High-throughput polyribosome fractionation

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ABSTRACT

Polyribosome sedimentation velocity centrifugation can be used to identify differential regulation of the translation of mRNAs. However, ultracentrifugation presents practical limitations on the number of sedimentation velocity gradients that can be run simultaneously. A method for sedimentation velocity analysis of polyribosomes is presented that is based on low-speed centrifugation of sucrose gradients prepared in deep 96-well plates, the advantage of which is that hundreds of polyribosome fractionations can be performed simultaneously in a tabletop centrifuge.

INTRODUCTION

Much of differential gene expression is controlled at transcription. Nevertheless, mRNA splicing (1,2), polyadenylation (3), transport from the nucleus (4,5), localization within the cell (6–8), mRNA decay (9) and translation (8,10–15) are all regulated, and all have been implicated in differential gene expression. While microarray analysis is an effective means of identifying changes in global transcript abundance, partitioning of RNAs, between the various processes of RNA metabolism and the various compartments of the cell, suggests a need for methods for isolating RNAs according to their intracellular distributions.

Translation is a rich substrate for differential regulation, yet translation is a complex process involving large and intricate macromolecular structures, and much of the regulation of translation remains obscure. Intact and functional polyribosomes can be isolated by sedimentation velocity centrifugation of cell lysates (16). Experiments focusing on the protein components of polyribosomes reveal polyribosome-bound and transcript-specific factors that influence translation efficiency (10,11), and experiments on fractionated subcellular components reveal the partitioning of specific polyribosome-bound mRNAs to different organelles (8,12). The distribution of transcripts in polyribosomes reflects the rate of synthesis of their corresponding proteins (13). Sedimentation has been used in conjunction with cDNA microarray analysis to study translation regulation (14,15). These experiments and many others show that mRNAs from different genes are translated with varying efficiencies.

Translation regulation plays an important role in normal biology and in pathology. For example, work in glioma

cell lines has demonstrated that activation of RAS and Akt has a larger effect on the translation of extant mRNAs than on transcription (17,18). This finding employed microarray analysis of mRNA abundances in polyribosome fractions from sucrose velocity sedimentation gradients. Von Hippel-Lindau protein (pVAL) is associated with polyribosomes, represses translation of TNF α and other transcripts (19), and increases translation of p53 by mediating HuR binding to p53 mRNA (20). Genes important in the cell cycle are regulated at the translational level, such as Cyclin D1, whose translation is regulated by prostaglandins through phosphorylation of the eukaryotic initiation factor-2 α (eIF-2 α), which can be observed in polyribosomes (21). Translation initiation factor eIF-4A1 is commonly over-expressed in melanomas, and antisense oligonucleotides directed toward eIF-4A1 retard the growth of melanoma cells (22). These studies show that some, perhaps many, aspects of the transformed phenotype of cancer are due to differential translation regulation, and a large and varied literature confirms the importance of translation regulation in normal cellular physiology. Some, or all, of these transcripts may translate efficiently under the appropriate biological circumstances, but there is no general experimental paradigm, short of extensive screening, that would be expected to reveal those circumstances.

Sedimentation velocity centrifugation of polyribosomes using an ultracentrifuge does not lend itself to extensive screening, because most swinging bucket rotors only have six buckets. However, ultracentrifugation is not necessary for the preparation of polyribosomes. We describe a method for preparing hundreds of polyribosome sedimentation gradients simultaneously. Sucrose gradients are prepared in 96-well or 384-well deep-well reaction blocks, spun in a standard low-speed tabletop centrifuge, and fractionated using a liquid handling robot. The distributions of specific mRNAs from these gradients can be determined by RT-PCR. The gradients can be fully fractionated, or samples can be taken, robotically, from various depths. This approach should make it possible to perform wide-ranging surveys of variables that may influence mRNA translation.

MATERIALS AND METHODS

Cell culture

HaCaT cells (German Cancer Research Center, Heidelberg, Germany) and HeLa Tet-OffTM cells (Clontech, BD Biosciences) were grown in Dulbecco's modified Eagle's

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medium (Irvine Scientific Sales Co., Inc) with 10% fetal bovine serum (Omega Scientific, Inc.), 100 units/ml penicillin (Omega Scientific, Inc.), 100 µg/ml streptomycin (Omega Scientific, Inc.) at 37°C in a 5% CO₂ humidified atmosphere. The cells were split 16 h before harvesting and 1×10^7 cells were put in each 150 mm dish. Polyribosomes were prepared when the cells were 80–90% confluent. Where indicated, cells were treated with 10 µg/ml of puromycin (ICN Biomedicals, Inc.) for 60 min or 100 ng/ml final concentration of rapamycin (EMD Biosciences, Inc.) for 2 h before harvesting. Puromycin and rapamycin stock solutions were prepared at 1000× in water or ethanol, respectively.

Cell lysis

Cell culture dishes were placed on ice, the medium was aspirated, and cells were washed three times with 10 ml ice cold PBS. Cells were lysed using 400 µl of lysis buffer (10 mM NaCl, 20 mM Tris–HCl pH 7.5, 3 mM MgCl₂, 1% Triton X-100) per plate, and transferred to an Eppendorf tube, on ice. After 10 min with occasional mixing, bench top centrifugation at 14 000 r.p.m. for 10 min at 4°C was used to pellet cell debris. The supernatant contains the polyribosomes. To chelate MgCl₂ and observe polyribosome disruption, 100 µl 0.5 M EDTA (pH 7.5) was added to 400 µl of cell lysate, prior to gradient centrifugation.

Preparation of sucrose gradients

96-well Deep Well plates (Nalge Nunc International, Cat. No. 270141) were used for sucrose gradients. These plates have wells 41.5 mm deep that hold 2 ml solution. A Biomek FX Laboratory Workstation (Beckman Coulter, Inc.) was used to set up sucrose gradients: 150 µl of a 60% sucrose solution (10 mM NaCl, 20 mM Tris–HCl pH 7.5 and 3 mM MgCl₂) was dispensed into the bottom of the well, followed by 250 µl 50, 40, 30, 20 and 10% sucrose solutions on each successive layer. Gradients were 31 mm tall. Disturbance of gradient interfaces was minimized by dispensing slowly (~9.7 µl/s), with the tips at 0.5 mm above the liquid level, and by maintaining this distance as the liquid level rose. Gradients were prepared 4–6 h before use, and kept at 4°C.

For ultracentrifugation, 25 × 89 mm tubes (Beckman Ultra-Clear Centrifuge tubes, Cat. No. 344058; 35 ml gradients) or 16 × 102 mm tubes (Beckman Polyallomer Centrifuge tubes, Cat. No. 337986; 12.5 ml gradients) were used. Equal volumes of sucrose solution were used per layer: 10% sucrose solution was loaded first and each subsequent layer was underlaid in the order 20, 30, 40 and 50%. Gradients were prepared ~16 h before use and kept at 4°C.

Sucrose gradient centrifugation

Cell lysate volumes ranging from 2–200 µl, depending on the experiment, were layered onto the gradients. Low-speed gradients were centrifuged at 6000 r.p.m. (5796 g) at 4°C for 40 h using a Sigma 4K15C centrifuge and a Qiagen plate rotor (09100/09366 rotor/bucket combination). Ultracentrifugation was performed at 26 000 r.p.m. (121 896 g for 35 ml gradients; 135 536 g for 12.5 ml gradients) at 4°C for 4 h using a Sorvall Ultra 80 centrifuge and an AH627 rotor, with the appropriate buckets.

Gradient fractionation

For ultracentrifuge gradients, UV absorption profiles of polyribosome gradients were measured, and fractions were collected, using a Bio-Rad Econo System (Bio-Rad Laboratories), comprising a UV monitor (Model EM-1), pump (Model EP-1) and fraction collector (Model 2110). Polyribosome profiles were recorded digitally using a DI-154RS (DATAQ Instrument) connected to the Econo System.

96-well Deep Well plate gradient fractions were collected using a Biomek FX Laboratory Workstation. Complete fractionation was performed by setting the pipette tip at the liquid level and moving it down with the descent of liquid level, for each 50 µl fraction. Alternatively, six 100 µl fractions were taken from 24, 20, 16, 12, 8 and 4 mm from the bottom of the 31 mm long gradient. The sucrose gradient was fractionated at ~9.7 µl/s.

Quantitative RT-PCR

First strand cDNA synthesis was performed on equivalent volumes of extracted total RNA from each fraction, without prior quantification of the RNA. DNase I digestion was performed at 37°C for 20 min, the reaction was inactivated at 70°C for 5 min, and the samples were placed on ice. Reverse transcription was done using 25 ng/µl oligo-dT₁₅, 50 mM Tris–HCl (pH 8.3 at room temperature), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM dNTPs, 2 units/µl of RNase Inhibitor (Roche) and 10 units/µl SuperScript II RNase H-Reverse Transcriptase (Invitrogen Corporation), at 42°C for 50 min, and the reaction was inactivated at 70°C for 15 min. For quantitative PCR cDNA was diluted 10-fold and 6 µl was used for analysis of each gene. Quantitative PCR was carried out in an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Each 25 µl reaction contained 6 µl diluted cDNA, 1× HotStartTaq PCR Buffer (with 1.5 mM MgCl₂, Qiagen, Inc.), 1:25 000 dilution of Sybr Green I (Molecular Probes), 0.35 µM 6-ROX (Molecular Probes),

Table 1. Oligonucleotide primers used in this study

Gene	Accession no.	Forward primer	Reverse primer
GAPDH	NM_002046	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
NTF5	NM_006179	TCCCCTCCGCCAGTACTTC	CCACCTTCCTCAGCGTTATCA
MAPK14	NM_001315	CAGGAGAGGCCACGTTCTA	TCGGGCACCTCCAGATT
PIN4	NM_006223	AATGAAGTGGCCGCACAGTAT	TGGTCATCCAACCCAAGTCA
EEF1G	NM_001404	GAGTCTGGGTCTTCCGAGGC	GTGTATGACTCGTAGTCCACCTGC
ATP6V1E1	NM_001969	CAAACCTGTATGTTGCCTAGGTTTCA	CCAGTATCTCCTACAGAATTCCAGTTAA
GNB2	NM_00573	AGCAGCTCCGGAACCAGAT	GGTCAGTGTTGAGTCCCCACAT

Sequences are 5'–3'.

Figure 1. Polyribosome fractionation from HaCaT cells. Polysome profile of HaCaT cells. (a) HaCaT cell lysate was loaded on the top of centrifuge tubes, centrifuged at 26 000 r.p.m. at 4°C for 4 h. (b) HaCaT cell lysate was loaded on the top of sucrose gradient of 96-well deep-well plate, centrifuged at 6000 r.p.m. at 4°C for 40 h. (c) HaCaT cell lysate, into which EDTA was added, was loaded on the top of sucrose gradient of 96-well deep-well plate, centrifuged at 6000 r.p.m. at 4°C for 40 h. Gradient bottom is to the left.

0.2 mM dNTPs, 4 mM $MgCl_2$, 0.025 unit/ μ l HotStartTaq DNA polymerase (Qiagen, Inc.) and 0.8 μ M of each primer. Quantitative PCR was done using 95°C for 15 min, followed by 50 cycles at 95°C for 15 s, 60°C for 15 s and 72°C for 30 s, followed by a temperature profile of 95°C 15 s, 60°C 15 s, 95°C 15 s to calculate the dissociation curve. Oligonucleotide primers for seven genes were designed for quantitative PCR using Primer Express (Applied Biosystems) and are given in Table 1.

RESULTS

Polyribosome gradients prepared using the standard ultracentrifugation approach were compared with polyribosome gradients prepared in 96-well deep-well plates and spun at low speed. Figure 1 shows a UV absorption profile of polyribosomes isolated from growing HaCaT cells. Figure 1a shows a standard polyribosome fractionation using a large gradient (35 ml) format and ultracentrifugation. Figure 1b shows polyribosomes from the same source resolved using a 2 ml gradient, prepared in a 96-well deep-well plate, and spun at low speed (6000 r.p.m.) in a tabletop centrifuge at 4°C for 40 h. Gradients were prepared by layering sucrose using a

Figure 2. Puromycin evidence. Polyribosome structure is perturbed by puromycin in HaCaT cells. Addition of puromycin causes disruption of polyribosomes by premature chain termination, and a shift of material out of the polyribosome envelope, in both high-speed (a and b) and low-speed (c and d) centrifugation.

Figure 3. Differential translation of NTF5. Quantitative RT-PCR shows that, in HaCaT cells, NTF5 (red, triangles) is inefficiently translated. This effect can be detected using either (a) high-speed, or (b) low-speed centrifugation. Other genes, GAPD (black circles), MAPK14 (blue crosses) and GNB2 (green $\times$'s) are more efficiently translated.

Figure 4. Number of cells needed. HaCaT gene translation profile with titration of loading sample amounts. Cell lysates from 5×10^4 cells (black circles), 5×10^5 cells (red triangles), 5×10^6 cells (blue crosses) were loaded onto low-speed gradients, and qRT-PCR was used to assess the positions of (a) PIN4 and (b) EEF1G mRNAs. PIN4 is more efficiently translated than EEF1G.

Biomek FX robot and storing at 4°C for 6 h. In this manner, we can perform up to 192 parallel fractionations in about two days using a two-bucket rotor and a single tabletop (i.e. inexpensive) centrifuge, or 384 parallel fractionations using a four-bucket rotor. 384-well deep-well plates can also be used, in which case quantitative measurements by RT-PCR had greater variance, due to the limited amount of lysate loaded onto the gradient (not shown). The low volume, low-speed preparation does not have the resolution of the large volume, high-speed gradient, especially for lower molecular weight materials nearer the top of the gradient. Toward the bottom of the high-speed gradient, polyribosomes of increasing size are resolved sufficiently to see individual peaks, and this resolution is not obtained in the low-speed gradient. The short length of the low-speed gradients, diffusion over the longer running times, and acceleration that is not purely radial for all positions in the deep-well plate, are all expected to limit resolution. Nevertheless, the major polyribosome packet is well-resolved from uninitiated ribosomes. The results of one standard test, disruption of polyribosomes using EDTA, in the

low-speed gradient is shown in Figure 1c. In another standard test (Fig. 2), the disruption of polyribosomes by premature chain termination using puromycin, in which the shift of UV absorbing material toward the top of the gradient is consistent with the low-speed fractionation of polyribosomes. These tests indicate that the low-speed polyribosome gradients recapitulated the major features of polyribosome gradients prepared by ultracentrifugation.

The obvious advantage of the low-speed approach is that it permits many gradients to be prepared simultaneously, thereby providing one of the several methodological components of a high-throughput screen for translation effects. However, is the resolution obtained with the low-speed approach sufficient to detect differential polyribosome loading on individual mRNAs? To address this, we examined polyribosome gradient fractions using quantitative RT-PCR (across splice junctions) for several transcripts that have been implicated in other cell lines to be translated with different efficiencies. Figure 3 shows the results of these experiments. We compared the polyribosome distributions of transcripts for

Figure 5. Partial fractionation of low-speed sucrose gradients. Low-speed gradients of polyribosomes from HeLa cells that were treated with rapamycin (black circles), or untreated (gray triangles) and analyzed by quantitative RT-PCR. (a and b) The distribution of PIN4 in polyribosomes shows rapamycin repression of translation, with both (a) 21 fractions comprising the whole gradient, and with (b) six samples taken at equidistant depths. (c and d) Parallel quantitative RT-PCR for ATP6V1E1 showed no such rapamycin-dependent translation repression.

NTF5, GAPD, GNB2 and MAPK14 in HaCaT cells. Note that quantitative RT-PCR is performed directly on sucrose gradient fractions, without further purification. The low translation initiation efficiency of NTF5 is observed in both ultracentrifugation and low-speed centrifugation.

We further show that, with quantitative RT-PCR, the distribution of an mRNA on polyribosomes can be followed from as few as 50 000 cells (Fig. 4). The goal of this work was to scale the number of cells to a number that can be grown conveniently in single wells of 96-well plates. Note, here, the identification of EEF1G as an inefficiently translated mRNA, relative to PIN4, in HaCaT cells.

An important issue regarding the development of high-throughput strategies for polyribosome analysis is the number of assays that need to be done, once resolution of the polyribosomes on sucrose gradients has been accomplished. If several hundred sucrose gradients are prepared, does this imply tens of thousands of fractions? It is not always necessary that every fraction be analyzed (e.g. by RT-PCR), and, in fact, the entire gradient does not always have to be fractionated. A liquid handling robot can be used to sample several fractions of a gradient from different levels. This is done by immersing the pipette tips to progressively lower points in the gradient, and taking a sample. This is convenient and fast, and is possible because the gradient resists mixing. The gradients could also be fractionated into several complete fractions with

larger volumes, in which case, analysis would comprise an average over the entire fraction, which has certain advantages. However, the scheme to sample at different levels has practical advantages, including speed, and convenient volumes, such that conventional 96-well plates can be used, and each sample is taken robotically with a single pipetting step, 96 gradients at a time. In Figure 5, we show that differential translation can be identified by performing quantitative RT-PCR on six progressively deeper fractions taken robotically and simultaneously from multiple gradients. This figure shows the regulation of PIN in HeLa cells by rapamycin, compared with a transcript that is not regulated by rapamycin, ATP6V1E1. Fractions 4 and 5 are not 'adjacent fractions', but 100 μ l fractions taken from depths of the gradient separated by 0.4 cm. In these gradients 0.4 cm corresponds to 190 μ l of volume, such that fractions 4 and 5 were separated by 90 μ l. This demonstrates that differences in translation efficiency can be detected by this convenient partial fractionation method.

DISCUSSION

This approach for parallel polyribosome fractionation makes possible high-throughput screening for effects of biological variables on the translation of specific transcripts. While the production of a protein can be followed using immunological

methods, the production of a specific antibody for every gene of interest, and for those whose importance is yet to be firmly established, presents a significant practical barrier. With the polyribosome approach, high confidence of differential translation can be attained for many genes under many conditions with comparatively little up-front investment of time, effort and money. There are several prominent sources of error in sedimentation velocity centrifugation. Sucrose gradients are relatively stable, but mixing inevitably occurs, due to diffusion, convection and mechanical disturbance; forming step gradients entails variable mixing at the interfaces and forming continuous gradients that are nearly identical is accompanied by similar challenges. The parallel configuration of the method presented should reduce measurement variance due to these factors, and also permit comparison of many samples that have been prepared simultaneously. This latter point becomes important when it is difficult to synchronize treatments, or when efficacious sample storage cannot be arranged. Ultracentrifugation has been used to separate other materials besides polyribosomes, and the method presented should be generally applicable, given an appropriate range of sedimentation coefficients. Shorter centrifugation times, higher resolution, and the resolution of smaller molecular species could all be achieved through the development of appropriate equipment.

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