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Exploring translation regulation by global analysis of ribosomal association

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ABSTRACT

Translation efficiency of an mRNA is related in most cases to its ribosomal association. This association can be readily measured through the separation of cellular complexes on sucrose gradients by velocity sedimentation, and identification of the sedimentation position of the mRNA in the gradient. Since ribosomes are the main driving force for mRNA sedimentation, sedimentation position is highly correlated with ribosomal association and thus translation efficiency. The advent of DNA microarrays allowed the determination of ribosomal association for many mRNAs in parallel through the combination of fractionation in a sucrose gradient followed by microarray analysis. This provided an enormous amount of novel information regarding translation control and regulation. Herein we provide a detailed protocol for performing such an analysis, indicating important points for consideration and discussing some of the advantages and limitations of this powerful approach.

1. Introduction

Velocity sedimentation of cellular extracts in sucrose gradients is the most common approach for determining the translational status of an mRNA *in vivo*. Since its introduction [1], this methods have been used to study translation of almost any cell type and various conditions (e.g., [2–5]). The main principle underlying this method is that during a short period of ultracentrifugation, complexes migrate mainly according to their mass. Since the mass of ribosomes engaged in translation is by far greater than the mass of their bound mRNA, mRNAs will sediment in the gradient according to the number of ribosomes that are bound to them [6]. In general, the number of ribosomes is proportional to the rate of protein synthesis. Thus, migration distance in the gradient serves as a good proxy for the translation status of an mRNA. However, it is important to note that the sedimentation in the gradient may not always be due to ribosomes; large cellular complexes that withstand the procedure will sediment in the gradient with their associated mRNAs (e.g., [7]). A Ribosome Affinity Purification (RAP) approach [8] may avoid these cases. Moreover, the number of ribosomes bound to the mRNA does not always correlate with its translational status. Most notable are cases of translation regulation by miRNA, in which ribosome-associated mRNAs do not produce a functional protein (e.g., [9–11]).

Following the mRNA distribution of a particular gene along a fractionated gradient provides valuable data regarding the fraction of transcripts engaged in translation (ribosomal occupancy) and the number of ribosomes per sequence length (ribosomal density) [12]. These characteristics define the translation efficiency of an mRNA and may vary greatly between different genes under different growth conditions or upon certain mutations. Deviation of these parameters with respect to the trend of most genes or with respect to specific reference mRNA may suggest regulation at the translation level.

In the past few years, genome-wide analyses of mRNA translation by DNA microarrays uncovered the translation status of thousands of mRNAs and provided new insights into the regulation of translation both globally and for specific mRNAs (e.g., [13–21]). Though experimental designs for studying the global translational status of a cell share similar steps (such as separation of complexes on a sucrose gradient, fractionation of the gradient, RNA purification from the fractions, cDNA labeling and hybridization to a DNA microarray), they differ markedly with respect to their resolution (the number of fractions to be analyzed), hybridization strategies, and data analysis scheme. Here we focus on one experimental design, which is the most commonly used and appears to identify most significant changes. In this design, the gradient is divided to non-polysomal and polysomal fractions, which contain mRNAs that are translated at low or high efficiency, respectively. The RNA is extracted from these fractions and labeled by fluorescent dyes. The two labeled samples are hybridized either to the same microarray or to two different microarrays with a common reference RNA.

2. Description of the method

We provide a detailed protocol for gradient preparation, separation of complexes into two fractions (non-polysomal and polysomal), RNA extraction and hybridization to spotted-microarrays.

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The protocol presented here was optimized for the isolation of polysomal mRNA from the yeast *Saccharomyces cerevisiae*, yet this procedure can be adapted easily to other organisms.

2.1. Gradient preparation

For the separation of eukaryotic polysomal complexes, we use linear sucrose gradients (10–50%) of total volume of 11 ml, onto which 1 ml of cells' lysate is laid. Gradients can be prepared by a gradient maker or by manually layering different concentrations of sucrose and allowing them to equilibrate at 4 °C for 12–24 h. We routinely utilized the latter method due to its simplicity and consistent results.

For each gradient, we prepare 3 ml mixes of 10%, 20%, 30%, 40% and 50% sucrose solutions in a gradient buffer containing 20 mM Tris–HCl pH 7.4, 140 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.1 mg/ml Cycloheximide (C-7698, Sigma) and 0.5 mg/ml Heparin (H-3393, Sigma). With a long Pasteur pipette, 2.2 ml of each mix are layered one on the other in a Beckman polyallomer tube (14 × 89 mm, #331372). We usually layer first the 10% sucrose mix and then, carefully underlay 2.2 ml of the 20% sucrose solution by inserting the pipette tip into the bottom of the tube and slowly pipetting the 20% sucrose solution under the 10% solution. The 30%, 40% and 50% sucrose solutions are then layered in the same manner. An alternative can be to first layer the 50% solution, freeze it on dry ice, then layer the 40%, etc. The gradients are stored overnight at 4 °C to establish a linear gradient. Alternately, the gradient can be stored at −80 °C for long periods and thawed 12–24 h at 4 °C before use.

2.2. Cell lysis

The protocol presented is designed for a 50–100 ml of liquid yeast culture grown to logarithmic phase (OD600 ranging from 0.4 to 0.8.) in rich media. Satisfactory signals on the microarray require at least 15 μg RNA. The amount of cells should therefore be adjusted with respect to the number of fractions to be analyzed, or to the specific growth condition used. It is important to perform all steps on ice or at 4 °C in order to minimize RNA degradation and polysomal dissociation.

Cycloheximide is added to a final concentration of 0.1 mg/ml and cells are spun immediately at 3500 g for 4 min. Cycloheximide inhibits ribosomal translocation yet does not lead to the disassembly of ribosomes from the mRNA. As an outcome, ribosomes remain associated with the mRNA throughout the procedure. Since the effect of cycloheximide on ribosome translocation is instantaneous, it is assumed that the ribosomal association obtained truly represents the intracellular association. The immediate centrifugation and removal of media prevents additional rounds of initiation, which may lead to accumulation of mRNAs having a single ribosome that were otherwise free of ribosomes. The cells' pellet is washed twice in 4 ml lysis buffer (20 mM Tris–HCl pH 7.4, 140 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.1 mg/ml Cycloheximide, 1 mg/ml Heparin, 1% Triton X-100) and then dissolved in 400 μl lysis buffer. To achieve efficient cell lysis, we add glass beads (0.45–0.55 mm) and vortex vigorously in a bead beater by two pulses of 90 s at maximum level. Standard vortex may also be used (5 pulses of 1 min with 1 min intervals in ice), but the RNA yields are usually lower.

The lysate is recovered from the beads, cell debris and large organelles are removed by centrifugation at 8000*g* for 5 min and the sample is carefully loaded on the sucrose gradient. The centrifuge tube should be filled almost to the top to minimize the chances of the tube collapsing during ultracentrifugation. If necessary, add lysis buffer to fill the tube. Polysomal complexes are resolved by centrifugation at 35,000 rpm for 160 min at 4 °C in a SW41 rotor. These settings are optimal for separation between polysomal and non-polysomal complexes (Fig. 1). The most effective forces on polysomes resolution are centrifugation time and the applied *g*. Density is a minor factor because different polysomal complexes are of similar density (they are all multiples of the same basic unit – the ribosome). Longer centrifugation times can be used to resolve small ribosomal complexes, but shorter times appear ineffective in improving the resolution of large polysomes. After

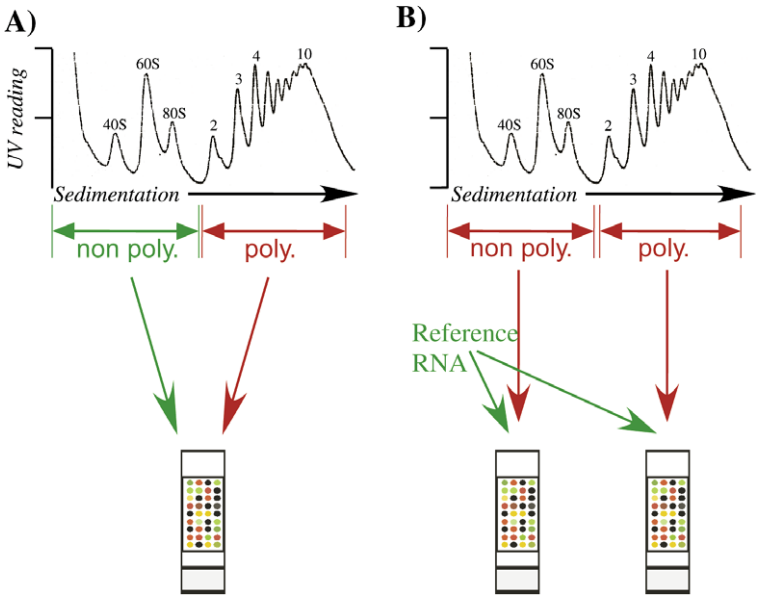

Fig. 1. Microarray analysis of fractionated RNA. (A) Direct comparison, in which the polysomal fraction is labeled with Cy5 (depicted in red) and the non-polysomal fraction is labeled with Cy3 (green). Both samples are hybridized to the same microarray. (B) Indirect comparison, in which the non-polysomal and polysomal fractions are labeled with Cy5, and each is hybridized to a different microarray together with Cy3 labeled reference RNA. Two microarrays are needed for this analysis, and therefore variations between microarrays may introduce an error. On the other hand, the reference RNA is expected to yield signal to all genes, and therefore genes with missing Cy5 signals may be interpreted as lacking transcripts in this fraction. The upper panel in each section is a representative UV-reading along a sucrose gradient that was treated as described in Section 2. Note the clear partition between the non-polysomal and polysomal fractions that is achieved by these settings.

centrifugation, gradients can be kept at 4 °C for 24 h with no apparent change in quality.

2.3. Fractions collection

Following centrifugation, the gradient is divided into two fractions that are collected based on the ribosomal complexes they contain. The use of a continuous ultraviolet (UV) detector, such as ISCO UA6, is highly recommended as it allows accurate determination of the sedimentation position of each complex (Fig. 1). Using this approach, the mRNA composition within each complex is comparable to equivalent fractions from other gradients, even if variations in gradient volumes or complexes sedimentation exist. The fractions (~6 ml each) are collected into Oak-ridge tubes and one volume of 8 M guanidinium–HCl, two volumes of 100% ethanol and spike-in controls (see below) are added to each fraction. It is important to mix the samples thoroughly before putting for an overnight incubation at −20 °C.

2.4. Adding spike-in controls to the fractions

Spike-in controls are *in vitro* transcribed RNAs that are added at known (and usually equal) amounts to each fraction at the time of collection. They are necessary for correcting variations between the collected fractions in all subsequent steps. These controls should have minimal cross-reactivity with the tested genome and should be labeled efficiently. The exact amount to be added should be such that their signals will be neither too low nor too high relative to the rest of the mRNA. Therefore it is recommended to use more than one type of RNA and to spike the different RNAs at different amounts in order to ensure a better coverage of sequences and expression levels.

We routinely use a mix of five mRNAs derived from lys (ATCC no. 87482), trp (ATCC no. 87485), dap (ATCC no. 87486), thr (ATCC no. 87484) and phe (ATCC no. 87483) clones from the bacterium *Bacillus subtilis*. These clones were inserted into an expression vector upstream to a stretch of adenines, therefore the transcribed RNA can be labeled with the oligo dT primer used during the RT reaction. The RNAs are generated by *in vitro* transcription reaction using a T3 *in vitro* transcription kit (e.g., MEGAscript from Ambion) and a spike-in mix stock solution (lys 80 pg/μl, trp 160 pg/μl, dap 200 pg/μl, thr 240 pg/μl and phe 320 pg/μl) is prepared. We typically add 70 μl of the spike-in mix into an entire sucrose gradient, whereby each fraction receives its relative share from that amount (i.e. 35 μl of the spike-in mix is added to each of the two fractions).

Probes corresponding to these clones should be present at multiple sites on the DNA microarray. We routinely put at least 20 spots for each of the five clones throughout the microarray.

2.5. RNA extraction from the collected fractions

The RNA from the collected fractions (Section 2.3) is precipitate by centrifugation at >10,000 rpm for 30 min in SS-34 rotor, washed with 5 ml ice-cold 75% ethanol and dissolved in 650 μl RNase-free water. It is important to ensure that all fractions are dissolved well, in particular the non-polysomal fraction which contains high amounts of Heparin (remnants of the lysis buffer). Proteins and DNA are extracted from the sample by addition of an equal volume of water-saturated phenol:chloroform (5:1), pH 4.7 (P1944, Sigma). The mixture is vortexed vigorously and spun at top speed for 5 min at room temperature. We usually take only 500 μl of the aqueous phase into a new microfuge tube, thereby avoiding contaminating phenol (which will inhibit the subsequent reverse transcriptase reaction) and DNA (which concentrates in the interphase).

To remove any residual Heparin, which may interfere with the labeling reaction, LiCl is added to a final concentration of 1.5 M. The sample is incubated overnight at −20 °C, thawed on ice and centrifuged at top speed for 20 min at 4 °C. The pellet is carefully washed with 200 μl of 75% ethanol and resuspended in 150 μl RNase-free water. Alternatively, some vendors provide RNA purification columns that remove Heparin; we use an RNA clean-up kit from Zymo Research (R1017).

Finally, the RNA is precipitated with 0.1 volume 3 M sodium acetate pH 5.2 and 2 volumes of 100% ethanol, washed with 75% ethanol and air dried. The RNA pellet is dissolved in 25 μl RNase-free water and stored at −80 °C.

2.6. Fluorescent labeling

Microarray analyses are usually performed as a comparison between two samples on the same microarray; one sample is labeled with red fluorophore (Cy5) and the other with green fluorophore (Cy3). Polysomal and non-polysomal fractions may be compared directly, whereby one is labeled with Cy5 and the other with Cy3, and both are hybridized to the same microarray (Fig. 1A). Alternatively, both fractions may be labeled with Cy5 and hybridized with Cy3-labeled unrelated reference RNA to two microarrays (Fig. 1B). Since the reference sample is the same for both hybridizations, it serves as a common denominator and a comparison between the Cy5-labeled samples can be made. The optimal reference is one that yields a strong and reliable signal for all genes so it is possible to assign a red-to-green ratio to each. We routinely use the "hot phenol" method to prepare a high quality reference RNA from yeast culture [22].

RNA labeling starts with a reverse transcription (RT) step during which amino–allyl linked nucleotides are incorporated into the cDNA. In a subsequent coupling step, a fluorescent dye (either Cy3 or Cy5) is coupled to the amino–allyl moiety. We favor this over protocols that utilize a fluorescently tagged nucleotide in the RT step since it is less expensive, it is more consistent with different dyes, and it should yield longer cDNAs due to low structural hindrance by the allyl moiety.

For one reaction, 15–50 μg of RNA is annealed to 5 μg Oligo dT (T20VN [V = any nucleotide except T]) in 15.5 μl nuclease-free water. The samples are incubated for 10 min at 70 °C and transferred to ice for 10 min. For the reference sample, the amounts should be multiplied according to the number of samples to be tested. The exact amount of RNA taken per sample is not critical since the spike-in RNA will correct for any variations between fractions.

cDNA synthesis is based on the Promega ImProm-II reverse transcription system, in which the annealed RNA is mixed with 6.0 μl 5× reaction buffer, 4.0 μl 25 mM $MgCl_2$, 1.2 μl 25× amino–allyl mix (12.5 mM of each dATP, dGTP and dCTP, 5 mM dTTP and 7.5 mM amino–allyl dUTP (Ambion #8439)), 3.0 μl reverse transcriptase and 0.3 μl nuclease-free water (total volume of the reaction is 30 μl). The reaction is performed at 42 °C for 2 h. The RNA is then degraded by adding 10 μl of 1 N NaOH and 10 μl of 0.5 M EDTA, and incubation at 65 °C for 15 min. To neutralize the pH, 25 μl of 1 M HEPES (pH 7.0) are added and the volume is increased to 100 μl by adding 25 μl of nuclease-free water. The cDNA is precipitated with sodium acetate (pH 5.2) and ethanol, and resuspend in 9 μl of nuclease-free water. Alternatively, one can use DNA clean and concentrator columns (e.g., D4004, Zymo Research). The samples can be stored at −80 °C for at least a month.

Coupling of the Cy dyes to the allyl moiety starts with addition of 1 μl of sodium bicarbonate 1 M (pH 9.0), and then the Cy3 or Cy5 dyes (Amersham Cat. #336219 or 335176, respectively). The dyes are usually dissolved in 12 μl DMSO and 1 μl is added to each sample. Incubation at room temperature for 1 h allows coupling of the

dye to the amino–allyl groups. It is important to minimize the exposure of the dyes or labeled samples to light. Unused dyes should be dried out completely and stored at 4 °C in a desiccator for later use.

Cy-labeled cDNA is purified from uncoupled dyes using a DNA clean and concentrator kit (D4004, Zymo Research), according to the kit manual. The final elution volume of the cDNA is 5 μl.

2.7. Microarray hybridization

Initially we used DNA microarrays that were spotted with PCR products made from the entire coding region of a gene. More recently, we have used DNA microarrays containing long oligonucleotides (70 mer) that are specific to each gene (Operon AROS for yeast). These oligos were designed to have similar hybridization characteristics with minimal cross hybridization. Both microarray platforms are prepared similarly for hybridization, except for UV-cross linking and DNA denaturation steps for the PCR-based microarray. The slides are stored routinely in the dark under desiccation and are handled with powder-free gloves. Before use, mark the array boundaries with a diamond pen on the back of the slide since the spotted DNA will not be visible during processing.

2.7.1. Hybridization to microarrays spotted with PCR products

PCR products are spotted on glass slides coated with amino-silane (Corning GAPS II). Immediately before hybridization, the slides should be subjected to UV cross-linking at 300 mJ and washes in 0.1% SDS solution for 30 s, distilled water heated to 95 °C for 3 min and 70% ethanol for 2 min. Thermo Shandon slides' racks #113 are convenient for these washing steps. The slides are spin-dried by centrifugation at 500 rpm for 5 min at room temperature.

Active sites on the slide are blocked by incubating for 1 h at 42 °C in preheated and filtered prehybridization buffer (1% BSA (A7906 Sigma), 5× SSC, and 1% SDS). The slides are then washed once in distilled water for few seconds and spin-dried. The slides are ready for hybridization and placed in a hybridization chamber.

Hybridization solution, which includes 5 μl of each of the Cy-labeled samples, 20 μl hybridization buffer (4.5× SSC, 75% formamide, and 0.15% SDS), 3 μl (30 μg) poly(A)-RNA (Sigma P-9403) and 3 μl of 10% BSA is prepared. The BSA is added last, after the rest of the mixture was denatured by incubation at 95 °C for 3 min. The hybridization solution is spun-down and 25 μl of it are put on the microarray. The slide is covered carefully with a dust-free 22 mm × 40 mm microscope glass coverslip. Large air bubbles can be moved away from the hybridization area by a gentle tapping on the coverslip with a yellow tip. Small air bubbles will be released during hybridization. Sterile water is added to the wells in both sides of the hybridization chamber to maintain a moist environment. The hybridization chamber is then closed and incubated for overnight in a water bath that has been preheated to 42 °C.

Following hybridization, unbound label is removed by three washes of increasing stringency: Wash buffer 1 (2× SSC and 0.05% SDS), Wash buffer 2 (1× SSC) and Wash buffer 3 (0.1× SSC). All washes are for 5 min at room temperature with agitation. The slides are spin-dried (500 rpm for 5 min at room temperature) and stored in a dark box until scanning.

2.7.2. Hybridization to oligo microarray

Glass slides that are spotted with oligonucleotides are coated with epoxy and subjected to prehybridization at 42 °C for 1 h with preheated and filtered prehybridization buffer (0.5% BSA (A7906 Sigma), 5× SSC, and 1% SDS). The slide is then washed three times with 0.1× SSC for 5 min each to remove the prehybridization buffer. Finally, the slide is rinsed in distilled water for 30 s and spin-dry by centrifugation at 500 rpm for 5 min at room temperature.

Hybridization is performed in 25 μl volume, which includes 5 μl of each labeled cDNAs, 12.5 μl 2× MWG buffer (Ocimum Biosolutions), 1 μl 10 μg/μl PolyA and 1.5 μl nuclease-free water, and was pre-heated at 95 °C for 3 min. The hybridization buffer is covered with a coverslip and incubation is at 42 °C for an overnight.

Following hybridization the slides are washed with: Wash 1 (2× SSC and 0. 2% SDS) Wash 2 (2× SSC) and Wash 3 (0.2× SSC), each wash for 12 min.

3. Data analysis

The washed DNA microarrays are scanned using one of the various microarray scanners (e.g., GenePix of Molecular Devices or Agilent microarray scanner), and the resulting red-to-green ratios are used to determine the relative mRNA translation. The spike-in controls allow normalization and the retrieval of values that represent the percent of mRNA in each fraction for every gene. It is important to note that obtaining these values requires a signal for every gene in both samples. This requirement is not always met, either due to technical reasons (the extensive purification protocol leads to losses of many mRNAs) or biological reasons (some mRNAs sediment in only one of the fractions). Overall, the number of genes that can be analyzed is usually lower than a standard transcriptome analysis.

The values obtained (e.g., ratio of polysomal to non-polysomal or percent in polysomes) are used to compare changes in translation efficiency between two cell populations. The comparison may be made using various statistical tools; the simplest way is to divide the values obtained by one condition with the values by another condition, and thus to get the fold-change for a gene. To select the most affected genes, a cut-off can be defined, which is arbitrary in most cases (e.g., changes by more than two standard deviations from the mean for all genes) or which could be relative to a known positive control. A more convenient way to identify affected genes is by a scatter plot representation, in which the values from one sample are plotted against the other (Fig. 2). The best-fit linear regression line and the standard deviations (SD) can be determined easily for such a plot. From the general trend line one can derive conclusions about the global translational response.

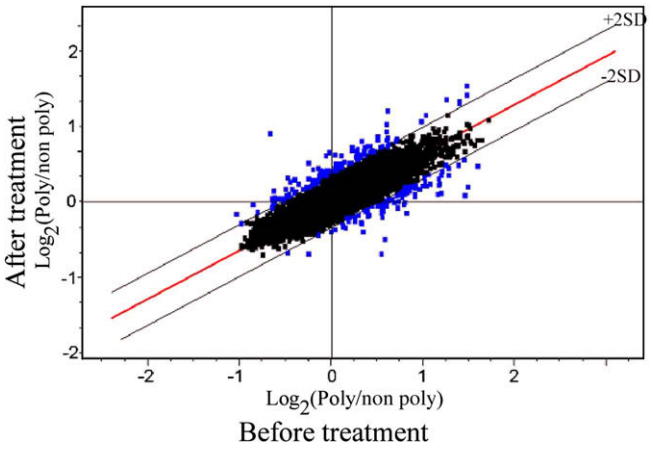

Fig. 2. Scatter plot representation of translational changes. In this scatter plot, each spot represents a gene, and its position in the graph is assigned according to the Log_2 value of its Poly/non Poly ratio before (*x*-axis) or after a treatment (*y*-axis). The best-fit trend line is presented in red, and the two dashed lines represent two standard deviations (2 SD) from it. Genes that deviate from the general trend by more then 2 SD are shown in blue and these are potential candidates for translation regulation.

In addition, genes that deviate by more than 2 SD (or any other selection cut-off) can be defined as having a translation status that is relatively different than most genes. The scatter plot representation also allows an easy separation of genes to groups based on the quadrate they are in; each quadrate represents a different type of effect and therefore has a biological relevance.

Dividing the signals in the polysomal fraction with those of the non-polysomal fraction is assumed to neutralize changes in transcript abundance, as these affect both fractions. Yet, this may not always be the case since changes in mRNA transcription or degradation may affect only one of the fractions, probably the non-polysomal one. For example, an increase in mRNA degradation rate, which occurs mainly at the untranslated mRNA pool, will increase the polysomal/non-polysomal ratio, even though there was not an actual increase in polysomal association. It is therefore highly recommended to supplement the translation analysis with a trancriptome analysis in which an unfractionated RNA is analyzed. Examining changes in translation with respect to changes in transcriptome may also add biological insights regarding the correlation between these two processes. For example, it was recently shown that in response to some environmental changes some genes increase their translational efficiency in addition to transcription elevation [15]. Such a potentiation effect appears to be advantageous when large amounts of a particular protein must be synthesized ("positive potentiation") or reduced ("negative potentiation") within a short period of time [23].

Analyses based on comparisons of *ratios* between two conditions (e.g., a change in the ratio between polysomal and non-polysomal fractions) may introduce an unavoidable bias whereby a small change in polysomal association will seem large for genes having an extreme mRNA distribution. For example, a shift of 1% from polysomes to non-polysomes for a gene having a high polysomal association (e.g., 99%) will seem more significant than a similar change for a gene having a lower association (e.g., 50%). [Calculation for 99% association: ratio before the 1% shift is 99/1 and the ratio after is 98/2, thus overall change is 2.02. For 50% association: ratio before the 1% shift is 50/50 and after is 49/51, therefore the ratio of change is 1.04, which may seem lower than 2.02.] Elaborate discussion on these effects is described elsewhere [22]. Although very high polysomal associations are biologically rare, one may wish to imply stringent selection criteria for such cases. Alternatively, other filtration criteria, such as changes only in the polysomal percentage, may be used.

Having identified some candidate mRNAs from the microarray analysis, it is important to verify them using other experimental approaches. Northern analysis and RT-PCR are the most common methods used for this purpose. RNAs from the spike-in mix can also be used for normalizing in these methods. In addition, it is advised to test the distribution of genes that cover all patterns of change (i.e., increase, decrease or no change in polysomal association).

Change in polysomal association of a particular mRNA may affect the synthesis rate of its cognate protein. Therefore, measuring changes in protein levels may support conclusions regarding an increase or decrease in translation rates. Various methods can be used to reveal protein levels; either gene-specific methods that utilize antibodies or quantitative global methods that utilize mass spectroscopy (e.g., SILAC or iTRAQ). It is very intriguing, however, that changes in mRNA polysomal association do not always correlate with changes in protein levels. Revealing the mechanisms that underlie this discrepancy is a major challenge for a complete understanding of translation control.

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