Published in final edited form as:
Methods. 2013 February ; 59(2): 180–187. doi:10.1016/j.ymeth.2012.09.003.

Translational Control by Negative-strand RNA Viruses: Methods for the Study of a Crucial Virus/Host Interaction

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Abstract

Protein synthesis is a vital step in the successful replication of negative-strand RNA viruses. Protein synthesis is also a critical step in the development of a successful antiviral response from the host. This makes understanding the interplay between host and viral translation an important aspect of defining the virus/host interaction. For the negative-strand RNA viruses there are disparate mechanism of how viruses interact with the host protein synthesis apparatus, ranging from the complete takeover of all protein synthesis to the subtle insertion of viral mRNAs into an otherwise unchanged protein synthesis pattern. In this article, we discuss different ways to investigate protein synthesis in virus-infected cells, ranging from the use of metabolic labeling for the study of general translation changes to using fluorescence-coupled labeling techniques that allow the pinpointing of any subcellular localization of protein synthesis during virus replication. We also discuss methods for analyzing the translation initiation factors that are frequently modified in virus-infected cells.

Keywords

Protein synthesis; Negative strand RNA virus; Alkyne labeling; Vesicular stomatitis virus; La Crosse virus; Virus/host interaction; eIF2α; 4E-BP1

1. Introduction

The translation of mRNA in a eukaryotic cell is a complex but indispensable process involving the orchestrated decoding of mRNA by the ribosome. The translation process is begun through the recruitment of capped and polyadenylated mRNAs from the cytoplasmic space to the ribosome (see Figure 1). This occurs through the coordinated action of protein complexes such as the capped-mRNA recruitment complex eIF4F (colored blue in Figure 1) [1]. Following recruitment to the ribosome, the translation start site of the mRNA is identified by other factors, including the initiation factor eIF2 (grey in Figure 1), which recruits the initiator tRNA charged with methionine that initiates the ribosome-catalyzed translation process [2]. These initiation processes are critical for effective protein synthesis and are also the sites of stress-induced regulation by cellular kinases and phosphatases [1, 3, 4].

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For negative-strand RNA viruses, protein synthesis represents an aspect of the viral life-cycle where the virus is highly dependent on the host cell. Negative-strand RNA viruses have evolved to encode proteins able to self-catalyze many aspects of their replication cycle, including genome replication, mRNA synthesis, capping (by cap-snatching and *de novo* synthesis) and polyadenylation [5]. However, they do not encode a ribosome. These dependencies on the host cell means the virus must insert viral mRNA into a translation apparatus that is specialized for the translation of host mRNA.

While negative-strand RNA viruses are similar in that they all make mRNA that is similar to host mRNA (contains a 5′ cap), how they interact with the protein synthesis apparatus is not at all uniform. Some negative-strand RNA viruses (RSV, PIV5) show only a minor disruption of host translation upon infection and both viral and host protein synthesis are observed throughout infection. In these situations, viral mRNA is translated as part of the general process of host protein synthesis. Other negative-strand RNA viruses (VSV, LaCrosse) force a major disruption of host translation following infection. In this latter case, translation of viral mRNA becomes dominant and little to no host protein synthesis is seen. The dramatic nature of this second phenotype is illustrated in Figure 2A, where host protein synthesis is illustrated in black/grey and viral protein synthesis is illustrated in red. In this panel, the decrease of host protein synthesis can be seen to decrease at approximately the same time that viral protein synthesis is strongly upregulated. Figure 2B shows a stylized quantitation of this change in virus and host protein synthesis, illustrating the coincident inhibition of host and rise of viral protein synthesis commonly seen during virus infection.

Understanding which of these phenotypes is displayed during infection is a relatively straightforward process that involves tracking protein synthesis using radioactive or chemically-modified amino acids. In cases where viral infection leads to the suppression of host protein synthesis, it is commonly found that translation initiation factors such as eIF2 or eIF4F are phosphorylated or disassembled. Methods for determining all of these changes are detailed below, with an initial focus on analyzing general protein synthesis, followed by the description of methods for analyzing stress-induced changes in host initiation factors.

2. Analysis of global protein synthesis during virus infection

2.1 Radioactive labeling of protein synthesis

2.1.1 Introduction to metabolic labeling—Metabolic labeling of protein synthesis allows an examination of global levels of translation in a population of cells over a defined period of time. To accurately understand the effect virus infection has on translation in virus-infected cells, a single-cycle infection must be used (MOI of 3 or higher). To best understand the host-viral interaction, it is best to examine translation at multiple time points that span all phases of viral infection, including early (prior to the production of new virions) to late (the time point that maximal viral titer is reached). To ensure consistency within the experiment, the infection should be carried out so that all time points are labeled and lysed at the same time. Mock-infected cells should be a vehicle-control for the longest time point examined.

2.1.2 Setup—This experiment is most easily carried out in 35mm dishes (or a 6-well plate). Plate cells the day prior to infection so that they are at optimal density for virus infection when the experiment begins. The efficiency of incorporation of the radiolabeled amino acid depends on first depleting the pools of free methionine and cysteine in the cells. This is done by incubating the cells in a cell culture medium lacking methionine and cysteine (depletion medium, eg: Life Technologies 21013-024). Additionally, free amino acids may be present in FBS. FBS should be dialyzed in a low MW-cutoff dialysis tubing (e.g. Spectrapor #132645) against sterile PBS overnight to remove potential methionine and

cysteine. This FBS should be used in the depletion media at the same percentage used in standard infection media.

2.1.3 Experimental Methods—Infect cells as appropriate for all time points to end simultaneously. Thirty minutes prior to the end of the time course, remove the media from the cells and wash twice with depletion media. All time points should end together so that all of the labeling can be done simultaneously. For infections that require time points longer than 12-24h, ensure that equal cell numbers are being infected at each time point. Leave the cells in the incubator in 500μL depletion media for 15 minutes. This incubation will reduce the methionine and cysteine pools in the cell and increase the amount of radiolabeled amino acids incorporated into new protein products.

While the cells are incubating, supplement depletion media with radiolabeled methionine and cysteine at 20μCi/mL (eg: Perkin-Elmer NEG772007MC). Thirty minutes prior to the end of the time course, replace the depletion media with 500μL of media containing radioactive amino acids. Return plate to the incubator for 15 minutes.

The labeling period may be extended to up to 6h following this protocol. To label low levels of protein synthesis, it may be desirable to increase radiolabeled amino acid to 100μCi/mL. In order to label infected cells for longer than 6h (e.g. to allow radioactive protein incorporation into virions released by the cell), supplement labeling media with 10% standard infection media (9 parts depletion media: 1 part complete media).

Note that in all following steps, the samples and any reagent or item that comes in contact with them should be considered radioactive. The use of barrier-filter tips is recommended to minimize pipette contamination. Appropriate disposal of all contaminated plasticware and liquid waste is required.

While the radiolabel-containing media is on the cells, add protease and phosphatase inhibitors to RIPA lysis buffer (Boston BioProducts BP-115-500; see 3.3 for description of inhibitors and lysis buffers). Remove the plate from the incubator and remove media to a radioactive-appropriate waste container. Wash cells twice with warm PBS to remove media. Completely aspirate PBS to a radioactive-appropriate waste container.

Add 300μL of complete lysis buffer to each well to lyse the cells. Place the plate on ice (or at 4 degrees), rocking occasionally. After 10 minutes, remove the lysate to a 1.5mL tube marked with radioactive tape. Spin samples at maximum speed in a refrigerated microcentrifuge for 10 minutes. A small white pellet should form at the bottom of the tube. This pellet contains cellular structures not able to be solubilized by the detergent, such as large pieces of membranes, nuclear components and the cytoskeleton. For the examination of total protein synthesis levels, the pellet does not need to be analyzed and should be avoided when removing lysate.

The protein concentration of each sample should be determined using a small-scale Bradford or Lowry assay (eg: Bio-Rad 500-006). Transfer 150μL of lysate to a new tube containing 30μl of 6× SDS-PAGE loading buffer (Boston BioProducts BP-111R). Load 20μg of total protein on a 10% SDS-PAGE gel alongside molecular weight standards (Life Technologies LC5800) for comparison. Global inhibition of protein synthesis by a virus over long periods may affect the total protein content of the cells. In these cases, ensure that equal numbers of cells are used for each condition and that all residual moisture is removed prior to addition of lysis buffer; equal volumes of each sample may then be used for comparison. Optimal separation of proteins can be attained by adjusting the mono:bis acrylamide ratio when preparing the gel.

When separating the radiolabeled proteins on an SDS-PAGE gel, running the dye-front off of the gel ensures that any free methionine/cysteine does not remain on the gel; however, small molecular weight proteins may also be lost. To examine the effects of virus infection on protein synthesis of small molecular weight proteins, it may be necessary to increase the acrylamide percentage of the gel. Running buffer should be disposed of as is appropriate for radioactive waste.

Stain the gel using colloidal coomassie or similar gel staining technique (Bio-Rad 161-0786). Verify that loading appears to be equal after destaining. The gel should then be dried at 80 degrees for one hour in a gel dryer and placed in a cassette for exposure to film or phosphorimager screen. Anything covering the gel, even plastic wrap, will greatly diminish the signal. Direct contact with film or screen is desirable. The exposure time needed for optimal signal detection will vary depending on the strength of host and viral protein synthesis. Between 6 and 24h is typically sufficient. To reduce the exposure time necessary for visualizing the labeled proteins, gels can be soaked in scintillation fluid (National Diagnostics LS-270) for one hour. The gel should then be washed in deionized water to precipitate the enhancer within the gel and dried as above.

2.1.4 Example Experiment—Figures 2C and 2D show two examples of metabolically-labeled translation products made over time during a virus infection. Vesicular stomatitis virus is a negative-strand RNA virus that shuts off host protein synthesis almost completely by 4hpi. Figure 2C shows protein synthesis in infected cells over a range of time points. Note that viral protein synthesis is very robust, with the relative levels of protein synthesis of the individual VSV proteins much higher than the level of protein synthesis of any of the host proteins. To contrast, Figure 2D shows the changes in protein synthesis seen with the La Crosse bunyavirus. While this virus also shuts down host cell translation during infection, the levels of protein synthesis of viral proteins during infection (right lanes) is significantly lower than the levels of translation seen in mock-infected cells (left lanes). This is not an uncommon aspect of viral inhibition of protein synthesis: viral protein synthesis may be at a significantly slower rate than is normal in uninfected cells.

The levels of protein synthesis can be quantitated if the virus inhibits host protein synthesis. Using any 1D gel quantitation software (ImageQuant, QuantityOne, ImageJ, etc), enclose portions of the gel to be measured. For host protein synthesis, use sections of gel where only host protein synthesis is seen. To subtract an appropriate background level, chose an area of the gel outside of any of the lanes that contained radioactivity. The volume/size of the boxes to be quantified should be equal across all samples. For viral protein synthesis, use sections of the gel containing viral protein products. To subtract an appropriate background, choose an area immediately above or below each viral band that reflects the intensity of host protein synthesis of that sample. While this is an imperfect approach to determine background, it is the best approach available using a single dimension electrophoresis technique.

2.1.5 Alternate Approach—Some viruses do not shut down host cellular translation, which makes the analysis of viral protein synthesis difficult, as distinguishing viral and host bands is virtually impossible to do with confidence. A common approach to circumvent this problem has been to immunoprecipitate viral proteins from radiolabeled cell lysates using antisera against the virus [6]. This additional step allows the selective analysis of viral protein products recognized by the antisera. Because only a fraction of the proteins immunoprecipitated will be radiolabeled, it can be necessary to incubate infected cells with radiolabel-containing media for longer periods of time (1-2 hours). These longer labeling conditions introduce additional influences on the level of protein found in cells, including protein degradation and loss of protein through virion budding, in addition to protein

synthesis. Thus, these experiments will not be representative of the "rate" of protein synthesis but an overall quantification of viral protein accumulation over the labeling period.

2.2 Metabolic labeling using non-radioactive techniques

2.2.1 Introduction to alkyne-based metabolic Labeling—Radioactive metabolic labeling is the most sensitive method for detecting protein synthesis during virus infection; however, radioactive techniques do not allow easy investigation at the single-cell level. Standard antibody-labeling techniques, such as flow cytometry and immunofluorescence, allow single-cell and localization studies but depend on the development of robust virus-specific antibodies. A labeling technique based on an alkyne-azide reaction pair combines the strengths of direct labeling but increases the options for detection and use (Life Technologies A10266).

Similar to metabolic labeling, a modified biomolecule is incorporated into newly-made cellular products by the cell. This modified biomolecule, in this case an amino acid (*L*-azidohomoalanine, AHA), contains an azide functional group. Once the period of labeling is complete, the cells are fixed and pearmeabilized, and an enzymatic reaction between the azide and a fluorescently-labeled alkyne is carried out (Click-It Reaction Cocktail). This reaction links the fluorescent molecule to the labeled proteins made in the cell. If the labeling is carried out on cells infected with a virus that shuts down host protein synthesis at the time point being examined, then the labeled proteins will be virus proteins. This technique allows direct detection of all of the proteins made [7]. In addition, labeled cells can then be stained for specific virus or host proteins using traditional immunofluorescent techniques, allowing detailed examination of the localization of virus proteins in infected cells [8].

2.2.2 Setup—To allow the best visualization of cells, this experiment should be carried out on cells plated onto glass coverslips. The wells of a 24-well plate hold 12mm round glass coverslips (Fisher Scientific 12-545-80) and allow room for easy manipulation. Autoclave coverslips and add to wells prior to plating cells. Poly-L-lysine, collagen or gelatin (e.g. R&D Systems 3438-100-01) is recommended for cell lines unable to adhere directly to glass. Plate the cells on the day prior to infection so that they are 50-60% confluent at the time of infection to allow for clear visualization.

Prepare Click-iT reagents as appropriate (Invitrogen C10269). Dilute 0.5 mg AHA reagent in 130μL DMSO to make a 5mM (1000×) labeling solution; store at 4°C. Make 1× Click-iT cell reaction buffer by diluting 4mL of component A into 36 mL deionized water; store at 4°C. Add 4mL deionized water to Click-iT buffer additive and store at −20°C.

Reagents needed for immunofluorescence include: 4% formaldehyde in PBS (kept frozen until use), ice-cold methanol and PBS supplemented with 5% horse serum.

2.2.3 Experimental Methods—Infect cells as appropriate for the time points to be examined. To infect all of the cells, use an MOI between 3 and10. To leave some cells uninfected as within-experiment controls, infect at an MOI between 0.5 and 1. If not all of the cells are expected to be infected, a viral-specific antibody should be used to identify infected cells.

Thirty minutes prior to labeling, remove the media from the cells and wash twice with depletion media (as in 2.1.2). If examining multiple time points, adjust infection times such that labeling is done simultaneously. Incubate the cells in depletion media for 15 minutes. Depletion time can be increased to up to 60 minutes for cells with high amino acid pools or low levels of protein synthesis.

While the cells are incubating, prepare labeling media by adding 50μM AHA reagent to depletion media (labeling media). Aspirate media from wells and replace with 250μL of labeling media before returning to incubator. To examine the localization of protein synthesis in infected cells, label cells for 10-15 minutes. To allow for protein complex formation and trafficking, label cells for at least 30 minutes and up to 4 hours. To investigate the localization of proteins, remove AHA reagent after labeling period and incubate cells in standard infection media for increasing "chase" periods.

Once the incubation is complete, wash cells twice with warm PBS. Fix cells by incubation with 4% formaldehyde in PBS for 15 minutes at room temperature with gentle shaking. Permeabilize cells by incubation with ice-cold methanol for 15 minutes at room temperature with gentle shaking. Alternative methods of permeabilization, such as Triton-X or saponin are also compatible with the Click-iT reagents and may be preferred with specific antibodies. Wash cells with PBS. Cells should be blocked in PBS + 5% Horse Serum for one hour at room temperature. Prepare Click-iT reaction cocktail, as described by the manufacturer. Add reagent to cells and incubate for 30 minutes at room temperature protected from light.

Wash cells with blocking solution. The samples can now be processed for antibody-based detection of viral and host proteins. Dilute antibody as appropriate in blocking solution and add to wells. Incubate for one hour at room temperature. Remove antibody solution and wash with PBS three times. Dilute secondary fluorescent antibody in blocking solution as appropriate, and incubate covered from light for one hour at room temperature with gentle shaking. Remove antibody and wash with PBS three times.

To mount coverslips onto slides, place one drop of ProLong Gold antifade reagent (Life Technologies P-36931) onto slide and gently place coverslip on top, cell side down. Allow sample to cure covered at room temperature for 24 hours. Seal with nail polish, if desired.

2.2.4 Sample Experiment—Figure 3 shows mock-infected and VSV-infected cells at 6hpi. DAPI is shown in blue. Click-iT protein synthesis labeling is shown in green, and antibody staining for a virus-specific protein (N) is shown in red. Mock-infected cells show robust protein synthesis over the 30-minute labeling period, and the proteins made localize to every part of the cell. No virus-specific staining using an antibody for the VSV N protein is seen in these cells. VSV-infected cells also show robust protein synthesis, which also localize to all parts of the cell. Virus-specific staining shows that the cell is infected and expressing the viral N protein. There is a notable difference in the brief labeling and the site of accumulation of viral N protein. This method allows specific examination of protein localization and easy labeling of all viral proteins produced over a specific time period.

2.3 *In vitro Translation*

2.3.1 Introduction to *in vitro* Translation—*In vitro* assays allow the focused examination of a biological process in the absence of other cellular processes. *In vitro* translation removes the rest of the cellular functions and allows determination of specific factors critical for protein synthesis through depletion of critical proteins [9].

2.3.2 Setup—This experiment uses spinner flasks in which to grow cells. Spinner flasks are used to grow specifically-adapted cells to high density in a minimum culture volume. HeLa S3 cells should be transferred to a spinner flask at a density of 1×10^5 cells/mL and allowed to grow until the density reaches $5\text{-}7\times10^5$ cells/mL. More media should then be added to reduce density to 1×10^5. Cells should be grown to a density of 5×10^5 cells/mL for lysate preparation. To produce sufficient lysate for multiple experiments, a 1L culture of cells (approximately 5×10^8 cells) is recommended.

This assay depends on mRNA translation by an active ribosome *in vitro*. All steps should be carried out under RNase-free conditions on ice, unless otherwise specified. All glassware used should be baked for four hours at 180°C or rinsed four times with 1N sodium hydroxide then rinsed thoroughly with RNase-free water to deactivate RNases. All solutions should be certified RNase-free or made with RNase-free water. RNase inhibitors are also included in the reaction mixture.

The protocol below provides instructions for making *in vitro* translation lysates for virus-infected cells. Lysates can also be made from mock-infected cells by skipping the virus infection step and carrying out the remainder of the protocol as described. To compare the effect of inhibitors on cellular and viral translation for viruses that shut down host translation, both types of lysates will be needed.

The individual reagents needed for *in* vitro translation may be made up beforehand and stored frozen. The following reagents should be stored in aliquots at −30°C: Translation Lysis Buffer (TLB; 100mM Hepes, pH 7.5; 120mM potassium acetate; 2.5mM magnesium acetate; 100mM sucrose), 100mM PMSF (in ethanol), 1M DTT, 0.5M Phosphocreatine (in 20mM Hepes) and 1M Spermidine. Creatine Phosphokinase should be resuspended in equal parts glycerol and 20mM Hepes at a final concentration of 5mg/mL and stored in aliquots at −80°C.

Additional reagents needed are: 1mM amino acid mixture without methionine (Promega L9961), 100mM ATP (Sigma A6559-20umo), 100mM GTP (Sigma G3376-24umo), RNasin (Promega N2511), ^{35}S-methionine/cysteine (Perkin-Elmer NEG772007MC) and RNase-free water.

2.3.3 Experimental Methods—Infect cells (approximately 5×10^8, see 2.3.2) at a high MOI (3 or greater) to ensure that every cells is infected. At the peak of virus translation (determined by metabolic labeling experiment above), pellet cells by centrifugation at 1000×g at 4°C for 10 minutes. Wash cells twice with cold PBS, taking note of the packed cell volume (PCV). Resuspend cells in 1 PCV of cold RNase-free water. Allow the cells to sit on ice for 10 minutes. Lyse cells with approximately 15 strokes with a dounce homogenizer. Complete lysis (>95%) can be determined by trypan blue exclusion after homogenization. Continue dounce homogenization until all cells have been lysed.

Move lysate to 1.5mL tubes, and centrifuge at maximum speed in a microcentrifuge for 10 minutes at 4°C. A small white pellet should form at the bottom of each tube. Aliquot lysate for quick freezing. For long-term storage, add glycerol to 10% final volume. Snap freeze samples in liquid nitrogen. Store at −80° until use. Lysates should not be exposed to multiple freeze-thaw cycles.

When preparing to carry out an *in vitro* translation assay, thaw all reagents except lysates on ice. Thaw lysates as quickly as possible without heating immediately prior to use. Add 15μL PMSF and 1.5μL DTT to 1.5mL TLB to make TLB-Complete. Dilute GTP to 10mM, creatine phosphokinase to 0.5mg/ml and spermidine to 0.01M using TLB-Complete. Make Translation Supplement Mix (TSM) by combining 5μL amino acid mixture –methionine, 0.4 μL 100mM ATP, 0.5 μL 10mM GTP, 1 μL 0.5 mg/mL creatine phosphokinase, 0.5μL 0.01M spermidine, 3μL 0.5M phosphocreatine, 1μL RNasin and 1μL ^{35}S-methionine/cysteine. To ensure consistency across samples, make up enough TSM for as many reactions as are being carried out and aliquot into individual reaction tubes. After the addition of the radioactive amino acids, all samples and plasticware should be treated as radioactive.

Combine 12.4 μL TSM, 27.6 μL TLB-Complete and 10μL protein lysate prepared above. Incubate sample at 30°C. To examine *in vitro* translation over time, remove 5μL of sample to a new tube containing 3μL SDS loading dye every 10 minutes. To examine the effects of inhibitors or drugs on viral translation, add the appropriate volume of drug or vehicle control prior to adding lysate to the reaction mixture. Reduce TLB-Complete volume to compensate. Incubate for one hour at 30°C, and stop reaction with 10μL SDS-PAGE loading dye for SDS-PAGE analysis.

Load equal volumes of each reaction mixture on a 10% SDS-PAGE gel alongside molecular weight standards for comparison. Separate proteins, stain gel and expose as in 2.1.3.

2.3.4 Example Experiment—Figure 4 shows an example of *in vitro* translation by VSV. The reaction was incubated for 60 minutes. After 10 minutes, very little protein synthesis is seen. By 20 minutes, four major viral protein bands can be detected. Note that glycoproteins and other proteins with a signal peptide will not be translated *in vitro* due to the removal of the endoplasmic reticulum by centrifugation during lysate preparation. The production of proteins increases over time up to 60 minutes (Figure 4B).

3. Analysis of host translational control mechanisms

3.1 *Introduction to Translation Initiation*

In understanding how viruses manage to dominate protein synthesis, it has become clear that many viruses do so by triggering host stress pathways that “help” the virus by reducing host mRNA recruitment into the ribosome or reducing the rate of “normal” translation initiation. There are many host proteins that are involved in initiating translation. These proteins coordinate the ordered recruitment of charged tRNA and mRNA to the 40S ribosome. An extensive list of the translation initiation proteins and functions are explained in detail in several reviews [1, 3, 4]. Of the protein complexes that are involved in the recruitment of mRNAs to the ribosome, two (eIF2, which brings the initiator methionine to the 40S ribosome, and eIF4F, which recruits capped mRNA to the ribosome) have a special significance because their activity can be endogenously regulated by protein phosphorylation (Figure 5, left panel).

The activity of the heterotrimer eIF2 is controlled by the phosphorylation of its alpha subunit, eIF2α. When eIF2α is phosphorylated (Figure 5, bottom right), the normal process of eIF2 recruitment of the initiator methionine is blocked, leading to the inability of the pre-initiation complex to properly place the first amino acid used to prime translation [2]. This phosphorylation can be carried out by several different cellular kinases, including virus-activated kinases. eIF2α phosphorylation is thought to play a major role in shutting down global protein synthesis [10].

The second cellular pre-initiation complex that can be controlled by phosphorylation is eIF4F, a multiprotein complex that includes a scaffolding protein eIF4G and the cap-binding protein eIF4E [11]. Direct phosphorylation of eIF4E is thought to control translation (Figure 5, top right), as increased levels of phosphorylated eIF4E are associated with increased translation and decreased levels with inhibition of translation [12]. In addition, eIF4E is controlled by eIF4E-binding protein 1 (eIF4E-BP1). eIF4E-BP1 binds eIF4E with high affinity [13]. This binding occurs at the same site that eIF4G binds to form the eIF4F cap-binding complex. When eIF4E-BP1 is not phosphorylated, it associates with eIF4E and prevents formation of the eIF4F complex. However, when eIF4E-BP1 is phosphorylated, the association with eIF4E is inhibited and translation initiation is able to proceed. Phosphorylation of eIF4E-BP1 is able to control translation indirectly; highly

phosphorylated eIF4E-BP1 is associated with increased translation and dephosphorylated 4E-BP1 is associated with an inhibition of translation [14].

It has been routinely noted that many viruses, including the negative strand RNA viruses, trigger changes in phosphorylation of one or both of these protein complexes [15-17], and that these changes are associated with the inhibition of host or viral protein synthesis. The association of phosphorylation state to specific functions of these proteins means that the effective study of both eIF2 alpha and eIF4E phosphorylation can be readily accomplished by means of immunoblot analysis. The simplest approach is to compare the relative level of phosphorylated protein to the level of total protein phosphorylation-specific antibodies and total protein detecting antibodies. Complete analysis is best done by selecting multiple time points that are well spaced around the time point of greatest interest, which may be determined by the metabolic labeling experiment described above.

The phosphorylation level of eIF4E-BP1 is also able to be directly measured using phosphorylation-specific antibodies for all four major sites of phosphorylation on eIF4E-BP1. However, a second approach allows investigation of global changes in the phosphorylation levels of eIF4E-BP1 in a more timely and less costly manner. It is well established that hyperphosphrylated forms of eIF4E-BP1 migrate more slowly than hypophosphorylated forms through high percentage SDS-PAGE gels [18]. Therefore, this approach allows complete description of the phosphorylation state of eIF4E-BP1 with a single antibody.

3.2 Setup

This experiment is most easily carried out in 35mm dishes (or a 6-well plate). Plate cells the day prior to infection so that they are at optimal density for virus infection when the experiment begins. A time course that includes time points from all stages in the virus life cycle will allow thorough investigation into the control of translation by these three translation initiation factors.

3.3 Experimental Methods

Infect cells as appropriate for the time points to be examined. Remove the plate from the incubator and aspirate media. Wash cells twice with warm PBS to remove any media and serum proteins. Immediately prior to the end of the infection, add protease inhibitors (100μl of 100mM PMSF and 100μl of 100mM Benzamidine) and phosphatase inhibitors (10μl of 1mM Microcystin and 10μL of 100μM Okadaic acid) to 10mL of lysis buffer and keep on ice. Lysis buffer containing NP-40 (Boston BioProducts BP-119-500) is sufficient for most routine applications. For examination of membrane proteins or proteins that localize to the nucleus or mitochondria, a more stringent detergent, eg RIPA (Boston BioProducts BP-115-500), is recommended.

Add 300μL of lysis buffer to each well to lyse the cells. A smaller volume (200 or 250 μL) can be used to maximize protein concentration. However, care should be taken to ensure the entire well is covered. Place the plate on ice for 10 minutes, rocking occasionally. After 10 minutes, remove the lysate to a 1.5mL tube and spin at maximum speed in a refrigerated microcentrifuge for 10 minutes. A small white pellet should form at the bottom of the tube.

The protein concentration of each sample should be determined using a small-scale Bradford or Lowry assay. Transfer 150μL of lysate to a new tube containing 30μL of 6× SDS-PAGE loading buffer. Load 20-50μg of total protein onto the appropriate gel (see below) alongside molecular weight standards for comparison.

For analysis of eIF2α (36kDa), a 10% acrylamide gel (or higher) is sufficient. For eIF4E, which migrates at an apparent molecular weight of 28kDa, a 12% gel should be used to avoid the possibility of running the protein off of the bottom of the gel. For eIF4E-BP1, a 15% gel made using a 40% 19:1 bis:mono acrylamide mixture (Bio-Rad 161-0144) is recommended to distinguish the phosphorylation variants of the protein.

Following electrophoresis, transfer the proteins from the gel to methanol-activated PVDF (Bio-Rad 162-0177) using either a wet-transfer or semi-dry transfer system. PVDF is preferred over nitrocellulose because of its superior binding capacity (Pluskal, et al., 1986). After transfer, block PVDF. For phosphorylation-specific antibodies, use either 5% BSA or 0.5% dry milk in TBS-T (Boston BioProducts 1BB-181). PBS should not be used for phosphorylation-specific antibodies.

Dilute antibodies as instructed by the manufacturer (typically 1:1000 is sufficient for strong signal and minor background) in the same solution used to block. Table 1 lists suggested antibodies. Incubate at room-temperature for one hour or overnight at 4°C with gentle rocking. The phosphorylation-specific antibodies should be used first and allowed to bind the blot overnight at 4 degrees with gently rocking. When the incubation is complete, wash the PVDF with TBS-T three times for five minutes each.

Dilute appropriate HRP-labeled secondary antibody as instructed by the manufacturer (typically 1:3000 is sufficient). Incubate at room-temperature for one hour or overnight at 4°C with gentle rocking. Wash the PVDF with TBS-T three times for five minutes each. Add chemiluminescent reagent (Perkin Elmer NEL101001EA) and expose to film or CCD camera. A typical exposure on film should take 1-5 minutes, though the weaker phosphorylation-specific antibodies may require more time. If background is high, reduce the amount of antibody used. If detection is weak, increase the amount of total protein lysate loaded into each well or increase the amount of primary (protein-specific) antibody used for detection.

Once exposure is complete, the antibody can be stripped from PVDF. Incubate PVDF membrane in 0.2M glycine, pH 2.3 for 30 minutes at room temperature with gentle shaking. The blot should then be washed in TBS-T and re-blocked as appropriate to allow reprobing with an additional antibody.

When detection of phosphorylation and total protein levels is complete, the bands should be quantified by densitometry. This will allow the calculation of the changes to phosphorylation levels over the course of virus infection. The levels of phosphorylation-specific signal should be normalized to total protein signal to account for any variability in total protein, either from changes in the total levels of protein during infection or from errors in gel loading. An unrelated loading control (actin or tubulin) should be used to investigate changes in total protein levels.

For the phosphorylation of eIF2α, which is not able to be detected in resting cells, the highest level of phosphorylation-specific signal should be set as 100% and all other samples compared to this value. For eIF4E, the signal detected in mock-infected cells should be normalized to 100% and the virus-infected cell samples be compared to this value. For eIF4E-BP1, the signal from the phosphorylated (slower-migrating) forms of the protein should be compared to the total signal. The slowest migrating form will run at approximately 23 kDa, while the fastest will run at approximately 19 kDa. If there is ongoing apoptosis during virus infection, an additional band can appear at approximately 17 kDa.

3.4 Example Experiment

Figure 6 is an example of the effects of viral infection on the translation initiation components. Panel A shows the phosphorylation of translation initiation proteins from cells mock infected or infected with LaCrosse virus. Mock infected HeLa cells show very low levels of eIF2α; HeLa cells treated with thapsigargin (which inhibits calcium pumps) phosphorylate eIF2α robustly while cells treated with LY294002 (a PI3 kinase inhibitor) show no phosphorylation of eIF2α. When cells are infected with LaCross virus, eIF2α phosphorylation increases over the time course of infection, detectable first at 4hpi and increasing up to 8hpi (Figure 6A, top panel). The level of phosphorylation of eIF4E in uninfected cells is high. The phosphorylation of eIF4E is not affected by thapsigargin or LY294002, but infection by LaCrosse virus causes dephosphorylation of eIF4E by 6hpi. There is no detectable phosphorylation at 12hpi (Figure 6A, bottom panel).

Figure 6B shows a western blot for eIF4E-BP1, whose phosphorylation level can be detected by migration on the gel. Mock infected cells contain mostly phosphorylated eIF4E-BP1, as can be seen by the strong gamma band seen in those lysates. When cells are infected with vesicular stomatitis virus, eIF4E-BP1 becomes dephosphorylated over the time course of infection, beginning at 4hpi and continuing until 6hpi. Actin is shown as a loading control for these experiments.

4. Conclusion

The obligate dependence of viruses on the protein synthesis machinery of a host cell makes the understanding of of viral mRNA translation by the infected cell an important aspect of the virus host interaction. Host-mediated interruption of viral translation is an effective means of blocking viral replication and limiting pathogenesis for many different viruses[19, 20], but the precise interaction of many negative sense RNA viruses with the translation apparatus has yet to be fully investigated.

The methods presented here illustrate the existing tools that exist to allow researchers to develop a full picture of the state of protein synthesis throughout the course viral infection. Global metabolic labeling of protein synthesis in infected cells provides the state of host protein synthesis, as well as the rate of viral protein synthesis (2.1). A similar approach using an alkyne-azide reaction pair to fluorescently label newly-made proteins provides insight into both the location of viral protein synthesis in the infected cell but also the localization of viral proteins (2.2). This method of directly labeling the viral proteins allows visualization of proteins for which antibodies are not available. An *in vitro* translation system allows the investigation of the effects of specific proteins and drug targets on viral translation without concern of bystander effects on other aspects of the virus life cycle (2.3).

In addition to examining the viral protein products made in a cell, determining the state of phosphorylation of key translation initiation components can also allow the researcher a more complete understanding of the state of the virus/host interaction at this critical level of translational control. Both viruses and host cells have evolved mechanism to promote (or limit) translation at the initiation step, and examination of the phosphorylation state of key translation initiation proteins can provide insight into pathways the virus is exploiting in order to promote viral translation (3).

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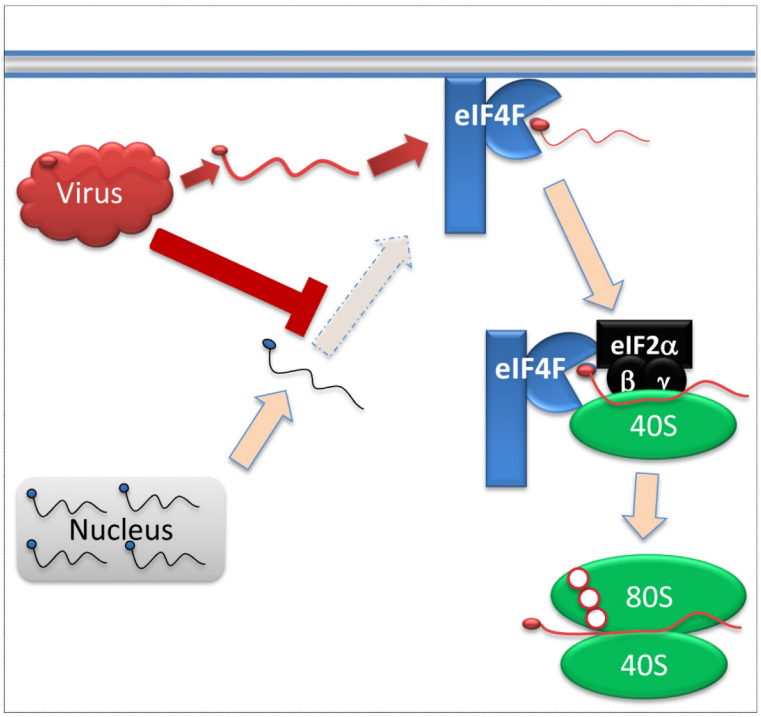

Figure 1.

Entry of negative strand RNA virus mRNA into the host translation machinery. Cartoon depicts the cycle of translation initiation for a host mRNA (depicted in black with a blue circle depicting the M7 guanine cap) from the nucleus to the cytoplasm where it is recruited by the cap-binding complex eIF4F through its cap moiety and delivered to the 40S ribosomal subunit, which is primed with the eIF2 complex bound to Methionine tRNA (tRNA not depicted). The final step in the process is the recruitment of the 80S ribosomal subunit and the initiation of translation. Negative strand RNA viruses produce capped mRNA that is generally presumed to be recruited to the ribosome through host machinery such as eIF4F. In this diagram, viral mRNA is shown in red, and is depicted as exiting from the site of viral mRNA synthesis to be recruited into the same pathway as host mRNAs. This cartoon depicts viral mRNA completely displacing host mRNA from the translation process, but varying degrees of host protein synthesis inhibition are seen during virus infect, suggesting different levels of dominance at the translation initiation step.

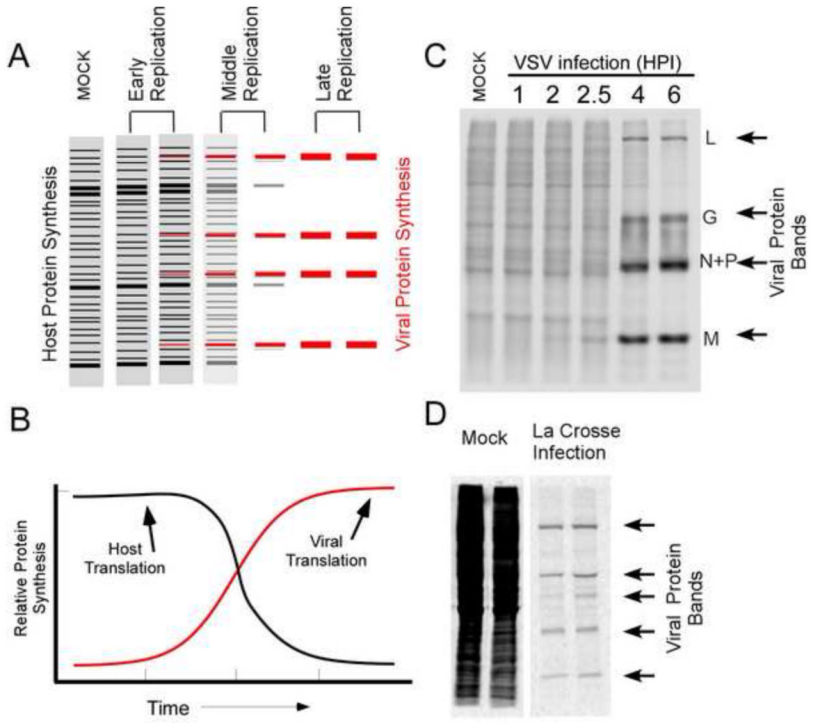

Figure 2.

Trace-label determination of protein synthesis. A) shows a cartoon representation of an SDS-PAGE gel experiment where cells were trace-labeled with 35S Methionine and Cysteine to determine rates of protein translation during infection with a virus that inhibits host protein synthesis while promoting synthesis of its own. On the far-left hand of Panel A, a gray smear with more prominent black bands is shown, depicting general host translation. This pattern predominates throughout early infection and then dissipates as infection process. Depicted in red are bands corresponding to viral proteins. These bands begin to be seen during the middle of the virus replication cycle and become the dominant bands at late times of infection. B) depicts the quantitation of both host protein synthesis (black line) and viral protein synthesis (red line). The increase in viral protein synthesis and decrease in host protein synthesis are normally inversely related, though the absolute value of protein synthesized may not be comparable. C) shows a representative autoradiograph of HeLa cells that were infected with vesicular stomatitis virus at a multiplicity of infection of 10 for the indicated times (listed in hours postinfection or HPI). Clearly visible on the left hand-side of the gel is host protein synthesis, while the right-hand side of the gel clearly shows viral protein synthesis only. At 2.5 hours postinfection, viral bands can be discerned in the overall protein synthesis pattern using the later timepoint synthesis as a guide. D) shows an autoradiograph of an SDS-PAGE gel showing radiolabeled lysates from HeLa cells that were either mock-infected or infected with La Crosse (H78) at a multiplicity of infection of 10 for 12 hours. Notable in this autoradiograph is that the relative level of protein synthesis in La Crosse virus infection is dramatically lower than that seen in mock-infected cells (see overexposed left-hand lanes).

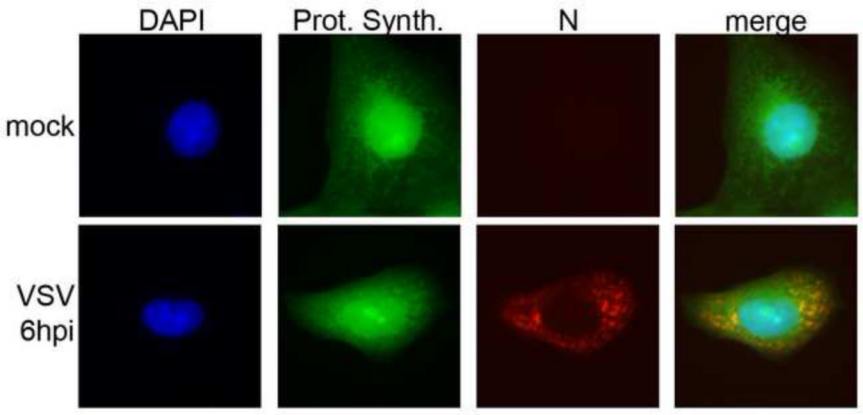

Figure 3.

In vivo labeling of translation with Click-iT. Vero cells were mock infected or infected with VSV for 6h and processed as described. Top panels show mock infected cells; bottom panels show VSV infected cells. DAPI is shown in blue. In green is Click-iT labeling of total protein synthesis from 5.5-6 hpi. Shown in red is antibody staining for viral nucleoprotein (N). Robust protein synthesis is seen in both mock and virus infected cells. Costaining with N demonstrates virus infection.

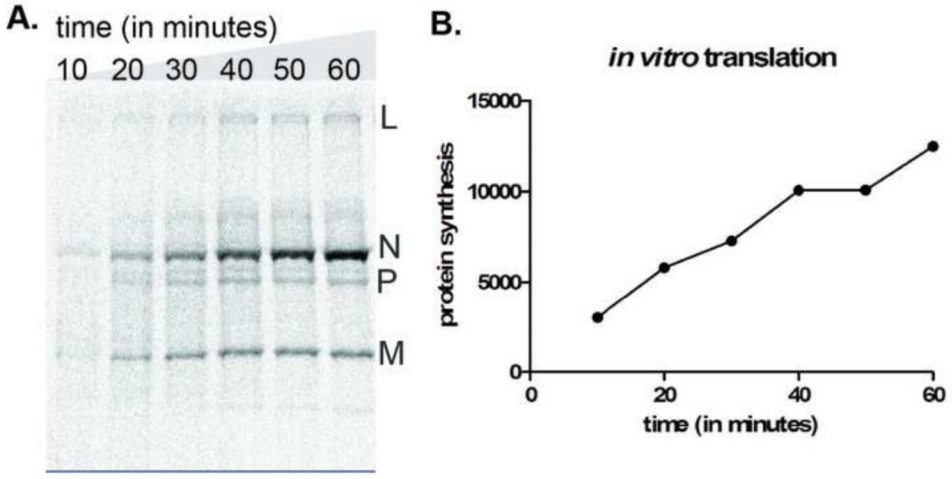

Figure 4.

In vitro synthesis of viral proteins. A) *In vitro* translation lysates prepared as described in the text were incubated at 30 degrees for increasing amounts of time. An autoradiograph of samples separated by SDS-PAGE is shown. B) Quantification of viral protein synthesis shown in A. Viral translation continues *in vitro* up to 60 minutes.

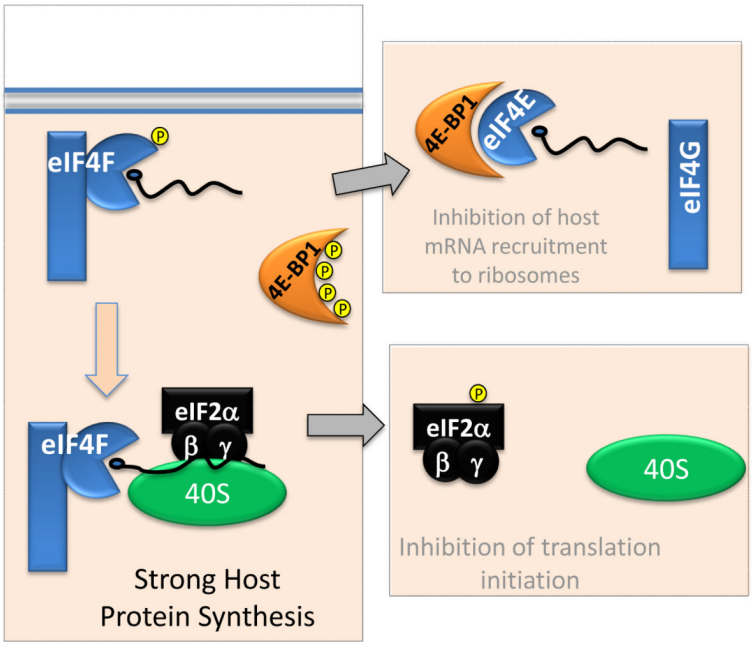

Figure 5.

Phosphorylation-mediated control of translation initiation. Cartoon illustrates three separate areas at which phosphorylation events can control the rate of translation initiation. Shown in the top panel is the phosphorylation status of translation initiation factors eIF4E (phosphorylated) 4E-BP1 (phosphorylated) and eIF2a (unphosphorylated) under conditions when translation of host proteins is unabated. Bottom panel shows conditions under which host protein synthesis is inhibited. Shown is the dephosphorylated form of 4E-BP1, which binds to eIF4E, removing it from the rest of the eIF4F complex (shown as eIF4G in this panel). eIF4E is also dephosphorylated. Both of these events are independently associated with the decreased protein synthesis. Also shown is the phosphorylation of eIF2a, which keeps the eIF2 complex from recycling to the 40S complex, thereby globally inhibiting translation initiation.

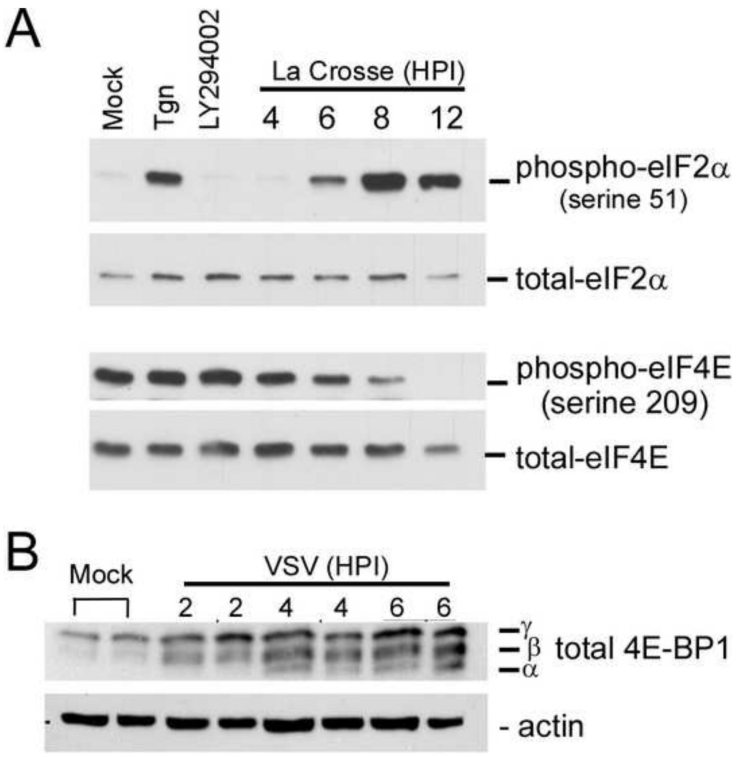

Figure 6.

Analysis of changes in translation initiation factor phosphorylation during virus infection. A) Top panel shows phosphospecific antibody detected changes in the phosphorylation of serine 51 of eIF2a in mock-infected HeLa cells, HeLa cells treated with 1μM thapsigargin (TGN) for 30 minutes or 10μM LY294004 for 1 hour. Also shown are several timepoints from an infection with LaCrosse virus at an MOI of 10. Second panel shows signal obtained from immunoblots with an antibody to total eIF2a. Third panel is the result of western blotting using an antibody that specifically recognizes serine 209 of eIF4E. The fourth panel shows total eIF4E signal on the same PVDF membrane. B shows a 4E-BP1 immunoblot of lysates from mock or VSV-infected cells. The image clearly shows the dephosphorylation of 4E-BP1, as evidenced by the appearance of the tripartite migration pattern of 4E-BP1 as infection proceeds.

Table 1

Useful antibodies that recognize phosphorylated or total populations of translation initiation factors

Table includes antibody catalog numbers for both Cell Signaling Technologies and Santa Cruz biotech. Antibodies shown have been successfully used in the laboratory. This list is not exhaustive, as several other companies also make high quality phosphorylation state-specific antibodies.

Target Protein	Cell Signaling	Santa Cruz
phos-eIF2α	9721S	sc-293100
total eIF2α	9722S	sc-12412-R
phos-eIF4E	9741S	sc-12885-R
total eIF4E	9742S	sc-13963
total eIF4E-BP1	9452S	sc-6936
actin	4967S	sc-47778
anti-mouse HRP	7076S	sc-2005
anti-rabbit HRP	7074S	sc-2004