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Studying translational control in non-model stressed organisms by polysomal profiling

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ABSTRACT

In stressed organisms, strategic proteins are selectively translated even if the global process of protein synthesis is compromised. The determination of protein concentrations in tissues of non-model organisms (thus with limited genomic information) is challenging due to the absence of specific antibodies. Moreover, estimating protein levels quantifying transcriptional responses may be misleading, because translational control mechanisms uncouple protein and mRNAs abundances. Translational control is increasingly recognized as a hub where regulation of gene expression converges to shape proteomes, but it is almost completely overlooked in molecular ecology studies. An interesting approach to study translation and its control mechanisms is the analysis of variations of gene-specific translational efficiencies by quantifying mRNAs associated to ribosomes. In this paper, we propose a robust and streamlined pipeline for purifying ribosome-associated mRNAs and calculating global and gene-specific translation efficiencies from non-model insect's species. This method might found applications in molecular ecology to study responses to environmental stressors in non-model organisms.

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1. Introduction

Over the past few decades, the integration of molecular and ecological approaches for unraveling both the genetic basis and the ecological importance of ecosystems has encountered increasing interest (Clark and Worland, 2008; Pauls et al., 2014). A pressing challenge for the ecological and evolutionary community is to understand the molecular basis of adaptation in ecosystems and predict how organisms reply to stresses such as climate change, habitat loss or fragmentation, exposure to chemicals or pesticides (Pauls et al., 2014). In particular, gene expression studies are of utmost importance to obtain a comprehensive view of biological processes in cells or tissues and are of particular interest in developmental biology, molecular physiology and ecotoxicology (Aardema and MacGregor, 2002). The measure of transcript variations has been widely used as truthful representation of changes in protein abundance in cells and tissues, but post-transcriptional and translational events are now recognized as fundamental players in shaping the cellular proteome (Vogel et al., 2010). In fact, the relevance of translational control in physiological and pathological conditions and the poor average correlation between proteins and transcripts (Vogel et al., 2010; Schwanhäusser et al., 2011; Scott et al., 2013), support the need of reliable methods for determining translational efficiencies of individual mRNAs (Powley et al., 2009; Ingolia et al., 2009). Translational control of gene expression is almost completely neglected in the study of natural populations subjected to environmental changes (Pauls et al., 2014). The majority of studies about molecular ecology of non-model organisms is exclusively focused on the transcriptional level of gene expression, given the lack of reliable techniques to describe the protein level. In fact, Western Blot analyses often bring to unclear if not unreliable results because specific antibodies are lacking.

Proteomics is barely applicable in non-model organisms due to the high amount of required starting material and the need of specific transcriptome or genome data for peptide annotation. The purification and identification of ribosome-associated mRNAs (translatome profiling (King and Gerber, 2014) i.e., polysome profiling (Grolleau et al., 2002), ribosome profiling (Ingolia et al.,

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2009) or translating ribosomes immunoprecipitation (Doyle et al., 2008), represents an interesting alternative approach to study protein changes that remain undetected in total mRNA profiles and unravel translational control of gene expression. This fact fosters a blooming explosion of a number of optimizations of the classical sucrose gradient separation of polysomes from several organisms (Esposito et al., 2010; Gandin et al., 2014; Faye et al., 2014; Coudert et al., 2014; Thomas et al., 2012). In fact, this approach is still the most robust choice for studying mRNAs engaged in translation. Though these protocols are quite similar, it appears that each organism or tissue requires a dedicated optimization and validation.

Recently, ribosomal genetic labeling has been proposed as a useful approach for studying translation using ribosome immunoprecipitation for isolation of cell-specific mRNAs in active translation from tissues (Heiman et al., 2014; Doyle et al., 2008; Thomas et al., 2012). This technique requires transgenic animal models, making its use very difficult for natural populations. The more classical technique, polysomal purification by means of sucrose gradient fractionation from biological samples (Scornik, 1973), allows coupling polysomal mRNA purification to RT-PCR or high-throughput assays (Arava, 2003) and can be used as an alternative method when classical biochemical techniques that assess individual protein levels cannot be employed.

Purification of polysomes from mammalian tissues has been performed using classical sucrose gradient fractionation (Del Prete et al., 2007; Lou et al., 2014) but both tissue preparation and RNA extraction are time consuming and unfriendly procedures. Sucrose gradient has been used in insects for obtaining polysomal profiles from the gut of the model invertebrate *Anopheles gambiae* (Mead et al., 2012), with unclear results. So far, polysome profiling has never been applied to non-model organisms and no dedicated protocol for polysome purification is available yet.

Here we propose a pipeline for translational studies formed by a method for polysome purification dedicated to non-model organisms and the calculation of two translational parameters (global translational efficiency and gene-specific translational efficiency). We set up the purification assay by using a simplified and optimized version of the classical assay (Scornik, 1973), taking advantage of additional adjustments used for solid mammalian tissues (Del Prete et al., 2007) and mammalian cells (Tebaldi et al., 2012). As experimental organism we employed the dipteran *Pseudodiamesa branickii* (Nowicki) (Chironomidae). *P. branickii* is a good bioindicator of climate change, as cold stenothermal species colonizing aquatic habitats (e.g., high mountain springs and streams) threatened by global warming (Lencioni and Rossaro, 2005). First, we demonstrated the presence of *P. branickii* polysomes in sucrose gradient fractions by classical molecular biology assays and Atomic Force Microscopy (AFM). We validated the presence of polysome-bound mRNAs of housekeeping and target genes by qPCR. Then, we purified polysomes from whole larvae shortly (1 h) exposed to higher temperatures (26 °C and 32 °C) than those normally experienced by this organism in nature (4 °C, Lencioni et al., 2008). In fact, it is well established that under heat stress conditions *P. branickii* develops a Heat Shock Response at the transcriptional level (Bernabò et al., 2011). Finally, taking advantage of the optimized purification, we measured the global and gene-specific translation efficiencies of Heat Shock Protein 70 (Hsp70) transcript, obtaining interesting results.

In summary, we improved the extraction of ribosome-associated mRNAs (polysomal profiling) from solid tissues and demonstrated that this method is effective for obtaining information on translational control of candidate genes in non-model organisms. We suggest that this streamlined pipeline can be conveniently applied to any non-model invertebrate specie to study variations of mRNAs recruitment onto the translation machinery.

2. Material and methods

The larvae of *P. branickii* were heat stressed (26 and 32 °C, 1 h) in the laboratory within 24 h from sampling. Alive specimens were flash frozen under liquid nitrogen and immediately stored at −80 °C. Approximately 5 larvae (10–20 mg altogether) were pulverized in a mortar with a pestle under liquid nitrogen. The obtained powder was lysed in 0.8 mL of lysis buffer (10 mM Tris–HCl at pH 7.5, 10 mM NaCl, 10 mM MgCl2, 1% Triton-X100, 1% Na-deoxycholate, 0.4 U/mL SUPERase-In RNase Inhibitor (Life Technologies), 1 mM DTT, 0.2 mg/mL cycloheximide, 5 U/mL Dnase I (Fermentas)), and cells were disrupted by vigorous pipetting. Tissue debris were removed from the lysate by centrifugation at 13.200 rpm for 1 min at 4 °C. Nuclei were removed from the precleared lysate by a second centrifugation at 13.200 rpm for 5 min at 4 °C. Supernatant was kept at 4 °C for 30 min to remove any genomic DNA contaminations. The polysome-containing lysate was stored at −80 °C or directly layered onto a linear sucrose gradient (15–50% sucrose [w/v], in 30 mM Tris–HCl at pH 7.5, 100 mM NaCl, 10 mM MgCl2) and centrifuged in a SW41Ti rotor (Beckman) for 100 min at 180,000 g at 4 °C. Ultracentrifugation allows the polysome separation according to the sedimentation coefficient of macromolecules. After ultracentrifugation, gradients can be fractionated and mRNAs in active translation, corresponding to polysome containing fractions, separated from translationally silent mRNAs. Fractions of 1 mL volume were collected with continuous monitoring absorbance at 254 nm using an ISCO UA-6 UV detector.

2.1. Protein extraction from sucrose gradient fractions and Western blot

Proteins were purified from sucrose gradient fractions using trichloroacetic acid/acetone precipitation according to Latorre et al. (2012). The proteins were resuspended in SDS protein gel loading solution, boiled for 5 min and separated on a 10% SDS–polyacrylamide gel. The high evolutionary conservation of most ribosomal proteins allowed us the use of a rabbit primary antibody against the human protein RPL24, which is part of the 60S subunit (Abcam, dilution 1:500). A goat anti-rabbit HRP conjugated was used as secondary antibody (Santa Cruz Biotechnology, dilution 1:1000). As control a mouse polyclonal antibody for human GAPDH (Santa Cruz Biotechnology, dilution 1:1000) was used. A goat anti-mouse HRP conjugated was used as secondary antibody (Santa Cruz Biotechnology, dilution 1:1000). Blotting was performed on nitrocellulose membrane and the signal was acquired by chemiluminescence using Chemidoc System (Biorad).

2.2. Atomic force microscopy

Few microliters (typically 20–40 μl) of sucrose fraction corresponding to polysomes and 80S were absorbed on freshly cleaved mica, previously washed 3–4 times with DEPC-water and treated with 1 mM Ni^{2+} in order to allow the interaction of the sample with the substrate. After 3 min of incubation, 200 μl of cold AFM buffer (10 mM Hepes, 10 mM MgCl2, 100 mM NaCl, 100 μg/ml cycloheximide, pH 7.4) were added on the sample and kept for 1 h at 4 °C. Finally, the solution was gently removed with DEPC-water in the presence of 100 μg/ml of cycloheximide and the sample was dried at room temperature. Images were acquired in air using a Cypher AFM (Asylum Research – Oxford Instruments, Santa Barbara, CA, USA) in AC mode using AC240TS (Olympus) tips with a nominal force constant of 2 N/m. Typical lateral resolution was estimated to be around 5–10 nm. Image acquisition was performed using an excitation frequency of about 70 kHz, with a scan rate of 1–2 Hz. Images were processed with Gwyddion (gwyddion.net).

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