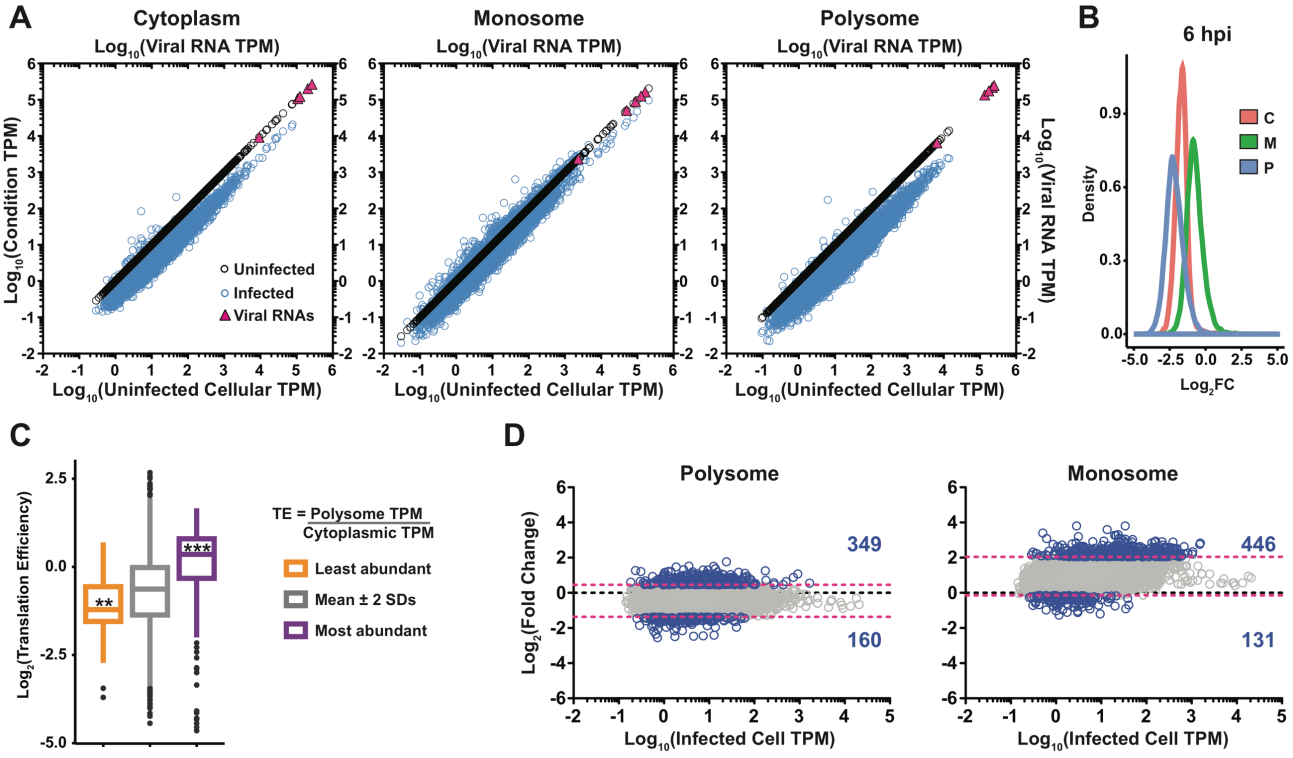

Figure 3.4 (Continued). Cytoplasmic abundance, and not translation regulation, is a key determinant of cellular gene expression during M51R infection.

We performed a similar analysis on the monosome-associated mRNA pool to identify cellular mRNAs differentially shifted to monosomes following infection. We first examined overall cytoplasmic abundance in M51R infected cells as a predictor of translation efficiency and confirmed that cytoplasmic abundance correlates with translatability for cellular mRNAs during M51R infection (Figure 3.4C).

We then plotted the fold change in translation efficiency at 2 and 6 hpi, with mRNAs exhibiting a fold change greater than two standard deviations of the mean highlighted in blue (Figure 3.3C & Figure 3.4D). This analysis revealed potential candidates for translation regulation at 2 and 6 hpi. However, we note that for the majority of genes at 2 hpi the fold change is <2-fold (Figure 3.3C). At 6 hpi we identified 160 candidates of negative translation regulation and 349 candidate mRNAs under positive translation regulation. However, as with the 2 hpi results, the majority of candidate mRNAs under positive translation regulation change <2-fold (Figure 3.4D). In contrast, in the monosome fraction at 6 hpi we identified 446 cellular mRNAs differentially shifted to monosomes, and observed an increase of >2-fold in monosome association for 4,459 cellular mRNAs (Figure 3.4D). These results are consistent with our previous observations during wild-type VSV infection, indicating that translation regulation does not account for differential abilities to inhibit cellular gene expression between wild-type and M51R VSV.

Interferon mRNAs are expressed and loaded on polysomes during M51R infection.

To address whether nuclear export of cellular mRNAs remains intact after infection with M51R, we performed differential expression analysis on sequencing reads

from all three fractions using DESeq2. Using this analysis, we found that interferons, and interferon-stimulated genes were the most upregulated cellular genes in the cytoplasm and on polysomes at 6 hpi (Figure 3.5A & 3.5C). There were no cellular genes identified as differentially expressed in the cytoplasm at 2 hpi, consistent with detection and response to viral infection being concurrent with replication. These data confirm that M51R is indeed defective in blocking nuclear mRNA export (Figure 3.5A). We validated the induction of the most highly upregulated cellular gene, IFN-λ by quantitative PCR (Figure 3.5B). IFN-λ mRNA was first detected at 4 hpi with M51R, representing a delay relative to viral gene expression, providing further support for the hypothesis that replication is required for immune activation (Figure 3.5B). Infection with VSV containing wild-type M did not induce IFN-λ at any time post-infection (Figure 3.5B).

Furthermore, in a single-round infection assay a VSV mutant, G4A, which is defective in performing both N-7 and 2′-O cap methylation, but retains a wild-type M protein, did not activate interferon at 6 hpi (Figure 3.6). The methylation of cellular mRNA caps at the 2′-O position is thought to aid in discriminating cellular “self” mRNA from viral “non-self” RNA.[431] Wild-type M is thus sufficient to block innate immune gene expression even in the presence of viral nucleic acids that are potent activators of innate immunity (Figure 3.6).

Consistent with interferons and interferon-stimulated genes being the most highly upregulated cellular genes in the cytoplasm of M51R infected cells, we found they were also the most highly upregulated mRNAs on polysomes at 6 hpi (Figure 3.5C). This provides further support for our conclusion that cytoplasmic abundance is a key

Figure 3.5. Interferon mRNAs are expressed and loaded on polysomes during M51R infection. (A) Top ten upregulated genes in the cytoplasm of M51R infected HeLa cells at 6 hpi. Differential expression analysis was performed in R using DESeq2. (B) RT-qPCR for VSV N, ACTB, and IFNL1 at indicated times post infection with VSV_{WT} (red) or VSV_{M51R} (blue). Reverse transcription was carried out on total RNA using oligod$(T)_{20}$ primers. Error bars denote SEM from three independent replicates. (C) Top ten results from differential expression analysis on polysome-associated mRNAs at 6 hpi with M51R. Genes are ordered by largest log_2 fold change. Differential expression analysis was performed as in A. (D) RT-qPCR across polysome gradients to determine the distribution of VSV N and IFNL1 mRNAs. Equal amounts, 500ng, of total RNA recovered from polysome fractions was reverse transcribed using oligod$(T)_{20}$ and subject to gene-specific qPCR. The fraction of the total mRNA recovered in the individual polysome fractions for each gene is plotted on the y-axis. Error bars denote the SEM from three independent biological replicates. A representative polysome profile of M51R infected HeLa cells at 6 hpi is shown in gray.

A

Gene Symbol	log2FC	p value	FDR p value
IFNL1	11.9	1.28E-15	6.74E-13
IFNL2	11.1	1.19E-13	5.40E-11
IFNL3	10.7	9.02E-13	3.21E-10
IFNB1	9.6	2.20E-07	2.69E-05
CXCL10	8.8	3.95E-31	7.43E-28
IFIT2	6.8	2.79E-07	3.16E-05
DDIT3	5.8	1.76E-56	1.16E-52
CXCL11	5.0	2.17E-15	1.10E-12
IFIT1	4.9	2.78E-09	5.91E-07
CHAC1	3.9	2.72E-37	8.96E-34

C

Gene Symbol	log2FC	p value	FDR p value
IFNL1	12.6	3.15E-17	1.13E-14
IFNL2	11.8	4.15E-15	1.05E-12
IFNL3	11.1	1.42E-13	2.70E-11
IFNB1	9.9	9.78E-08	3.67E-06
IFIT2	7.5	7.89E-05	1.00E-03
IFNL3P1	7.2	1.19E-06	3.00E-05
CXCL10	7.0	2.63E-37	7.20E-34
DDIT3	6.9	1.58E-80	2.16E-76
IFIT3	5.4	4.84E-03	2.68E-02
CXCL11	5.0	7.60E-09	3.74E-07

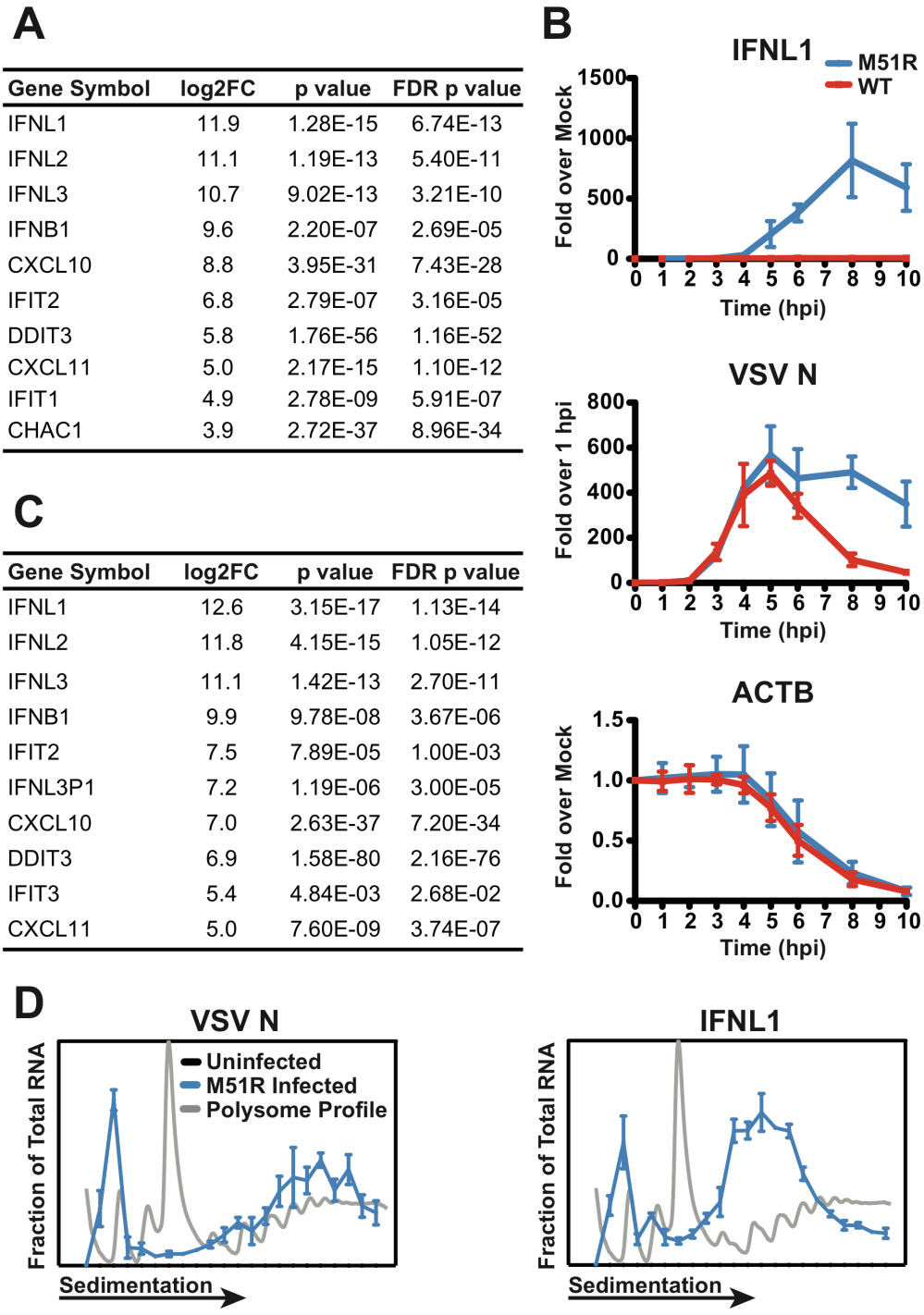

Figure 3.5 (Continued). Interferon mRNAs are expressed and loaded on polysomes during M51R infection.

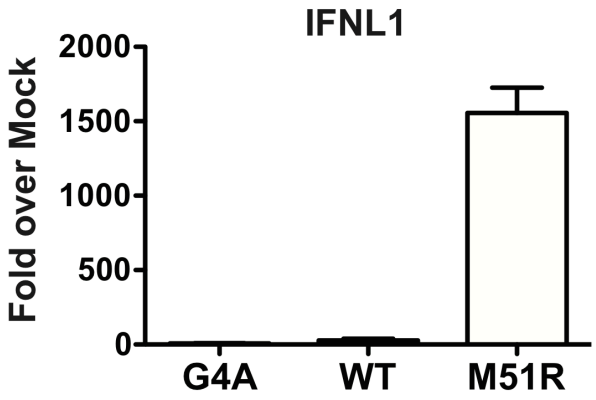

Figure 3.6. A VSV mutant defective in 2′-O cap methylation does not induce interferon when wild-type M is present. IFNL1 expression at 6 hpi from cells infected with G4A, WT, or M51R VSV. Results are presented as fold induction of IFL1 over uninfected cells. Error bars denote standard deviation from two independent replicates.

determinant of polysome association (Figure 3.1C & Figure 3.4A). We next determined the polysome distribution of IFN-λ and found that it is polysome-associated by 6 hpi, but in smaller polysomes than viral transcripts (Figure 3.5D).

IRF1 mRNA is induced during M51R infection, and is associated with actively translating ribosomes at 6 hpi.

We extended our analysis by identifying an additional cellular gene upregulated in our DESeq2 data sets, IRF1. Our sequence read data indicated that while there is a basal level of cytoplasmic and polysome-associated IRF1 in uninfected cells, the mRNA is induced >2-fold in both fractions at 6 hpi with M51R. We confirmed this observation by quantitative PCR for IRF1 mRNA following infection with M51R or wild-type VSV (Figure 3.7A & 3.7B). IRF1 mRNA was re-localized to small polysome fractions and sub-polysome fractions during wild-type infection but remained associated with large polysomes at 6 hpi with M51R (Figure 3.7B). We should note that this analysis only interrogates the polysome distribution of the mRNA and does not account for the increase in total mRNA level. Therefore, it underrepresents the induction of IRF1 in polysomes during M51R infection (Figure 3.7B).

Western blotting for IRF1 confirmed a basal level of IRF1 expression in uninfected cells (Figure 3.7C). In wild-type infected cells, IRF1 protein was absent, indicating the protein had been turned over and that no new protein was synthesized due to VSV-induced host shutoff (Figure 3.7C). In contrast, we observed IRF1 protein at 6 hpi with M51R (Figure 3.7C). Although western blots measure steady protein levels,

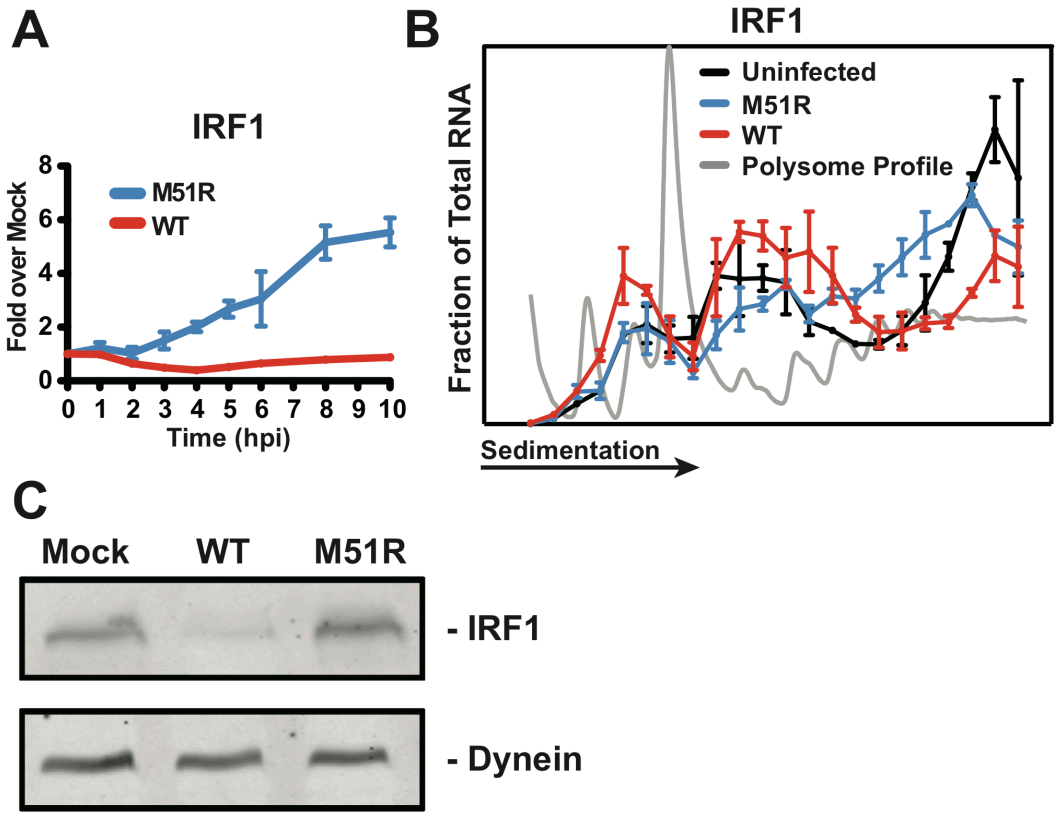

Figure 3.7. IRF1 mRNA is induced during M51R infection, and is associated with actively translating ribosomes. (A) RT-qPCR for IRF1 at different times post-infection with VSV_{M51R} (blue) or VSV_{WT} (red). Total RNA was used for reverse transcription using oligod$(T)_{20}$. (B) Polysome distribution of IRF1 mRNA in uninfected cells (black) or cells infected for 6 hours with VSV_{M51R} (blue) or VSV_{WT} (red). Total RNA from each individual polysome fraction was reverse transcribed using oligod$(T)_{20}$ and probed for IRF1. The fraction of the total IRF1 mRNA recovered in each polysome fraction is presented on the y-axis. Error bars denote SEM from three independent biological replicates. A representative polysome profile at 6 hpi with M51R is shown in gray. (C) Representative western blot for IRF1 levels in uninfected HeLa cells, or cells infected with VSV_{WT} or VSV_{M51R} for 6 hours. Dynein levels are used to show equal loading.

the half-life of IRF1 protein has been measured to be ~30-40 minutes, suggesting that IRF1 mRNA is associated with active ribosomes during M51R infection.[450-452]

Discussion

Activation of innate immunity when viral antagonism of host cell gene expression is disrupted.

The results presented here expand upon previous work seeking to understand the mechanism of host shutoff during VSV infection. Our principal finding confirms that viral mRNA abundance contributes to host cell shutoff. We also obtained evidence that inhibition of host transcription and nuclear export of mRNPs blunts the innate immune response. We found that during infection with VSV_{M51R}, but not VSV_{WT}, the most highly upregulated cellular mRNAs in the cytoplasm were interferons and interferon-stimulated genes (Figure 3.5A). This result confirms crystallographic data suggesting that residue 51 in VSV M is critical to inhibiting nuclear mRNP export.[389] The appearance of IFN and ISG gene transcripts in the cytoplasm of infected cells by 6 hpi demonstrates functional nuclear export of mRNPs during M51R infection. Interferons and ISGs were not differentially expressed at 2 hpi, but our qPCR results demonstrate an increase between 2 and 4 hours (Figure 3.5B). One hypothesis for why immune activation lags behind viral transcription is that stimulatory ligands must accumulate past a certain threshold to trigger detection. However, we observed that viral mRNA abundance was similar to the most highly expressed cellular mRNAs by 2 hpi. This indicates that the viral polymerase makes few mistakes during transcription or that the threshold for innate immune activation is very high (Figure 3.3A).

We did not observe cytoplasmic interferons and ISGs during infection with VSV_{WT}, which we interpret to reflect the inhibitory effect of M on nuclear export of cellular mRNAs. This is consistent with the observation that infection of HeLa cells with

VSV containing wild-type M leads to nuclear retention of poly(A) mRNA.[374] Infection of MEFs with VSV containing wild-type M, but not M51R, also led to the nuclear accumulation of IFN-β mRNA.[424] However, we cannot formally rule out effects of the M51R mutation on the ability of M to inhibit cellular transcription as being responsible for this observation. Regardless, the role of M in inhibiting transcription and blocking nuclear export likely represents functionally redundant mechanisms to ensure innate immune genes are suppressed in infected cells.

The PAMP detected in VSV infected cells

The timing of the innate immune response to infection suggests that genome replication is required for activation. RIG-I is clearly important in detecting VSV infection, but the physiologically relevant ligand remains unclear.[411, 418] The genome and antigenome have 5′ terminal triphosphates but are completely encased in a proteinaceous, nucleocapsid sheath that is added concomitant with their synthesis.[38, 67, 425-427] Our results indicate that VSV N is one of the most abundant mRNAs on polysomes by 2 hpi, suggesting there is large supply of N produced early during infection to support this encapsidation (Figure 3.3A). The two short leader RNAs contain triphosphate but are not significantly structured which may render them less effective ligands for RIG-I.[426] During mRNA synthesis, the coupling of mRNA cap addition to transcription elongation would also suppress production of triphosphate ligands for RIG-I. Clearly ligands are produced, as wild type M suppresses the downstream consequences of RIG-I activation, but the timing of interferon induction suggests that their production is a rare event and depends upon RNA replication.

This interpretation is consistent with the idea that defective-interfering (DI) particles produced during RNA replication may well serve as source for such PAMPs. DI particles are known to induce a potent interferon response, and they are made at high frequency during VSV infection.[436] Therefore, secondary infection of bystander cells with DI particles and detection in those cells, is likely the source of the IFN response in VSV infected animals.

Viral mRNA abundance and host shutoff.

We previously demonstrated support for the hypothesis proposed by Lodish and Porter in 1980, that viral mRNA abundance contributes to host shutoff by leading to competition for ribosome binding, and a redistribution of ribosomes onto viral messages.[87] The results described here support and extend this conclusion. We found that >50% of the cytoplasmic and polysome-associated mRNA was viral by 6 hpi with M51R, despite nuclear export of mRNPs remaining intact (Figure 3.1C and Figure 3.5A). This indicates that blocking nuclear export of cellular mRNPs does not contribute significantly to inhibiting bulk cellular translation during VSV infection.

Furthermore, polysome formation was enhanced following M51R infection, despite the translation efficiency of >90% of cellular mRNAs remaining unchanged (Figure 3.1B and Figure 3.4D). These results do not support a significant contribution to host cell shutoff by translational regulation, and they suggest that additional mechanisms beyond mRNA abundance contribute to modulation of cellular gene expression. We interpret the increased monosome association of many cellular mRNAs to reflect a subtle role for eIF4E-BP1 activation in shifting cellular mRNAs out of

polysomes (Figure 3.1C & 3.4D). We propose that the only mechanism which can reasonably explain the inhibition of host cell translation during M51R infection is viral mRNA abundance.

Further support for this hypothesis comes from the induction of IFNs and ISGs during M51R infection. We confirmed that two of these transcripts, IFN-λ and IRF1, are expressed following infection, and become polysome-associated, and at least one, IRF1, is associated with translating ribosomes (Figure 3.5 and Figure 3.7). This is direct evidence that cytoplasmic availability determines polysome association, because the cytoplasmic appearance of IFN-λ results in polysome association (Figure 3.5). However, we have not formally ruled out that IRF1 protein is stabilized during M51R infection. Additionally, despite the upregulation of IRF1 mRNA at 6 hpi, its polysome distribution is shifted towards smaller polysomes, likely reflecting a shift in cellular mRNAs out of large polysomes as was observed with wild-type VSV infection (Figure 3.7A & 3.7B).

Together, these results suggest that successful pathogenesis by VSV depends primarily on the robustness of transcription by the viral polymerase, and the ability of M to block nuclear export of interferon response genes. This may help explain the ability of VSV to replicate in a broad array of cell types, and because VSV naturally cycles through insect vectors, comparing our results from infected mammalian cells with viral replication in insect cells will be of significant interest.

Materials and Methods

Cells and Viruses

HeLa S3, Vero, and BSRT7 cells were grown at 37°C in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Tissue Culture Biologicals, Tulare, CA). Viral stocks were grown on Syrian golden hamster kidney BSRT7 cells (a gift from K. Conzelmann), and titered on African green monkey kidney Vero cells (ATCC, CCL-81), as previously described.[32]

Infections and Polysome Profiling

For polysome profiling, 12 x 10^6 HeLa S3 cells were plated on a 15cm dish 24 hours prior to infection with VSV. Cells were washed once with Hank's balanced salt solution (HBSS) and infected in 5 ml serum free DMEM at MOI 10 for 1 hour at 37°C. At 1 hour post-infection the inoculum was removed, cells were washed again with HBSS, and 20 mL of DMEM supplemented with 10% FBS was added for 2 or 6 hours. At the indicated time post-infection the medium was removed and replaced with fresh media containing 100 μg/ml cycloheximide, and the cells incubated at 37°C for 3 minutes. Cells were then washed once with ice cold 1X phosphate buffered saline (PBS) containing 100 μg/ml cycloheximide, and all subsequent steps were performed at 4°C or on ice. Cells were scraped into 1 ml 1X PBS with 100 μg/ml cycloheximide and pelleted at 300 × *g* for 10 minutes. Cell pellets were resuspended in 300 μl hypotonic lysis buffer containing 5mM Tris (pH 7.4), 2.5mM $MgCl_2$, 1.5mM KCl, and RNAsin (Promega, Madison, WI). Cycloheximide and DL-Dithiothreitol (DTT) were added sequentially to 100ug/ml and 3uM, respectively. The resuspended cells were briefly vortexed, and

Triton X-100 and sodium deoxycholate were added sequentially to 0.5%. Cells were pulse vortexed and incubated on ice for 15 min. Polysome extracts were clarified by centrifugation for 2 minutes at 12,000 × *g*. Lysates were layered on a 10-50% w/v sucrose gradient prepared using a Gradient Master Station (Biocomp, Fredericton, Canada). Sucrose was dissolved in a buffer containing 15mM Tris (pH 7.4), 15mM $MgCl_2$, and 150mM NaCl. RNAsin and 100ug/ml cycloheximide were added immediately before gradient preparation, and prepared gradients were cooled to 4°C before loading. Gradients were centrifuged for 2 hours at 40,000 × *g* in a Beckman Coulter ultracentrifuge using an SW40Ti rotor, and 500 μl fractions were collected while monitoring UV254 absorbance on a Gradient Master Station.

RNA Extraction, Library Preparation, and RNAseq

RNA was extracted using Trizol LS Reagent (Invitrogen), following the manufacturer's protocol. Equal amounts of total RNA as determined on a Nanodrop 2000 (Thermo Fisher, Waltham, MA) were used for library preparation using the Illumina TruSeq vII RNA Library Preparation Kit (Illumina, San Diego, CA). Samples were sequenced at the Whitehead Institute (Cambridge, MA) on an Illumina HiSeq2500 system. Reads were mapped to a concatenated hg38 and M51R genome using CLC Genomics Workbench (Qiagen, Redwood City, CA).

Translation Efficiency and Differential Gene Expression Analysis

Translation efficiency for cellular mRNAs was calculated using the expression value Transcripts per Kilobase Million (TPM) as determined from co-mapping to the human and viral genomes using the following formula:

$$\text{Translation Efficiency (TE)} = \frac{\text{TPM from Polysome Library}}{\text{TPM from Cytoplasmic Library}}$$

The change in this ratio following infection was presented as the $\log_2$ fold change.

Differential gene expression analysis was performed using DESeq2.

Statistical analysis of TE and mRNA abundance was performed using the calculated TE and cytoplasmic TPM from the data sets presented here. Translation efficiency of mRNAs with high or low abundance (determined as genes with abundance measurements outside 2 standard deviations of the mean abundance) was compared to mRNAs with abundance measurements within the 95% confidence interval of the mean abundance using the Wilcoxon rank sum test in R. Analysis of $\log_2$ fold change density was performed using the “geom_density” function in cowplot in R.

RT-qPCR

RNA was recovered from polysome fractions using Trizol LS Reagent (Invitrogen), per the manufacturer’s instructions. For reverse transcription, 500ng total recovered RNA as determined on a Nanodrop 2000 (Thermo Fisher) was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen) and oligod$(T)_{20}$ priming at 50°C for 1 hour. The resulting cDNA was treated with RNAseA and RNAseH for 15 minutes at 37°C and diluted for qPCR using gene specific primers and Power

Sybr Green (Thermo Fisher). Relative quantitation was determined by the $\Delta\Delta C_t$ method using the following primer pairs:

ACTB Forward: 5′ ACCCAGCACAATGAAGATCA 3′

Reverse: 5′ CTCGTCATACTCCTGCTTGC 3′

IFNλ Forward: 5′ CACATTGGCAGGTTCAAATC 3′

Reverse: 5′ AAGCCTCAGGTCCCAATTC 3′

IRF1 Forward: 5′ CCAAGCATGGCTGGGACATC 3′

Reverse: 5′ TGCTTTGTATCGGGCCTGTGTG 3′

VSV N Forward: 5′ GAGTGGGCAGAACACAAATG 3′

Reverse: 5′ CTTCTGGCACAAGAGGTTCA 3′

VSV N Forward: 5′ CTCTGCCGACTTGGCACAAC 3′

Reverse: 5′ TTCAAACCATCCGAGCCATTCG 3′

VSV G Forward: 5′ GTGGGATGACTGGGCTCCAT 3′

Reverse: 5′ CTGCGAAGCAGCGTCTTGAA 3′

Polysome distribution is presented as the fraction of the total amount of a given mRNA recovered in each polysome fraction.

For time courses of interferon and ISG induction, HeLa cells in 60mm dishes were infected with VSV and harvested at the indicated time post infection by scraping into 1X PBS. Cells were pelleted at 4°C, for 2 minutes at 845 × *g* and resuspended in Trizol LS Reagent. Total RNA was extracted using Trizol LS Reagent following the manufacturer's protocol and treated with 5U RQ1 DNAse (Promega) for 30 minutes at 37°C. RNA was re-extracted using the phenol:chloroform method and precipitated by

ethanol precipitation overnight at -20°C. cDNA synthesis and qPCR were performed as described above except 100ng total RNA was used as the input for cDNA synthesis.

Western Blots

For western blots, 10cm dishes of HeLa S3 cells were infected for 6 hours, washed with 1X PBS and lysed in Rose Lysis Buffer (10mM Tris-HCl, pH 7.4, 66mM EDTA, 1.0% v/v NP-40, and 0.4% w/v sodium deoxycholate) containing Pierce EDTA-Free Protease Inhibitor (Thermo Fisher) on ice for 15 minutes. Lysates were clarified by centrifugation for 1 minute at 4°C at 16,363 × *g*. Protein input was normalized by Bradford assay and separated on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes at 100v for 1.5 hours, washed in 1X PBS for 2 minutes, and blocked in Odyssey Blocking Buffer (LI-COR Biosciences) for 1 hour at room temperature. Membranes were incubated with the following primary antibodies diluted in Odyssey Blocking Buffer (LI-COR Biosciences) overnight at 4°C; anti-IRF1 1:1000 (Cell Signaling, 8478) and anti-Dynein 1:2,000 (Millipore, MAB1618). Membranes were washed 3 times with 1X PBS with 0.1% Tween, and incubated with the following secondary antibodies; goat anti-mouse 1:10,000 (LI-COR Biosciences, 925-68070) and goat anti-rabbit 1:10,000 (LI-COR Biosciences, 925-32211) diluted in Odyssey Blocking Buffer (LI-COR Biosciences). Membranes were washed 3 times with 1X PBS with 0.1% Tween, and washed a final time with 1X PBS without tween. Detection was by the LI-COR fluorescent based system.

Chapter 4: Discussion

Summary of Results

We have reported here a time-resolved, global analysis of ribosome-associated mRNA during VSV infection. These results demonstrate that viral mRNA dominates the pool of cytoplasmic and polysome-associated mRNA by 6 hours post-infection. This supports the overwhelming abundance model first proposed by Lodish and Porter to describe the mechanism by which VSV shuts off host cell protein synthesis, while maintaining efficient viral translation. Our finding that cellular mRNAs are differentially shifted to monosomes during infection reconciles the model of Lodish and Porter with observations that the activity of the cellular initiation factor cap-binding complex is modified during the course of infection.

We also tested the specific contribution of the viral matrix protein in blocking nuclear export of cellular mRNPs to host shutoff. The findings from these experiments provide further evidence that viral mRNA abundance is a key determinant of host shutoff, and highlight the function of the matrix protein in blunting the innate immune response to viral replication. The contribution of these studies to our understanding of VSV replication and viral-host interactions is discussed below.

Model for VSV-mediated host shutoff

The mechanism by which VSV shuts off host cell protein synthesis, while maintaining efficient viral protein synthesis has remained an unresolved question. To interrogate this question, we took the global approach of massively parallel sequencing cytoplasmic and ribosome-associated mRNA at early and late times post-infection. We found that >60% of the cytoplasmic mRNA mapped to the 5 viral mRNAs at 6 hours

post-infection, and that this corresponded with viral mRNA abundance on polysomes. We observed an underrepresentation of viral mRNA in monosome fractions compared with the abundance of viral mRNA in the cytoplasm and on polysomes and an overrepresentation of cellular mRNA in monosome fractions. This suggests that additional factors may account for the shifting of cellular mRNAs into monosomes. Finally, we tested the contribution of the viral matrix protein in blocking nuclear export of cellular mRNPs to mRNA abundance and innate immune activation. These results have led us to propose a model, depicted in Figure 4.1 that unifies the observations of Lodish and Porter with roles for the modulation of translation initiation machinery and the functions of the matrix protein in suppressing cellular gene expression.

In our model, the exponential amplification of viral mRNA levels resulting from genome replication and secondary transcription that occurs between 2 and 6 hours post-infection leads to competition for limiting pools of cellular ribosomes and the redistribution of ribosomes from cellular transcripts onto viral messages, Figure 4.1. This follows the model framed by Lodish and Porter, but with a few accommodations.[87] We found that viral mRNAs were underrepresented in monosomes compared with their cytoplasmic and polysome abundance. If competition for ribosomes were the sole factor determining ribosome occupancy, we would expect to see similar levels of viral mRNA across all 3 fractions. Because we did not observe this, additional mechanisms must contribute to host shutoff. We have interpreted the overrepresentation of cellular mRNAs in the monosome fraction to reflect the effects of eIF4E-BP1 activation and subsequent eIF4E sequestration during infection.[293]

Figure 4.1. Model of VSV-mediated host shutoff during efficient viral protein synthesis. In uninfected cells (top panel), fully processed cellular mRNAs are exported out of the nucleus and translated in the cytoplasm. Translation is initiated by eIF4E recognition of the m7G cap on cellular transcripts. The negative regulator of eIF4E, eIF4E-BP1 is maintained in a phosphorylated and inactive state. At early time points post-infection with VSV (middle panel) viral mRNAs, depicted in red, may be translated in an eIF4E-dependent manner. The regulator of eIF4E, eIF4E-BP1 is still phosphorylated and inactive. A fraction of the viral matrix protein that enters with the infecting virion, in addition to newly synthesized matrix, associates with the nuclear pore complex and sterically blocks the export of cellular mRNAs. At late times post-infection with VSV (bottom panel) viral mRNA overabundance leads to competition and a redistribution of ribosomes onto viral messages. Activation of eIF4E-BP1 leads to its association with eIF4E, thereby sequestering and inactivating the cellular cap-binding protein. This differentially shifts cellular mRNAs into monosomes. Viral mRNAs, which exhibit a reduced requirement for eIF4E remain associated with large polysomes. The ability of viral mRNAs to utilize additional initiation strategies dependent on rpl40 or m6A may contribute to this effect. The continued synthesis of matrix protein blocks all nuclear export of cellular mRNAs. While this leads to the natural decay of mRNAs with short half-lives (black dotted line), it principally serves to suppress the expression of interferons and interferon-stimulated genes.

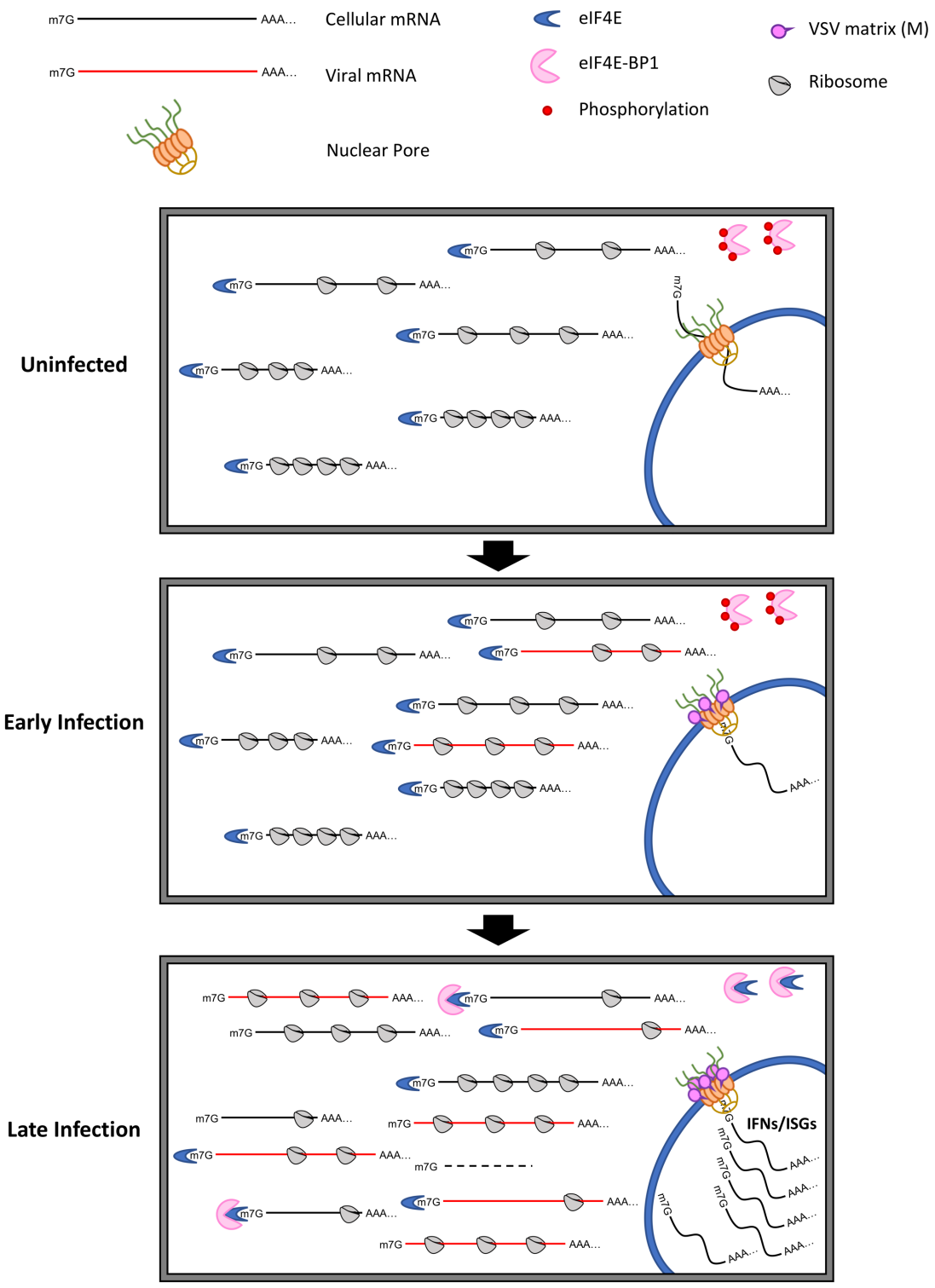

Figure 4.1 (Continued). Model of VSV-mediated host shutoff during efficient viral protein synthesis.

Further mining of our data set revealed that cellular mRNAs with high translation efficiency at 6 hours post-infection were more abundant and had longer open reading frames than mRNAs with low translation efficiency. These results directly follow the predictions of the Lodish and Porter model. In a competition setting, we would expect long, abundant mRNAs to be bound by more ribosomes. A comparison of these data with the results we obtained by infecting cells with the viral mutant M51R enhances and extends our understanding of the competition model.

We found that viral mRNA dominates the cytoplasmic mRNA pool under conditions where host cell export of mRNA to the cytoplasm remains intact. We interpret these results to mean that the blocking of nuclear mRNP export by the viral matrix protein does not contribute significantly to the translational shutoff of the host. However, wild-type matrix protein was completely able to ablate the cytoplasmic expression of interferons, even during infection with viral mutants defective in performing mRNA cap methylation. Therefore, we propose that a principal function of M, aside from its established role in viral assembly, is to inhibit interferon gene expression. This function of M likely precedes its role in virion assembly during infection, and could be initiated by M that enters with the infecting particle, and amplified by M synthesized from primary viral transcripts.

One prediction from our model is that viral mutants producing less mRNA should be less capable of inducing host shutoff. Although such viral mutants have been described, we have not tested those mutants directly. We did, however, use a mutant G4A whose kinetics of replication are substantially impaired, and levels of mRNA reduced 4-fold. This mutant was able to shut off cellular translation as evidenced by the

collapse of large polysomes during infection. Based on our sequencing results, we predict that transcript levels would need to be reduced >10-fold to observe an impact on host shutoff. The results we presented were obtained using infection of mammalian cells. However, VSV naturally infects insect cells as part of the virus transmission cycle.[1] In cultured insect cells, VSV does not induce host shutoff.[453] This may be due, in part, to temperature because increasing the incubation temperature of mosquito cells infected with VSV from 28°C to 34°C increases viral mRNA production and protein synthesis.[454] The effect of raising the temperature of the insect cells on host shutoff is less clear, because cells cultured in serum rapidly shut down both viral and cellular translation at 34°C, likely through eIF2α phosphorylation.[454, 455] How the insect RNA interference pathway relates to these observations is currently unknown. A global analysis of cytoplasmic and polysome-associated mRNA during VSV infection of insect and HeLa cells at 28°C would provide a useful comparison to interrogate how viral mRNA abundance relates to differential host shutoff between species, as well as differences in the transcriptional and/or translational responses to infection between the species.

Viral RNA abundance as a conserved mechanism of host shutoff

During the course of this study, work with influenza A, a segmented negative strand RNA virus; a mouse coronavirus, a positive sense RNA virus; and vaccinia virus, a DNA virus found evidence in support of a similar competition model.[217, 440, 441] Those studies performed ribosome footprinting, instead of polysome profiling, and they concluded from sequencing ribosome-protected fragments and cytoplasmic mRNA that

viral RNA dominates the cytoplasmic pool of mRNA during infection.[217, 440, 441] Similar to VSV, these viruses all induce host shutoff in infected cells, but in contrast to VSV, influenza, coronaviruses, and vaccinia virus target cellular mRNAs for endonucleolytic cleavage and degradation.[216, 245-247, 249, 250, 255, 259, 260, 350-352, 357, 358] So, while viral mRNA abundance may represent a common strategy used by diverse viruses to overwhelm the host, VSV appears to be the only virus so far that is known to accomplish this feat solely through the enzymatic activities of the viral polymerase. This underscores the robustness of VSV polymerase activity and the exponential amplification of viral RNA as a result of genome replication and secondary transcription. Furthermore, VSV, influenza, coronaviruses, and vaccinia virus all manipulate the cellular translation initiation pathway, so viral mRNA abundance represents one of multiple mechanisms by which these viruses subvert host cell gene expression.

During infection with influenza virus, cellular mRNAs encoding for genes involved in oxidative phosphorylation were found to be more resistant to host shutoff.[217] This was correlated with decreased mRNA length, and higher GC content, leading the authors to conclude that reduced RNA secondary structure is correlated with efficient degradation.[217] RNAs encoding for genes involved in oxidative phosphorylation were found to be translationally upregulated during vaccinia virus infection. [441] Further experiments demonstrated that this was dependent on shorter 5′ UTR length, although a precise mechanism for how shorter 5′ UTR length confers enhanced translation efficiency during vaccinia infection remains unknown.[441] The authors speculate that because many vaccinia virus mRNAs have short 5′ UTRs, this may also help explain

their efficient association with ribosomes.[441] These studies highlight the importance of cellular energy production for efficient viral replication and suggest that the shorter mRNA length in genes enriched for this function confers an advantage during host shutoff and competition for cellular ribosomes.

In contrast to these two studies, we identified longer, AU-rich mRNAs as having increased polysome association during VSV infection. An analysis of the functional categories for translationally enhanced RNAs identified RNA-binding functions, but not functions in oxidative phosphorylation. This suggests that not all viruses differentially manipulate mRNAs encoding genes involved in oxidative phosphorylation. This may reflect the kinetics of the VSV replication cycle, which is fast even compared to other highly cytopathic viruses. These results may also reflect differences in the techniques used. We sequenced pooled polysome fractions corresponding to large polysomes, so we may not have been able to resolve the shifting of shorter mRNAs, which due to length constraints cannot be bound by as many ribosomes.

Our results support the hypothesis that while some features may be shared between efficiently translated cellular and viral mRNAs such as AU content during VSV infection, or 5′ UTR length during vaccinia virus infection, there is not a single mRNA feature that predicts translatability. This highlights that we still do not entirely understand what defines efficient translatability, although it is somehow correlated with mRNA abundance. Furthermore, influenza, vaccinia, and coronaviruses all induce additional alterations to cellular translation machinery that likely affect the translation of subsets of mRNAs after infection.

Role of eIF4E-BP1 and eIF4E during VSV infection

The inhibition of cellular protein synthesis during VSV infection has been attributed to sequestering of the eIF4E cap-binding protein by activated eIF4E-BP1.[293] This hypothesis would predict that eIF4E target mRNAs should be differentially depleted from polysomes during VSV infection. Our analysis of cellular mRNAs with decreased translation efficiency at 6 hours post-infection did not identify classical features associated with eIF4E dependence such as long, structured 5′ UTRs.[121] This could reflect our use of UTR annotations from published databases, which may not accurately predict authentic UTR usage in our cells. Future experiments should be designed to intersect the results of our deep sequencing with published gene annotations, to more accurately map the UTRs expressed in our cells.

However, the generality of 5′ UTR length and propensity to form secondary structure as defining features of eIF4E sensitive RNAs has been questioned by several recent genome-wide studies.[93, 456] The results of analyses from eIF4E-depleted cells did not find a correlation between long, structured 5′ UTRs and eIF4E susceptibility.[93, 456] While some of these studies have identified putative sequence elements that may define eIF4E target mRNAs, the mechanism by which these elements direct eIF4E is unclear.[93, 103, 104] Our results are therefore consistent with additional determinants beyond 5′ UTR length and structure leading to differential eIF4E sensitivity. Indeed, our results are entirely consistent with cellular mRNAs exhibiting a wide range of eIF4E sensitivities.

We identified differential shifting of cellular mRNAs out of polysomes and into monosome fractions, which could reflect a range of eIF4E requirements for cellular

mRNA. Further support for this comes from individual distributions of cellular mRNAs across polysome profiles, where we observed that some cellular mRNAs are shifted more towards smaller polysomes than other mRNAs. Viral mRNAs, which are known to be less sensitive to eIF4E depletion, remain associated with large polysomes, as do some cellular mRNAs.[293, 395, 403] This occurs despite the collapse of large polysomes. While abundance and coding length are correlated with increased polysome association after VSV infection, we believe the range of polysome and monosome distributions indicates that differential sensitivities to eIF4E also contribute to the movement of cellular mRNAs within polysomes following infection.

Although we did not observe a correlation between long, GC-rich 5′ UTRs and reduced translatability during VSV infection, we did identify AU enrichment as a general feature among translationally enhanced cellular mRNAs. VSV mRNAs are AU-rich, and are thought to be relatively unstructured in the cell. Viral mRNAs are also efficiently translated during infection. One implication of this is that translation initiation proceeds efficiently on viral mRNAs because there is a reduced propensity to form hairpins and stem loops that could impede ribosome movement. We have now extended this observation to cellular mRNAs and suggest that for cellular mRNAs with enhanced translation efficiency during VSV infection, AU enrichment contributes to efficient translation in a similar manner by reducing the likelihood that secondary RNA structures impede ribosome movement.

The role of matrix protein during VSV infection

The matrix protein of VSV has multiple functions during infection, including inhibiting transcription from cellular polymerases, blocking nuclear export of cellular mRNA, and promoting assembly and budding of virions.[30, 31, 374, 375, 380-386, 392, 424] We tested the specific contribution of blocking nuclear export to host shutoff by using the mutant in M, M51R, which is defective in blocking nuclear export of cellular mRNPs.[386] We found that >50% of the cytoplasmic and polysome-associated mRNA was viral, indicating that continued export of cellular mRNA did not impact viral mRNA dominance in the cytoplasm and on polysomes. Furthermore, we observed that polysomes remained intact in M51R infected cells at 6 hpi. This is consistent with observations by multiple groups that bulk protein synthesis increases in cells infected with viruses containing the M51R mutation.[420, 421] Although bulk protein synthesis increases, cellular translation is inhibited, albeit to a lesser extent than in wild-type infected cells.[293, 338, 391, 421] Viral protein synthesis must account for the difference. Although multiple mechanisms likely contribute to this effect, we believe it predominantly reflects the overwhelming abundance of viral mRNA in the cytoplasm, and its corresponding abundance on polysomes during M51R infection.

The increase in monosomes and polysomes at 6 hours post-infection with M51R is reminiscent of the increase in monosomes and polysomes we observed in wild-type infected cells at 2 hours post-infection. One interpretation of these results is that they reflect a kinetic delay in host shutoff during M51R infection. Despite M51R being defective in shutting off host cell protein synthesis, the published literature shows that it

progressively inhibits cellular translation with increasing time post-infection.[293, 338, 391, 420, 421] Therefore, we propose that the M51R polysome profiles at 6 hours post-infection, may reflect what polysome traces in wild-type infected cells would look like between 2 and 3 hours post-infection. Collectively, these results highlight that the function of M in blocking nuclear export does not play a major role in inhibiting cellular protein synthesis. We identified a key function of M in inhibiting interferon and interferon-stimulated gene expression.

Evasion of innate immune sensors and the interferon response

VSV employs at least two mechanisms to evade detection by cytosolic sensors of foreign nucleic acids, 1) viral mRNAs are modified by VSV L such that they are structurally indistinguishable from cellular transcripts, and 2) by encasing the viral genome and antigenome RNAs in a proteinaceous nucleocapsid sheath. Despite these mechanisms, we found that interferons and interferon-stimulated genes were upregulated at 6 hours post-infection in the cytoplasm of M51R infected HeLa cells. Interferons and interferon-stimulated genes were not upregulated in cells infected with a virus harboring wild-type M. These observations indicate that VSV does not evade detection by cytosolic cellular sensors during infection, but rather that the cellular response to infection is impeded in wild-type infected cells. Because the M51R mutant is impaired for blocking nuclear export of cellular mRNPs, we conclude that a principal function of M is to prevent the transport of interferon and interferon-stimulated gene mRNAs out of the nucleus following cellular sensing of VSV.

One implication of this model is that wild-type VSV is still sensed, but interferon mRNAs are sequestered in the nucleus. We did not sequence the nuclear fraction from infected cells, but further experiments should address this possibility. Complementary to sequencing, quantitative PCR for interferon mRNAs in the nuclear fraction would reveal whether the mRNAs are transcribed, but subsequently sequestered by M. Results from MEFs infected with VSV harboring the M51R mutation suggest that this is the case.[424] To confirm that wild-type VSV is sensed, the phosphorylation and nuclear translocation of the cellular transcription factors IRF3, IRF7, and NF-κB could be investigated by western blotting and immunofluorescence at different times post-infection. Based on our finding that type III interferons are detected at 4 hours post-infection, we would predict that activation and nuclear translocation of IRF3, IRF7 or NF-κB would occur between 2 and 4 hours post-infection.

The timing of interferon induction during M51R infection at 4 hours post-infection suggests that viral replication is the source of ligands responsible for activating innate immune sensors. We did not find interferons expressed in the cytoplasm of M51R infected cells at 2 hours post-infection, a time when viral mRNAs are as abundant as highly expressed cellular mRNAs in the cytoplasm and on polysomes. These results suggest that primary transcription by infecting viral cores is not a significant source of stimulatory ligands. What then is the ligand recognized by cytosolic sensors during replication in a VSV infected cell?

Several RNA molecules produced during VSV replication have been proposed to be ligands for detection by RIG-I, including double-stranded RNA and the 5′ leader.[412, 415] Although the 5′ leader is a short RNA with a terminal triphosphate and thus seems

an ideal RIG-I ligand, it has a low propensity to form RNA structure. The viral genome and antigenome are completely coated with N, and thus not accessible for recognition by cytosolic sensors.[38, 67, 426-430] Our sequencing results also indicate that nucleocapsid (N) is already one of the most abundant mRNAs on polysomes at 2 hours post-infection, so it is difficult to reconcile the abundance of N protein with incomplete encapsidation. Moreover, it is established that inhibiting N protein expression arrests replication and releases aborted encapsidated partial genomes.[457, 458] The encapsidation of genome and antigenome RNA also prevents any potential for hybridization between complementary RNA sequences. The viral polymerase itself requires a cofactor P to allow it to copy the bases of the template RNA, because they are inaccessible otherwise. It is therefore difficult to envision how significant levels of virus-derived double-stranded RNA could accumulate in infected cells.[459] Alternatively, the RNA species detected may not be viral RNAs, but cellular RNAs released by dying cells late during infection. Further experimentation is required to resolve these possibilities.

While our results cannot definitively identify any viral RNA species as the molecule detected by the innate immune system, we propose that the timing of interferon induction suggests improperly capped viral mRNA species are a potential source of RIG-I ligands. Capping by VSV L occurs when transcripts have reached 30 nucleotides in length.[46] Failure to cap viral mRNAs results in premature termination of transcription, which would produce a short, improperly processed RNA molecule harboring a terminal triphosphate and capable of potently activating RIG-I.[42, 460-462]

The production of aberrant, uncapped mRNAs would be expected to increase concurrent with the amplification of transcription as a result of replication.

Aberrant products from viral transcription seem unlikely candidates to activate innate immunity during infection of an animal with VSV. If a principal function of M is to indeed block interferon gene expression, we expect that it will carry out this function in the cells of an infected animal. This means the interferon response observed in infected animals does not originate from the primary infected cell. Rather, we propose that it is the production of defective-interfering particles that is responsible for initiating an interferon response in an infected animal. It is known that defective-interfering particles are produced at high frequency during VSV infection and that they can potently stimulate the innate immune response.[436] It therefore seems probable that secondary infection of bystander cells with defective-interfering particles leads to detection of DI genomes by RIG-I and the induction of interferon gene expression.

The function of viral mRNA cap methylation

Our observation that viral mRNA transcribed by VSV mutants defective in N-7 only or both N-7 and 2′-O cap methylation remains associated with large polysomes at 6 hours post-infection, suggests that N-7 cap methylation is not required for efficient viral protein synthesis. This result contradicts previous results obtained from *in vitro* experiments that reported N-7 methylation on viral mRNAs was required for binding to cellular ribosomes.[72, 73, 433, 434] This apparent discrepancy can be explained by the excess of viral mRNA transcribed in an infected cell, which could compensate for reduced eIF4E affinity. Furthermore, given the apparent insensitivity of VSV to eIF4E

levels, N-7 methylation may serve a more mediatory, rather than obligatory role in an infected cell. How a failure to perform N-7 cap methylation would affect the polysome association of viral mRNAs earlier than 2 hours post-infection has not been addressed directly, but our results, interpreted with previous results published for these mutants suggest there is not a significant delay in viral protein expression by 6 hours post-infection.[54] One caveat to these studies, is that the methylation status of viral mRNAs from N-7 mutant infected cells has not been confirmed, so it cannot be ruled out that a cellular methyltransferase compensates for the loss of VSV methyltransferase activity.[54]

Previous work on VSV mutants defective in performing cap methylation demonstrated differential restriction to viral replication among cell lines from varying species.[463, 464] Viruses which capped, but failed to methylate the caps, were highly restricted in cell lines of human origin but retained the ability to replicate in Syrian hamster kidney (BHK) cells.[463, 464] Viral mRNAs isolated from infected BHK, but not the human HEp-2 cell line were found to contain N-7 cap methylation, suggesting that a cellular methyltransferase present in BHK cells was at least partially active on viral mRNAs.[465] HeLa cells were also found to be restrictive to methylation-defective viruses, but not to the same extent as HEp-2 cells.[463] Future experiments should determine the methylation status of the viral mRNAs from HeLa cells infected with our methylation-defective viruses.

The presence of 2′-O cap methylation is thought to distinguish cellular “self” mRNAs, from pathogen-derived “non-self” mRNAs.[431] The cellular IFIT family of proteins is responsible for this discrimination, and replication of vaccinia and

coronavirus mutants defective in 2′-O cap methylation is impaired in cells expressing these proteins.[431] These proteins were highly upregulated in our differential gene analysis of VSV M51R infected cells, consistent with their categorization as ISGs. However, our results demonstrate that wild-type VSV M efficiently blocks interferon gene expression during infection, suggesting that 2′-O methylation may be redundant to the function of M. Indeed, the viral mutant G4A, which is defective in both N-7 and 2′-O cap methylation, retains pathogenicity in mice.[54, 435] If viral mRNA abundance can compensate for N-7 effects on ribosome association and 2′-O methylation is redundant with M inhibition of interferon expression, what selective pressure imposed the requirement for a methyltransferase domain in VSV L?

Our experiments were performed in HeLa cells using 10 plaque forming units (PFUs) per cell, as determined by plaque assay on Vero cells. PFU does not account for the ratio of particles to plaque forming units, and the particle to PFU ratio for the methylation mutant viruses is currently unknown. Therefore, we could be challenging cells with significantly higher numbers of methylation mutant particles than we did with wild-type or even VSV_{M51R}. If this is the case, it would suggest that viral mRNA methylation is important for establishing productive infection.

Towards a unifying model for VSV mRNA translation

We deep-sequenced mRNA from VSV-infected cells at 2 hours post-infection and found that viral mRNAs were already as abundant on polysomes as highly expressed cellular mRNAs. This indicates that the association of viral mRNA with polysomes is efficient at early times post infection, and we have hypothesized that this

may depend on the proper methylation of the transcripts. One challenge to presenting a unified model for translation in a VSV infected cell is incorporating multiple observations that VSV mRNA translation has unusual dependencies and insensitivities relative to canonical cellular transcripts.

We hypothesize that the initial rounds of viral mRNA translation following infection require proper cap methylation and are likely eIF4E-dependent. Additional modifications on viral mRNAs may further increase the chance of productive ribosome association. These modifications identified on viral mRNAs from infected cells include *N6*-methyladenosine at the first two nucleotides of viral UTRs.[49, 65, 66] The m6A mark has been shown to directly recruit eIF3 and bypass the requirement for eIF4E when present in the 5′ UTR of some cellular transcripts.[107-109] It is possible that these modifications in VSV UTRs are also capable of directing eIF4E-independent translation, providing the virus with additional mechanisms through which ribosomal complexes can be assembled. The flexibility to initiate translation through eIF4E, or through eIF3 would increase the efficiency with which viral mRNAs associate with polysomes early during infection, and aide the establishment of productive infection.

Once infection is established, viral mRNA levels rise quickly, and cellular eIF4E is sequestered. A reduced dependency for viral mRNAs on eIF4E through m6A modifications would ensure efficient viral protein synthesis until viral mRNA abundance reaches a sufficient level to drive host shutoff and maintain robust polysome association. Our results from infected HeLa cells at 6 hours post-infection support that viral mRNA abundance contributes to host shutoff and efficient viral protein synthesis, but they do not exclude effects from the relative eIF4E insensitivity of viral transcripts.

Work from our laboratory previously reported that VSV mRNAs are hypersensitive to RNAi-mediated depletion of rpl40, and also to the loss of eIF3 subunits.[78, 437] The relationship between those observations and the present work is unclear. Rpl40 is a solvent-exposed protein on the large ribosomal subunit, and through its localization it may be able to contact mRNA or RNA-binding proteins, and direct initiation on select transcripts from the ribosome.[78, 82] An alternative hypothesis is that the proposed function of rpl40 in regulating large subunit joining renders rpl40 depleted ribosomes defective in assembling 80S complexes. This phenotype may cause mRNAs with short, unstructured UTRs to exhibit hypersensitivity to rpl40 depletion if stabilizing interactions between the mRNA, initiation factors and ribosomes are modified or unable to occur due to short 5′ UTR length. This may result in 80S ribosomal complexes forming less efficiently, or exhibiting a greater propensity to dissociate. As the loss of rpl40 dramatically suppresses viral gene expression, it would be of interest to compare the transcriptomes and polysome-associated mRNAs from cells depleted of rpl40 and infected with VSV.

Our results from deep-sequencing cytoplasmic and polysome-associated mRNAs at early and late time points post-infection with VSV have allowed us to build a model for the early events required to establish productive infection. In addition, they have provided two fundamental insights into the mechanism by which VSV inhibits host cell gene expression, 1) viral mRNA abundance is a key determinant for the inhibition of cellular translation, and 2) the viral matrix protein plays a critical role in blocking the innate immune response to viral infection. This expands the mechanisms by which

highly cytopathic viruses dominate the cytoplasmic mRNA pool and highlights the robust activity of the VSV polymerase.

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