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Investigating Myc-Dependent Translational Regulation in Normal and Cancer Cells

John T. Cunningham, Michael Pourdehnad, Craig R. Stumpf, and Davide Ruggero

Abstract

There is an increasing realization that a primary role for Myc in driving cellular growth and cell cycle progression relies on Myc's ability to increase the rate of protein synthesis. Myc induces myriad changes in both global and specific mRNA translation. Herein, we present three assays that allow researchers to measure changes in protein synthesis at the global level as well as alterations in the translation of specific mRNAs. Metabolic labeling of cells with ^{35}S -containing methionine and cysteine is presented as a method to measure the overall rate of global protein synthesis. The bicistronic reporter assay is employed to determine levels of cap-dependent and cap-independent translation initiation in the cell. Finally, isolation of polysome-associated mRNAs followed by next-generation sequencing, microarray or quantitative real-time PCR (qRT-PCR) analysis is utilized to detect changes in the abundance of specific mRNAs that are regulated upon Myc hyperactivation. The protocols described in this chapter can be used to understand how and to what extent Myc-dependent regulation of translation influences normal cellular functions as well as tumorigenesis.

Keywords

Myc; Translation; Protein synthesis; Ribosome

1 Introduction

Myc has an evolutionarily conserved role in regulating cell growth. This function of Myc is dependent on its ability to increase protein synthesis. Importantly, numerous studies have demonstrated that Myc directly regulates the expression of many components of the protein synthesis machinery. Myc transcriptional target genes include translation initiation and elongation factors, tRNA synthetases, Pol III, nucleolar assembly components, and proteins belonging to the small and large ribosomal subunits [1–3]. As such, one of the major functions of Myc is to control ribosome biogenesis and mRNA translation. The role of Myc-dependent regulation of protein synthesis in physiologic cellular processes or under pathological conditions is not well understood. However, the importance of alterations in translation induced by Myc has been highlighted in the setting of oncogenic Myc activity, where restoring protein synthesis to normal levels leads to suppression of cell growth and decreased Myc-driven tumorigenesis [4, 5]. Additionally, Myc oncogenic activity has been

shown to deregulate the translational control of specific mRNAs during mitosis leading to genomic instability [4]. Therefore, Myc-dependent alterations in mRNA translation direct an oncogenic program, involving distinct cellular processes, which is active during multiple stages of cancer initiation and progression [6–8].

The purpose of this chapter is to describe in detail several methods used to investigate the effects of Myc on protein synthesis. The first protocol explains *³⁵S metabolic labeling*, which allows the measurement of global protein synthesis rates by quantifying the incorporation of ³⁵S-labeled methionine and cysteine into newly translated proteins (Fig. 1a). Increased uptake of ³⁵S methionine in neoplasms compared to normal tissue was first described in the 1950s [9]. Subsequent studies demonstrated that increased Myc activity led to increased protein synthesis, which was coupled to increased cell mass [4, 10–12]. Importantly, these and other studies demonstrated that Myc has an evolutionarily conserved role across multiple cell types in promoting protein synthesis, which leads to cell growth, cell division, and, when deregulated, can lead to cancer [4, 5, 13].

The second protocol describes *measuring cap-dependent and IRES-dependent translation initiation using the bicistronic reporter assay* (Fig. 1b). The best studied mechanism of translation initiation is cap-dependent, which requires the assembly of an initiation complex on the 5' end of mRNAs that recruits the ribosome and scans along the 5' untranslated region prior to initiation of translation at the start codon. Most mRNAs are translated by this method of initiation. IRES-dependent translation, on the other hand, is an alternative method of translation initiation in which the ribosome is recruited by an RNA structural element, known as the internal ribosome entry site (IRES). It is postulated that IRES-mediated translation is an important alternative mode of translation that can differentially regulate the translation of specific mRNAs during distinct physiological contexts (e.g., during cell cycle progression and development) or in response to certain stimuli (e.g., hypoxia and endoplasmic reticulum stress) [4, 7, 14–17]. There is no existing structure or sequence prediction method that can accurately identify IRES elements in an mRNA. Therefore, translation initiation from these elements must be validated experimentally via methods such as the bicistronic reporter assay. This approach measures the levels of cap-dependent and IRES-dependent translation in cells. Changes in the relative levels of cap-dependent and IRES-dependent translation have been observed during various stages of tumorigenesis [7, 18]. In this regard, several translation initiation factors are transcriptional targets of Myc, including eIF4E, and have been shown to cooperate with Myc-driven tumorigenesis [19, 20]. Additionally, Myc has been shown to deregulate the switch between cap- and IRES-dependent translation that occurs during the mitotic phase of the cell cycle [15, 16, 21]. Specifically, Myc hyperactivation leads to decreased expression of the endogenous IRES-dependent isoform of Cdk11 (p58-PITSLRE) during mitosis, which leads to genomic instability [4]. The mechanism for maintaining the normal balance between these modes of translation as well as how oncogenic signaling pathways impinge on this translational control is not well understood.

The third protocol describes *analyzing gene-specific changes in mRNA translation by isolation of polysome-associated mRNA* (Fig. 1c). While the ³⁵S methionine incorporation assay measures changes in global protein synthesis, analyzing the fraction of mRNAs

associated with multiple ribosomes (polysome-associated) can be used to detect changes in the translation of specific mRNAs [22, 23]. Polysome-associated mRNAs undergo high levels of translation, while mRNAs that are not polysome-associated are less efficiently translated. After their isolation, polysome-associated mRNAs can be analyzed by qRT-PCR to determine the relative level of translation for each specific mRNA. This approach has uncovered Myc-dependent changes in the translation of specific genes. For example, B cells overexpressing Myc specifically upregulate the translation of VEGF, while VEGF mRNA levels are unchanged [22]. Two approaches utilizing next-generation RNA sequencing can be used to identify genome-wide changes in polysome-associated mRNAs. Polysome-associated mRNAs can be characterized by RNA sequencing to identify translationally regulated genes [24]. Alternatively, ribosome profiling is a novel method to analyze genome-wide translation (Fig. 1c) [25]. Recent studies have used this technology to characterize the differential translation of mRNAs downstream of an oncogenic signaling pathway [26]. The ribosome profiling protocol has been recently published and a detailed protocol specifically for analyzing polysome-associated mRNAs is provided below [27]. These technologies can be used to address a major unresolved question by identifying the translational landscape of mRNAs that are regulated by Myc.

Taken together, these protocols provide tools to better understand the effects of Myc on global protein synthesis rates, the regulation of different modes of translation, and the translational regulation of specific mRNAs. These protocols can be utilized across tissue and cell types and under various physiological and pathological conditions to unravel outstanding questions regarding the role of Myc-dependent translation regulation in normal cellular processes and cancer.

2 Materials

2.1 ³⁵S Metabolic Labeling Assay

1. Radioactivity license.
2. Tissue culture equipment (e.g., laminar flow hood, incubator, tissue culture dishes).
3. Methionine- and cysteine-free culture medium (Sigma).
4. ³⁵S-Express Protein Labeling Mix (Perkin-Elmer).
5. Dialyzed fetal bovine serum (FBS).
6. Charcoal filters.
7. Rubber policeman.
8. Gel electrophoresis equipment and buffers.
9. Autoradiography equipment.
10. Image analysis software.
11. Bradford Assay kit.

12. RIPA buffer: 50 mM Tris, pH 7.4, 150 mM NaCl, 0.1 % sodium dodecyl sulfate, 0.5 % sodium deoxycholate, 1 % Triton X-100, 10 mM β -glycerophosphate, 50 mM NaF.
13. Complete protease inhibitors (Roche).
14. 1 μ M phenylmethanesulfonyl fluoride (PMSF).
15. Phosphate buffered saline (PBS).
16. Primary antibodies: tubulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β -actin.
17. Secondary anti-mouse and anti-rabbit antibody conjugated to Horseradish peroxidase (HRP).

2.2 Measuring Cap-Dependent and Cap-Independent Translation Initiation Using the Bicistronic Reporter Assay

1. Bicistronic plasmid containing Renilla and firefly luciferase under the control of a T7 promoter.
2. Restriction endonuclease.
3. mMessage mMachine T7 transcription kit (Ambion).
4. TransMessenger mRNA transfection reagent (Qiagen).
5. Dual-Luciferase assay kit (Promega).
6. Luminometer.

2.3 Analyzing Gene-Specific Changes in mRNA Translation by Isolation of Polysome-Associated mRNA

1. Sucrose.
2. Gradient buffer: 25 mM Tris pH 7.4, 25 mM NaCl, 5 mM MgCl₂.
3. Heparin: 50 mg/ml in DEPC water.
4. DTT: 1 M in DEPC water.
5. Cycloheximide: 50 mg/ml in ethanol.
6. PBS.
7. Refrigerated microcentrifuge.
8. Ultracentrifuge with SW40 (or equivalent) rotor and matching tubes.
9. Lysis buffer: 10 mM Tris, pH 8.0, 140 mM NaCl, 1.5 mM MgCl₂, 0.25 % NP-40, 0.1 % Triton X-100, 20 mM DTT, 0.15 mg/ml cycloheximide, 0.6 U/ml RNasin.
10. Trizol solution (Life Technologies).
11. PureLink RNA miniprep kit (Life Technologies).
12. Optional: automated gradient maker and fraction collector, microwave, trypan blue.

3 Methods

3.1 ³⁵S Metabolic Labeling Assay

1. On the day before labeling, seed cells on 6-well tissue culture dishes such that the dish is approximately 70–85 % confluent at the time of labeling (*see* Note 1).
2. Aspirate medium and replace with 1.5 ml of methionine- and cysteine-free medium supplemented with dialyzed FBS. Incubate in methionine- and cysteine-free medium for 30 min (*see* Note 2).
3. Add 3 μ L (33 μ Ci) of Express Protein Labeling Mix directly to each well of the 6-well tissue culture dish. Return dish to tissue culture incubator and incubate for 1 h. Follow proper institutional guidelines on handling radioactive materials such as co-incubating cells with activated charcoal filters to absorb any volatile ³⁵S compounds.
4. Using institutional guidelines governing handling and disposal of radioactive materials, harvest cells in 1 ml/well ice-cold PBS using a rubber policeman and collect in Eppendorf tubes.
5. Lyse cells in RIPA lysis buffer supplemented with 1 $\times$ complete protease inhibitors and 1 mM PMSF. Approximately 20–100 μ L of RIPA lysis buffer per sample should be used. Incubate lysis for 15–20 min on ice. Centrifuge the lysed cells at maximum speed for 5 min and collect the supernatant.
6. Measure the protein concentration of the lysates using a Bradford Assay.
7. Run a standard 10 % SDS-PAGE gel using 10–30 μ g of total protein from each of the samples per well. *See* Note 3 for discussion of alternative approaches to detection of ³⁵S incorporation.
8. Transfer gel to PVDF membrane.

¹Several variables need to be considered at this step. ³⁵S labeling should be performed on metabolically active cells. Therefore, take care to seed cells at a density that will permit this condition to be met (i.e., ensure cells are not too confluent). Myc-overexpressing cells typically proliferate faster and may be larger than normal counterparts. In this respect, different amounts of cells may be initially plated such that the density and total number of cells is equal at the time of ³⁵S labeling. To quantify cell size prior to plating, a Coulter Z2 Particle Count and Size Analyzer or flow cytometer may be used.

Myc-dependent increases in global protein synthesis rates are mediated through a coordinated transcriptional response involving genes encoding ribosome biogenesis factors, ribosomal proteins, initiation factors, as well as the ribosomal RNA and transfer RNAs. Therefore, if using an inducible system of Myc activation such as MycER, or Tet-inducible Myc overexpression, care should be taken to optimize the timing between activation of Myc and incubation of cells with ³⁵S-containing methionine and cysteine. Typically, using the MycER system, we routinely observe increased target gene expression within 3–6 h and increased protein synthesis rates within 24 h following 4-hydroxytamoxifen administration.

Labeling experiments using cells freshly isolated from living tissue should be performed using the minimal amount of time necessary between harvest and ³⁵S labeling and should begin with 30 min of culture in methionine- and cysteine-free medium.

²Methionine and cysteine starvation is performed solely to increase the uptake and incorporation of ³⁵S-labeled methionine and cysteine. The incubation time necessary to achieve proper starvation is dependent on cellular metabolism. Care should be taken not to incubate cells too long in the absence of methionine and cysteine, as this could elicit an amino acid starvation response through GCN2, which phosphorylates eIF2 α and suppresses global protein synthesis. The levels of methionine and cysteine in serum are typically several hundredfold below that of common medium formulations such as DMEM and RPMI; therefore using dialyzed FBS is not absolutely necessary.

³To measure ³⁵S incorporation into proteins, many suitable methods exist. We have presented our preferred method, but alternative approaches may also be utilized. In addition to the method presented above, other researchers have precipitated whole cell lysates with trichloroacetic acid and used a scintillation counter to measure ³⁵S incorporation. Using this method, data is expressed as ³⁵S signal intensity per cell or per μ g of protein. Alternatively, instead of transferring protein from acrylamide gel to PVDF membrane, gel may be stained with Coomassie to ensure equal loading between samples followed by gel drying and exposing to film.

9. Remove PVDF membrane containing bound ^{35}S -labeled proteins and immediately wrap in cling wrap and expose to film in a cassette (*see* Note 4). Use a Geiger counter to help ascertain the exposure time necessary for obtaining the appropriate signal intensity. Typical exposure times will range from 2 to 48 h depending on the metabolic activity of the cells used.
10. Once a suitable exposure within the linear intensity range of film has been obtained, perform western blot analysis with tubulin, GAPDH, or β -actin antibodies for internal loading control.
11. Use Image J or other imaging software to calculate the intensity of scanned images (*see* Note 5). Express data as a ratio of ^{35}S signal intensity/loading control signal intensity.

3.2 Measuring Cap-Dependent and Cap-Independent Translational Initiation Using the Bicistronic Reporter Assay

1. Linearize bicistronic plasmid by digesting with a restriction endonuclease downstream of the firefly luciferase gene (*see* Note 6).
2. Transcribe capped mRNA using the mMessage mMachine in vitro transcription kit. Confirm the transcript is the proper length by gel electrophoresis and phenol extract the RNA.
3. Transfect 2 μg mRNA per 100,000 cells following the TransMessenger protocol.
4. Incubate cells for 6–8 h to allow for translation of the luciferase genes.
5. Harvest the cells and perform the Dual-Luciferase assay according to the manufacturer's protocol.
6. Normalize the luciferase readings to the amount of reporter mRNA as measured by quantitative PCR or northern blot.

3.3 Analyzing Gene-Specific Changes in mRNA Translation by Isolation of Polysome-Associated mRNA

1. Prepare 10 % and 50 % sucrose solutions in gradient buffer. Add 0.1 mg/ml heparin and 2 mM DTT. Prepare 10–50 % linear sucrose gradient in an ultracentrifuge tube (*see* Note 7).

⁴Avoid allowing the PVDF membrane to dry out, as this will interfere with downstream western blotting applications. If the membrane happens to dry out, a quick rinse in methanol should be performed to prepare the membrane for western blotting. Use of a phosphor imaging screen as an alternative to film can significantly reduce exposure times and expand the linear range of detection.

⁵In Image J (<http://rsbweb.nih.gov/ij/>), use the rectangle tool to outline the lanes of interest on a grayscale image of your autoradiograph. Use Ctrl + 1 to select the first lane followed by dragging the outlined region to the next lane and pressing Ctrl + 2 to select each additional lane. Once all lanes have been established in this manner, press Ctrl + 3 to plot the intensity of the signal within the lanes. Use the wand tool to select the area underneath the peaks within each lane to quantify the overall signal intensity for each lane. It is important to note that many gels will have some degree of background signal and it may be necessary to manually draw a horizontal baseline using the straight line tool in each of the plots of the lanes to account for this prior to using the wand tool. Quantify both the ^{35}S autoradiograph and the image of the loading control western blot (e.g., tubulin, β -actin, GAPDH). Data should be expressed as ^{35}S signal intensity/loading control signal intensity for each lane.

⁶In addition to this protocol for analyzing cap-dependent and IRES-dependent translation in cell culture, a mouse model harboring a widely expressed bicistronic reporter mRNA has also been developed [28].

2. Treat cells with 0.1 mg/ml cycloheximide (*see* Note 8).
3. Wash cells in PBS containing 0.1 mg/ml cycloheximide.
4. Resuspend in lysis buffer (~ 100 μ l per 10 million cells). Lyse cells at 4 °C for 30 min. Vortex cells every 10 min during the lysis (*see* Note 9).
5. Centrifuge lysate at 10,000 $\times$ g at 4 °C for 5 min.
6. Transfer cleared lysate to clean RNase-free microfuge tube. Save 1 % of the lysate to analyze total cellular RNA levels.
7. Carefully layer the lysate onto the 10–50 % sucrose gradient. Place the centrifuge tube in the appropriate bucket, ensuring that opposing buckets are balanced.
8. Centrifuge samples at 243,000 $\times$ g (at maximum radius) at 4 °C for 2.5 h in SW40 rotor.
9. After centrifugation, collect 12–24 fractions from each sample (*see* Note 10).
10. Isolate RNA from each fraction using the Trizol modification of the PureLink RNA miniprep kit protocol.
11. Analyze RNA levels of genes of interest by standard quantitative PCR or analyze global polysome-associated mRNA profiles by next-generation RNA sequencing or microarray (*see* Notes 11 and ¹²).

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⁷Use of an automated gradient maker (e.g., Teledyne ISCO) is recommended for producing uniform gradients. Alternatively, gradients can be made by manually layering the lower percentage sucrose solution over the higher percentage sucrose solution, capping the tube, and carefully storing the tube on its side for 1–4 h. Store gradients at 4 °C until samples are ready to load.

⁸A single cell suspension must be generated from tissue or cultured cells prior to beginning the lysis protocol. Ideally start with greater than 10 million cells.

⁹Lysis usually takes approximately 30–45 min depending on cell type. Check for completion of lysis by staining a small aliquot with trypan blue.

¹⁰Optional: An automated fraction collector will increase the reproducibility of collecting fractions. Prepare fraction collector according to manufacturers specifications. For the ISCO fraction collector, dissolve 60 g sucrose to 100 ml DEPC water to push gradient into UV detector/fraction collector. Alternatively, fractions can be collected by pipetting off 0.5–1.0 ml fractions from the top of the gradient into collection tubes. Measuring and plotting the absorbance at 260 nm across the fractions will give a graph representing the presence of ribosomes among the fractions.

¹¹It is generally recommended to correlate changes in polysome association to changes in cellular protein levels measured by western blot analysis to confirm translational regulation.

¹²Alternative: Ribosome profiling is an emerging technology that can be used as an alternative to global polysome-associated mRNA analysis by RNA-seq. Ribosome profiling characterizes the fragment of mRNA protected by the ribosome during RNase digestion. The advantage to using the ribosome profiling approach is the precise, codon level, positioning information along each individual mRNA. This positional data may provide unique mechanistic insights into specific regulatory elements important for the proper translation of mRNAs, such as upstream open reading frames or ribosome pause sites [29].

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Fig. 1. Methodologies presented to dissect translational changes downstream of Myc hyperactivation. **(a)** Flowchart of ³⁵S data acquisition and processing in wild-type (WT) or Myc-overexpressing (Myc) cells. **(b)** Illustration of a bicistronic cap/IRES translation initiation reporter. In this reporter mRNA, translation of the *Renilla* luciferase open reading frame is driven by a 5' 7-methylguanosine cap-dependent mechanism, whereas the firefly luciferase protein is expressed via translation initiation that relies on ribosome recruitment through an IRES-dependent mechanism. **(c)** Sucrose gradient fractionation of cytoplasmic RNA reveals translationally inactive (40S, 60S, 80S/monosome) and active (polysome) fractions (*left*). A schema depicting the steps involved in ribosome profiling is presented (*right*). Both procedures can be used to generate sequencing libraries for analysis of the genome-wide changes in translation upon Myc hyperactivation (*bottom*)