Video Article

Eukaryotic Polyribosome Profile Analysis

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Abstract

Protein synthesis is a complex cellular process that is regulated at many levels. For example, global translation can be inhibited at the initiation phase or the elongation phase by a variety of cellular stresses such as amino acid starvation or growth factor withdrawal. Alternatively, translation of individual mRNAs can be regulated by mRNA localization or the presence of cognate microRNAs. Studies of protein synthesis frequently utilize polyribosome analysis to shed light on the mechanisms of translation regulation or defects in protein synthesis. In this assay, mRNA/ribosome complexes are isolated from eukaryotic cells. A sucrose density gradient separates mRNAs bound to multiple ribosomes known as polyribosomes from mRNAs bound to a single ribosome or monosome. Fractionation of the gradients allows isolation and quantification of the different ribosomal populations and their associated mRNAs or proteins. Differences in the ratio of polyribosomes to monosomes under defined conditions can be indicative of defects in either translation initiation or elongation/termination. Examination of the mRNAs present in the polyribosome fractions can reveal whether the cohort of individual mRNAs being translated changes with experimental conditions. In addition, ribosome assembly can be monitored by analysis of the small and large ribosomal subunit peaks which are also separated by the gradient. In this video, we present a method for the preparation of crude ribosomal extracts from yeast cells, separation of the extract by sucrose gradient and interpretation of the results. This procedure is readily adaptable to mammalian cells.

Video Link

The video component of this article can be found at http://www.jove.com/video/1948/

Protocol

1. Preparation of 7-47% sucrose gradients

1. Prepare sucrose gradients one day before use to allow gradients to become continuous. Make sterile 7% and 47% sucrose solutions containing 50mM NH4Cl, 50mM Tris-Acetate pH 7.0 and 12mM MgCl2 and store at 4°C.

2. For 6 gradients mix the 7% and 47% sucrose stock solutions to make 48 mL of each of the following sucrose concentrations. Add DTT (1M stock) to each sucrose solution to a final concentration of 1mM.

| Final Concentration | 7% sucrose | 47% sucrose |
|---------------------|------------|-------------|
| 7%                  | 48 mL      | 0           |
| 17%                 | 36 mL      | 12 mL       |
| 27%                 | 24 mL      | 24 mL       |
| 37%                 | 12 mL      | 36 mL       |
| 47%                 | 0          | 48 mL       |

3. In order to pour the gradients, attach a Pasteur pipette to a 20 mL syringe by securing and sealing with Parafilm or use a long needle. Add 7 mL of 7% sucrose to the bottom of a 1 x 3.5" polycarbonate ultracentrifuge tube. Next add 7 mL of 17% sucrose solution under the 7% solution by placing the Pasteur pipette tip near bottom of tube and pipetting no faster than 0.3 mL/sec. Repeat with 7 mL each of 27%, 37% and 47% sucrose solutions. You should observe clear lines between the layers, indicating that minimal mixing has occurred. Store gradients at 4°C overnight along with the rotors, bottles and tubes that will be used to harvest samples.

2. Preparation of the yeast extract

1. Grow 125 mL of yeast culture to an OD600 of 0.8-1.0. Add cycloheximide to the culture to a final concentration of 100 μg/mL and continue shaking at 30°C for 15 min.

2. Meanwhile, prepare lysis buffer containing 80 μg/mL cycloheximide, 200 μg/mL heparin, 0.2% DEPC, and 10 mM Tris-HCl pH 7.5, 0.1M NaCl, 30mM MgCl2. Keep everything on ice from this point on.
3. Preparation of extracts from mammalian cells

1. For adherent cells, grow in 100 mm dish to ~70% confluence. You will need one 100 mm dish per 11 ml gradient (typical yield is ~20 OD

2. Prior to lysis, add cycloheximide to 100 μg/mL. Incubate at 37°C for 15 min. Transfer plates to ice and rinse cells twice in ice cold PBS. All

3. Remove all traces of PBS by aspiration (let plates drain on an angled bed of ice) and add 1 mL lysis buffer, scrape and transfer to pre-chilled

4. Spin at 14,000 x g for 5 min in refrigerated microcentrifuge. Transfer fresh lysate to the sucrose gradient (for mammalian cells this is made up

5. Collection of data and fractions

1. Approximately 30 min prior to the completion of the 4 hr spin attach the needle to the Model 184 tube piercer. Attach the pen to the online

2. Fill the syringe pump with 50mL of Fluorinert using the reverse flow, rapid setting. Make sure that there are no air bubbles present. Connect

3. Place a waste beaker or series of tubes if collecting fractions under the hose at the end of the flow cell to collect sample run off.

4. Carefully remove the polyallomer centrifuge tubes containing the sucrose gradient from centrifuge rotor and place them on ice (pre-form the

5. When the end of the polyribosome profile has been reached, turn off the flow of Fluorinert to the centrifuge tube and stop the chart movement

6. Once the Fluorinert has begun flowing into the tube, monitor the movement of the needle on the online absorbance monitor. Once the needle

7. When the end of the polyribosome profile has been reached, turn off the flow of Fluorinert to the centrifuge tube and stop the chart movement

8. Retreat the Fluorinert from the centrifuge tube into the syringe by beginning the flow in the reverse direction at a rapid rate making sure not to
draw sucrose from the tube into the syringe. Unscrew the centrifuge tube from the tube piercer, lower the needle, and remove the tube.

9. Repeat steps 5.5 through 5.8 for each sucrose gradient.

10. When all sucrose gradients have been analyzed, retract the Fluorinert from the hose back into the syringe. Remove each part of fractionator
(tube piercer and flow cell) including the waste hose located at the top and rinse well with water.
6. Representative Results

Figure 1. Representative trace of ribosome extract prepared from yeast strain BY4741 (\textit{mat}a \textit{his3}\Delta1 \textit{leu2}\Delta0 \textit{met15}\Delta0 \textit{ura3}\Delta0) in the presence of cycloheximide. The ribosomal extract was fractionated utilizing a 7-47\% sucrose gradient and analyzed using a pump syringe apparatus attached to a UV detector. Peaks containing polyribosomes and 80S, 60S and 40S ribosomes are indicated.

Discussion

The information obtained from the polyribosome profile can provide valuable insight into the translational status of the cell. In addition, the status of the assembly of ribosomal subunits themselves can be determined\(^2\). For example the presence of halfmers or 80S and larger peaks with a slight shoulder to the right on the profile indicates a bound 40S subunit awaiting 60S subunit joining. Performing the experiment in the absence of any added cycloheximide or other inhibitor of elongation allows for analysis of the rate of run off, which indicates whether or not elongation is altered\(^3\). The fractions themselves are a rich source of reagents for the subsequent determination of the association of a specific mRNA or protein with ribosomal subpopulations by Northern or Western blotting of the fractions. The total pool of mRNAs associated with active ribosomes can be determined via an associated microarray\(^4\) or deep sequencing analysis\(^5\). The protein associations can also be stabilized by the appropriate addition of a cross linking reagent\(^6\).

Polyribosome analysis from mammalian cells is also worth mentioning as a distinct protocol in terms of the apparent difficulties in establishing the conditions required to obtain stable polysomes. The buffer systems reported in the literature are widely variable\(^7\)-\(^10\), thus there may be a requirement for cell-type specific optimization of the lysis buffer, or it is equally plausible that the buffer system is not as important as keen attention to working with fresh lysates kept at low temperatures. To our knowledge a systematic evaluation of the parameters affecting mammalian polysome formation has not been reported.

Disclosures

No conflicts of interest declared.

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References

1. Baim, S. B., Pietras, D. F., Eustice, D. C. & Sherman, F. A mutation allowing an mRNA secondary structure diminishes translation of \textit{Saccharomyces cerevisiae} iso-1-cytochrome C. \textit{Mol. Cell. Biol.} 5, 1839-1846 (1985).
2. Ripmaster, T. L., Vaughn, G. P. & Woolford, J. L. DRS1 to DRS7, novel genes required for ribosome assembly and function in \textit{Saccharomyces cerevisiae}. \textit{Mol. Cell. Biol.} 13, 7901-7912 (1993).
3. Anand, M., Chakraburthy, K., Marton, M. J., Hinnebusch, A. G. & Kinzy, T. G. Functional interactions between yeast translation eukaryotic elongation factor (eEF) 1A and eEF3. \textit{J Biol Chem} 278, 6985-6991 (2003).
4. Serikawa, K. A. et al. The transcriptome and its translation during recovery from cell cycle arrest in Saccharomyces cerevisiae. *Mol Cell Proteomics* **2**, 191-204 (2003).

5. Ingolia, N. T., Ghaemmaghami, S., Newman, J. R. & Weissman, J. S. Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. *Science* **324**, 218-223 (2009).

6. Valasek, L., Szamecz, B., Hinnebusch, A. G. & Nielsen, K. H. In vivo stabilization of preinitiation complexes by formaldehyde cross-linking. *Methods Enzymol* **429**, 163-183 (2007).

7. Fletcher, J. E., Copeland, P. R. & Driscoll, D. M. Polysome distribution of phospholipid hydroperoxide glutathione peroxidase mRNA: evidence for a block in elongation at the UGA/selenocysteine codon. *Rna* **6**, 1573-1584 (2000).

8. Ruan, H. J., Brown, C. Y. & Morris, D. R. in *Analysis of mRNA formation and function* (ed J.D. Richter) 305-321 (Academic Press, 1997).

9. Martin, G. W., 3rd & Berry, M. J. Selenocysteine codons decrease polysome association on endogenous selenoprotein mRNAs. *Genes Cells* **6**, 121-129 (2001).

10. Lee, Y. Y., Cevallos, R. C. & Jan, E. An upstream open reading frame regulates translation of GADD34 during cellular stresses that induce eIF2alpha phosphorylation. *J Biol Chem* **284**, 6661-6673 (2009).