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Affinity purification of ribosomes to access the translatome

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ABSTRACT

We describe ribosome affinity purification (RAP), a method that allows rapid purification of ribosomes and associated messages from the yeast *Saccharomyces cerevisiae*. The method relies on the expression of protein A tagged versions of the ribosomal protein Rpl16, which is used to efficiently recover endogenously formed ribosomes and polysomes from cellular extracts with IgG-coupled spherical microbeads. This approach can be applied to profile reactions of the translatome, which refers to all messages associated with ribosomes, with those of the transcriptome using DNA microarrays. In addition, ribosomal proteins, their modifications, and/or other associated proteins can be mapped with mass spectrometry. Finally, application of this method in other organisms provides a valuable tool to decipher cell-type specific gene expression patterns.

1. Introduction

The control of protein synthesis plays pivotal roles in diverse physiological processes such as development, neurogenesis, memory formation, and aging [1–3]. Misregulation of translation can cause problems for the cell to maintain proper cell function or to adapt to changing environmental conditions, and thus, can lead to a variety of human diseases [4,5]. Therefore, besides studying the mechanism of translation, deciphering the messages that undergo translational control is crucial to get insight into essential features of the gene expression regulatory program [6,7].

A reliable measure for translation of cellular mRNA is the degree of its association with ribosomes. Since the rate of initiation usually limits translation, most translational responses will alter the ribosome density on a given mRNA [8]. Actively translated mRNAs are typically bound by several ribosomes (polysomes) and can be separated from the small (40S) and the large (60S) ribosomal subunits and the 80S monosomes by sucrose gradient centrifugation (Fig. 1). In classical experiments, total RNA is isolated from different fractions of the polysomal gradient and assayed for the mRNA of interest by Northern blot analysis. Several laboratories have further extended this technique by using DNA microarray technology to perform genome-wide analysis of mRNAs in polysomes in yeast, *Drosophila* and mammals [6,7,9] (see also the report by Melamed et al. in this issue of *Methods*). Although sucrose density fractionation is recognized as the "gold" standard to monitor active translation, there are also some drawbacks: it requires special equipment (e.g. ultracentrifuge, gradient fractionation system) that may not be available in every laboratory; the procedure is time consuming and does not allow handling of many samples in parallel; and the samples are diluted in sucrose solution containing heparin, making more elaborate precipitation steps necessary to isolate RNA of sufficient quality to perform downstream experiments such as microarray analysis or reverse-transcription polymerase chain reactions (RT-PCR). Moreover, polysomal fractions may be contaminated by other high molecular weight complexes that are not an integral part of ribosomes such as lipid rafts, P-body components or pseudo-polysomes [10,11]. In this report, we therefore present a detailed protocol for an alternative approach, termed ribosome affinity purification (RAP), which allows rapid access to the cell's translatome without need for sucrose density fractionation. Besides the study of translational regulation upon changing environmental conditions, this method can also be applied to study cell-specific gene expression in complex tissues of model organisms [12–14].

2. Description of the ribosome affinity purification (RAP) method

RAP is based on experiences with epitope-tagged RNA-binding proteins for the purification of ribonucleoprotein complexes (RNPs) to systematically analyze RNA targets with DNA microarrays (Fig. 1) [15–20]. In RAP, a tagged ribosomal protein (RP) of the large ribosomal subunit is used to capture fully assembled ribosomes on a matrix [12,21]. To increase the specificity of the reaction, we used an affinity-tag that can be cleaved-off from the ribosomal protein with a site-specific protease from the tobacco-etch virus (TEV). The eluate can then, on the one hand, be analyzed by mass spectrometry to identify ribosomal proteins, post-transla-

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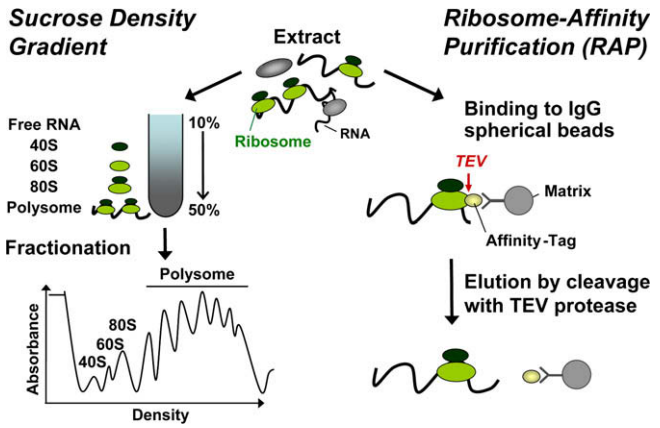

Fig. 1. Experimental schemes to study the translatome. (Left) "Classical" sucrose density fractionation. Cell-extracts are prepared in the presence of cycloheximide (a potent inhibitor of translational elongation) and separated by ultracentrifugation through a linear 10–50% sucrose density gradient. The gradient is then fractionated while continuously monitoring the absorbance at 254 nm allowing the separation of "free" RNA, the small (40S) and large (60S) ribosomal subunits, monosomes (80S), and polysomes. RNA is isolated from individual gradient fractions and pooled for subsequent microarray analysis. The relative position of a message in this profile is an indicator for its translational activity. (Right) RAP procedure. Affinity-tagged (e.g. Protein A) ribosomes are captured from extracts with IgG-coupled spherical microspheres (matrix) and released from the matrix with a site-specific protease from tobacco-etch virus (TEV). To profile the translatome, RNAs associated with the ribosomes are analyzed with DNA microarrays.

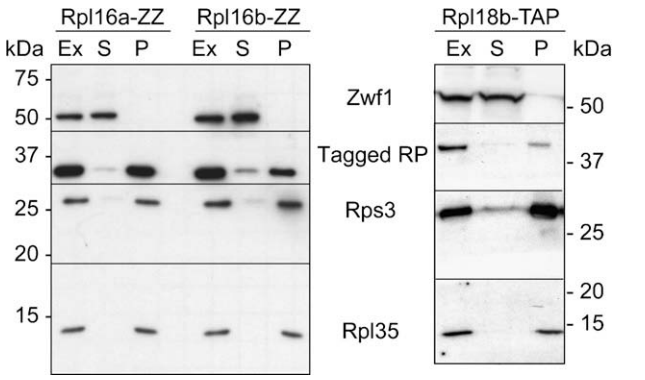

Fig. 2. Immunoblot analysis of sucrose cushions to evaluate incorporation of tagged RPs into ribosomes. Fractions of the extract (Ex), the supernatant (S) and the pellet containing ribosomes (P) were analyzed with specific antibodies detecting a cytoplasmic protein not expected to be present in the ribosome pellet (rabbit anti-Zwf1, Sigma; 1:5000), the tagged RP (peroxidase anti-peroxidase soluble complex [PAP], Sigma; 1:5000), and a protein of the small (rabbit anti-Rps3; 1:100,000) and of the large ribosomal subunit (rabbit anti-Rpl35; 1:20,000) [29]. The tagged RP under investigation is indicated on the top. The specific protein for analysis is indicated between the panels, molecular size markers are marked in kilodaltons (kDa) on the side.

tional modifications, and additional ribosome-associated proteins (see also [11]). On the other hand, RNAs associated with ribosomes, which we further refer to as the translatome, can be isolated from the purified ribosomes and quantitatively analyzed by quantitative reverse-transcription PCR (RT-qPCR), DNA microarrays, or may be sequenced. In this respect, we have successfully performed such an analysis and compared the relative changes of global transcript levels (corresponding to RNA isolated from the extract) with that of the translatome (corresponding to messages associated with affinity-captured ribosomes) after application of diverse stresses to cells [22]. Besides previously known translationally regulated messages such as *GCN4*, which codes for a transcription factor that is increasingly expressed upon depletion of amino-acids from the medium, we identified, and in some cases verified, additional messages that likely undergo translational regulation, underlining the applicability of RAP to study translational regulation [22].

In the following, we highlight critical steps for establishing the procedure including the selection of a suitable tagged ribosomal protein and the choice of the matrix for affinity purification, and we provide a detailed protocol for RAP. Furthermore, we include a brief description of how to analyze the translatome with yeast oligo microarrays.

2.1. Establishing RAP

2.1.1. Selection of tagged ribosomal proteins

The yeast ribosome consists of 78 RPs and four ribosomal RNAs (rRNAs) [23]. Fifty-nine of the 78 RPs are encoded by two paralogous genes (termed a- or b-copy). Therefore, a careful selection procedure is necessary to find a suitable RP for affinity purification. Most importantly, the fusion tag should not interfere with RP function, since this could affect the cell's physiology (i.e. cell growth) and/or promote specific stress programs that significantly alter the cells' translatome. We evaluated six RPs that are preferentially located at the solvent accessible side of ribosomes [24]. This includes four proteins with paralogs (Rpl7, Rpl12, Rpl16, and Rpl18) and two non-duplicated proteins (Rpl25, Rpl30). To achieve stable expression of tagged RPs under the control of their endogenous promoters, we used the ZZ/TAP-tag integrated at the original chromosomal location by homologous recombination, more specifically, at the C-terminus of the corresponding open reading frame in BY4741 (MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*) wild-type yeast cells. The ZZ-tag, which comprises part of the tandem-affinity purification (TAP)-tag [25], contains two protein A IgG-binding units and a tobacco-etch virus (TEV) protease recognition sequence allowing elution of the bound material from the affinity resin [16].

We first tested strains bearing tagged versions of the respective RP genes for growth defects. Six strains showed moderate to severe growth defects when cultured in rich or synthetic media (Rpl7a-TAP, Rpl12a-TAP, Rpl12b-TAP, Rpl16b-TAP, Rpl25-TAP, and Rpl30-TAP) and hence, were not further considered to be suitable for RAP (data not shown). Four strains had no growth defect (Rpl16a-ZZ, Rpl16a-TAP, Rpl16b-ZZ, and Rpl18b-TAP). We next investigated whether tagged RPs are efficiently incorporated into the ribosome which can be easily assessed by sedimentation of extracts through a sucrose cushion (details of the procedure are described below). We found that three tagged proteins Rpl16a-ZZ, Rpl16b-ZZ, and Rpl18b-TAP were efficiently (>80%) incorporated into ribosomes and hence, are valuable candidates for affinity purification of ribosomes (Fig. 2). On the other hand, Rpl16a-TAP as well as Rpl30-TAP and Rpl12a-TAP for which respective strains had slight growth defects, were only to about 50% incorporated into ribosomes (data not shown). The reason for the partial incorporation is not known but may be due to steric hindrance by the TAP-tag. Possibly, the use of a smaller tag than the relatively large TAP-tag (21 kDa) may be favored in these cases. For instance, Inada et al. used a FLAG-$(His)_6$ epitope-tagged Rpl25 which did not seriously compromise cell growth. However, the degree of incorporation of tagged-Rpl25p into actively translating ribosomes was not directly investigated [21].

2.1.2. Affinity purification – the matrix matters

Protein A-tagged ribosomes can be recovered from cell lysates by affinity selection on IgG-coupled beads. However, we observed that with regular IgG-coupled 4% agarose beads (Sigma) only a maximum of about 10–30% of the tagged ribosomes could be captured from the extract (Fig. 3A and B), with large polysomes being underrepresented in the purified fraction (Fig. 3C). Even an