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The use of *Saccharomyces cerevisiae* proteomic libraries to identify RNA-modifying proteins

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Abstract

Biochemical assay of proteomic libraries derived from the *S. cerevisiae* genome provides a powerful new tool for the assignment of activities to proteins. Particular advantages of this approach include the speed with which a protein can be identified, and the generality for any biological activity for which an assay can be developed. We discuss the utility of this approach for the identification of RNA modifying enzymes using a yeast proteomic library derived from a genomic set of strains expressing GST-ORF fusion proteins. This technique is also broadly applicable to other classes of RNA-protein interactions, including RNA binding and RNA degradation, and can be used with any of the proteomic libraries that are available.

Keywords

S. cerevisiae; proteomics; RNA modification; RNA processing; biochemical genomics approach

1. Introduction

The recent explosion in genomic sequencing projects has resulted in the creation of the new field of proteomics, one goal of which is to classify the biochemical activities of proteins on a genome-wide scale. Thus, the entire set of proteins encoded by the genome of an organism can be queried in a single experiment, to identify a protein associated with a given activity. The advantages of a proteomic approach for assignment of protein function include its speed, generality, comprehensiveness and sensitivity. Once a particular activity has been shown to exist in an organism or extract, and an assay has been established, the protein responsible can often be identified in a matter of days. Moreover, this approach inherently broadens the scope of proteins that can be identified, since all proteins are in principle examined without bias. It also does not depend on any prior knowledge of the protein responsible for an activity, or of its catalytic motifs or domains, as determined by bioinformatic analysis. Therefore this approach is well-suited to the identification of novel proteins. Finally, the method has very high sensitivity, particularly for enzymatic activities; thus one can obtain numerous proteins with particular biochemical activities in a single experiment.

A large number of proteins interact with RNA, including those that participate in transcription, translation, RNA processing, assembly or maintenance of ribonucleoproteins, and RNA trafficking. Proteins that interact with RNA can function as chaperones or helicases, as degradation, processing, editing or modifying enzymes or as various structural components of ribonucleoprotein complexes.

The biochemical genomics approach involves the preparation of purified proteins from pools and sub-pools of strains derived from proteomic expression libraries, and the subsequent biochemical assay of the resulting pools of purified proteins, to identify previously unknown proteins (1,2). This approach has been applied to several proteomic expression libraries derived from the yeast *Saccharomyces cerevisiae* (3,4), and has resulted in the identification of a number of proteins that act on RNA, several of which have potentially novel catalytic and/or binding motifs. Some of these include: a two-component methyltransferase required for formation of m⁷G₄₆ of tRNA (5); a tRNA m¹G₉ methyltransferase that is phylogenetically distinct from other classes of methyltransferases (6); a family of four dihydrouridine synthases with specificity for different sites (7,8); a highly unusual guanylyltransferase responsible for addition of a GMP residue to the 5' end of tRNA^{His} (9); a pseudouridylase responsible for modification of U2 RNA (10); a cyclic phosphodiesterase involved in the metabolism of the nucleotide byproduct of tRNA splicing (4), and a 2'-O-methyltransferase that acts at position 4 of a diverse set of tRNA species (11). The biochemical genomics approach is generally applicable to any catalytic activity acting on RNA, and can be easily extended to RNA binding reactions by using the appropriate assay. We illustrate the utility of this technique by describing the example of Trm5, a tRNA m¹G₃₇ methyltransferase previously identified by other methods (12).

2. Materials

1. Radiolabeled tRNA substrate (produced by site-specific labeling techniques (13), or by standard in vitro transcription with T7 RNA polymerase).
2. Enzymes for production of site-specifically labeled RNA: RNaseH, alkaline phosphatase, T4 Polynucleotide kinase and their respective reaction buffers (Roche).
3. [γ -³²P] ATP: labeling grade, 7000 Ci/mmol (MPX Biomedicals).
4. Urea-polyacrylamide gel equipment for purification of RNA substrates.
5. Methyltransferase reaction buffer: 100 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 2 mM dithiothreitol (DTT), 100 mM ammonium acetate, 0.1 mM ethylenediamine-tetraacetic acid (EDTA) and 1 mM S-adenosylmethionine.
6. 1.0 M Tris-HCl, pH 8.0 for termination of methyltransferase reactions.
7. Ribonuclease T2 (RNase T2) digestion buffer: 20 mM sodium acetate, pH 5.2, 1 mM EDTA.
8. S-adenosylmethionine (Sigma): 20 mM stock solution made in 1 mM HCl, stored at -80°C and added as final component to methyltransferase reaction buffer.
9. Bulk yeast RNA: 10 mg/ml stock solution in water.
10. Phenol saturated with 0.5 M Tris-HCl, pH 7.5: the preparation of buffer-saturated phenol can take several days and is as follows. A fresh bottle of phenol is thawed at room temperature and approximately 0.5 volumes of 0.5 M Tris-HCl, pH 7.5 is added to it and mixed thoroughly by inversion. The phases are allowed to separate, the aqueous phase is removed and a fresh layer of 0.5 M Tris-HCl, pH 7.5 is added. This is repeated until the pH of the aqueous phase is around 7.5 (usually 2–3 times) and then a small amount of 8-hydroxyquinoline is added as a preservative. The resulting phenol is stored at 4 °C protected from light for up to 6 months.
11. Phenol:chloroform:isoamyl alcohol (PCA solution): 25:24:1 v:v solution made using buffer-saturated phenol (see above), and commercially available chloroform and isoamyl alcohol with no further purification. 0.5 M Tris-HCl, pH 7.5 (1/10th of the

final volume) is added to the final solution to maintain proper pH and the resulting solution is stored at 4°C protected from light for up to 6 months.

12. RNase T2 (Invitrogen): 1 U/μL stock solution made in 50 mM sodium acetate pH 5.2 and stored at −80°C. The solution is stable for several years; however, small aliquots are used to avoid repeated freeze-thawing.
13. Thin-layer chromatography (TLC) tank and plastic-backed polyethyleneimine (PEI)-F cellulose and cellulose TLC plates (EM Science).
14. Chromatography solvent: isobutyric acid: water: ammonium hydroxide (66:33:1, v/v/v).
15. Phosphorimager and Phosphor screens (Molecular Dynamics).
16. *Saccharomyces cerevisiae* GST-ORF library strain collection, or equivalent genomic library of strains (3) (see Note 5).
17. Affinity-purified preparations of pools (64 each) of 96 GST-ORF strains. Detailed methods for the preparation of the protein pools are provided elsewhere (4,14). Briefly, the library strains are pooled according to their location on the 96-well plates such that each pool of cells contains all of the library strains from a given plate. The pools strains are grown, the expression of proteins is induced (in the case of the GST-ORF library, by the addition of copper sulfate), the cells are harvested after induction, and the resulting proteins from each pool of strains are purified. Purification of the proteins from the GST-ORF library is performed on glutathione-agarose affinity resin.

3. Methods

The procedures that follow outline: 1) the development of an assay to detect the interaction with the targeted RNA, 2) the biochemical assay of the proteomic expression library and subsequent identification of a positive signal and 3) assignment of that signal to a single ORF by deconvolution and confirmation of the identity of the ORF by standard biochemical techniques.

3.1 Development of m¹G₃₇ methyltransferase activity assay

3.1.1. Preparation of radiolabeled tRNA^{Leu} substrate—The main concern when designing an assay to use with a proteomic expression library is the sensitivity of the detection system; the signal produced from the desired activity should be strong enough that it can be detected when the desired protein is present as only one component of a mixture of purified proteins. Fortunately, for analysis of protein interactions with nucleic acids, this is fairly straightforward given the many readily accessible and well-known techniques of labeling the RNA at the position of interest with ³²P. Thus, when looking for an activity that modifies a G-residue in tRNA, tRNA could be simply transcribed with T7 RNA polymerase in the presence of [α-³²P]-GTP, resulting in the incorporation of ³²P throughout the backbone of the tRNA substrate. Likewise, another labeled nucleotide could be used in transcription for detection of

⁵The proteomic expression library used for the example shown here is the yeast GST-ORF library developed by Martzen *et al.* (4) and the proteins represented in it are derived from the yeast genome annotation ca. 1996. In recent years, the annotation of the genome has been updated to add some new ORFs as well as remove some sequences that had been incorrectly described. Therefore, although this earlier yeast expression library has been successfully used to identify at least 10 previously unknown RNA-interacting proteins, this collection does not contain all known yeast proteins as of 2007. A more recently described *S. cerevisiae* proteomic expression library, the MORF (Moveable Open Reading Frame) library, has several advantages over the earlier GST-ORF library in that it is derived from the most recently annotated version of the yeast genome, it allows for tight control of protein expression under a galactose-inducible promoter, and it is extensively sequence-verified. The ORFs in the MORF library contain a tripartite tag at the C-terminus that allows for purification by either IgG-sepharose affinity columns or immobilized metal-ion affinity chromatography, as well as for antibody detection through an HA epitope and cleavage of part of the tag at a 3C protease site (3).

modifications of other residues. An alternative, and sometimes advantageous, approach (see Note 1) is to use a site-specifically labeled substrate, such as the substrate used for the assay described here, which consists of tRNA^{Leu} specifically labeled with ³²P at the phosphate 3' of the G residue at position 37 (the position known to be methylated to m¹G₃₇ *in vivo* (15)). This substrate will be referred to as G37*Leu (Fig. 1). Site-specific labeling of RNA is accomplished by 1) cleavage of the RNA substrate with RNaseH directed at the appropriate position by annealing with a chimeric 2'-O-methylated/deoxyoligonucleotide, 2) post-cleavage labeling of the resulting 3'-half molecule with [γ -³²P] ATP and 3) religation of the RNA pieces to yield G37*Leu tRNA (for specific details see (6,13)).

3.1.2. m¹G₃₇ methyltransferase activity assay—Methyltransferase reactions are first performed with yeast crude extract as a source of Trm5 methyltransferase activity to determine the limits of detection of activity using this assay. After incubation of G37*Leu tRNA with the crude extract in the presence of SAM, the RNA is digested to 3'-phosphorylated nucleotides with ribonuclease T2. The resulting Gp (from unreacted substrate) and m¹Gp (from methylated product) are resolved by thin-layer chromatography (Fig. 1, see Note 2).

1. Decreasing concentrations of yeast crude extract (typically approximately 1 mg/ml to 10 μ g/ml total protein, by factors of 4–5) are incubated with 10,000 cpm of G37*Leu tRNA in methyltransferase reaction buffer at 30 °C for 4–16 hrs. Reaction mixtures containing all components except crude extract are assembled on ice in a volume of 8 μ L and reactions are started by the addition of 2 μ L of each crude extract dilution.
2. Add to each reaction: 50 μ L of 1.0 M Tris-HCl, pH 8.0, 38 μ L of water and 2 μ L of 10 mg/ml bulk yeast RNA (see Note 3).
3. Add 100 μ L of 0.5 M Tris-HCl, pH 7.5-saturated phenol:chloroform:isoamyl alcohol (25:24:1) and vortex. Spin in microcentrifuge at 13,000 rpm for 5 min at room temperature, and remove aqueous layer to new microfuge tube.
4. Add 200 μ L of 100% ethanol to aqueous layer, mix, and incubate at –20 °C for at least 30 minutes. Spin in microcentrifuge at 13,000 rpm for 20 min at 4 °C and remove supernatant, being careful not to disturb the RNA pellet.
5. Add 100 μ L 70% ethanol, mix, and spin in microcentrifuge at 13,000 rpm for 5 min at 4 °C. Remove supernatant, again being careful not to disturb the RNA pellet and air dry.
6. Resuspend dried pellet in 4 μ L of RNase T2 digestion buffer and 2 units of RNase T2. Incubate at 50 °C for 30 min–1 hr.

¹Use of a uniformly labeled RNA, such as would result from *in vitro* transcription in the presence of [α -³²P]-NTPs, is simple and can be a good alternative to creating more complicated site-specifically labeled substrates. However, use of a uniquely-labeled RNA molecule as the substrate for assay of the proteomic expression library improves the sensitivity of the assay 15–25-fold when looking for an enzyme that modifies a known position. This increase in sensitivity can be crucial when looking for a single protein within a pool of purified proteins, such as in the first step of the biochemical genomics approach where the entire expression library is assayed (see section 3.2). Nonetheless, there may be cases where observation of a specific RNA residue is not the goal of the assay, particularly when looking for general RNA-binding activities; in these cases, uniformly labeled RNA substrates may be preferred.

²The assay procedure described here could, in principle, be used for any nucleotide modification of any RNA substrate. For uniformly labeled RNA, digestion of the substrate after reaction with library proteins would be performed with P1 nuclease, since the radiolabeled phosphate would be 5' of the nucleotide of interest. Also, the TLC system to be used in the assay may be different for different modified nucleotides in order to be able to resolve the original unmodified nucleotide from its modified form. Other TLC systems that have been used successfully to visualize modified nucleotides include cellulose TLC systems resolved in one or two dimensions (21) or systems using PEI-cellulose plates resolved in formate buffer or lithium chloride (22,23).

³The inclusion of bulk yeast RNA makes the pellet from the ethanol precipitation step readily visible, and has the additional advantage of permitting visualization of the digestion products by fluorescence quenching after chromatography when the TLC plates are fluorescently labeled.

7. Spot 2 μ L of the digested reaction to flexible plastic-backed cellulose TLC plates and allow to air dry.
8. Thin layer plates are developed (for at least 12 hours) by chromatography in a solvent system of isobutyric acid: water: ammonium hydroxide (66:33:1 v/v/v).
9. After chromatography, the TLC plates are air-dried, exposed to phosphor screens and visualized by Phosphorimager (Fig. 2).

Trm5 methyltransferase activity in this assay is visible in yeast crude extract even at a total protein concentration of approximately 15 μ g/mL (Fig. 2, Lane 5). This is typical of the level of activity visible in crude extract for several of the RNA modifying enzymes which have been found using proteomic expression libraries, and is sufficient for detection of Trm5 as well, as demonstrated below (see Note 4).

3.2. Assay of the yeast proteomic expression library

To identify an activity using the biochemical genomics approach, individual yeast strains are combined into multiple pools of 96 proteins each (derived from 96-well microtiter plates) which are grown and purified together (3,4). The resulting pools contain 96 different proteins with a final concentration of about 1–2 μ g/mL per individual protein (Fig. 3). The GST-ORF library used for the test case described here consists of a set of approximately 6400 yeast strains each expressing a single N-terminally GST-tagged yeast open reading frame, and therefore is assayed in a set of 64 pools (see Note 5). To determine which pool contains the protein of interest, in this case the tRNA m^1G_{37} methyltransferase Trm5, G37*Leu tRNA is assayed exactly as described above (section 3.1.2, steps 1–9), except with 2 μ L of each pool of the GST-ORF library replacing the yeast crude extract as the source of enzymatic activity, resulting in an initial assay consisting of 64 total tubes (Fig. 4A). The known tRNA m^1G_{37} methyltransferase, Trm5 (YHR070w), is located on plate 14 of the GST-ORF library. As expected, this assay yields a positive response for m^1G_{37} methyltransferase activity in Pool 14 (Fig. 4A); thus in one experiment narrowing down the possible candidates for the m^1G_{37} methyltransferase, or a protein that copurifies with the methyltransferase, to 96 proteins out of the initial 6400. Similar approaches to identify novel protein activities have been used with other proteomic libraries derived from *S. cerevisiae* (11–16)(see Note 5), and the biochemical genomics approach can also be easily applied to proteomic libraries from other organisms (17–19).

3.3. Assignment of the positive signal to Trm5 and verification of the identity of the ORF

Once the positive signal is observed in a given protein pool, the individual yeast strain containing the ORF with the desired activity can be readily identified. In this case, the 96-well plate of strains comprising pool 14 was regrouped into intersecting sets of strains representing the rows (A–H) or columns (1–12) of the plate, and proteins from these sub-pools were purified in the same way as the original library (Fig. 3). In this experiment, the number of proteins in an individual pool has been decreased to 8 (columns) or 12 (rows). Again, G37*Leu tRNA is assayed using the standard methyltransferase protocol. The strongest signals are found in row B (data not shown), and column 12 (Fig. 4B). The intersection of these two pools should be the position on the 96-well plate of the individual ORF expressing m^1G_{37} methyltransferase activity or of an ORF that copurifies with the protein that expresses this activity (Fig. 3). In

⁴If the activity observed in the titration of crude extract is significantly weaker than that shown here, it may still be possible to observe the activity in the proteomic expression library. Purification of the library may remove contaminating proteins that compete with the desired protein in a crude extract; this may be particularly important when looking for RNA binding activities for which nonspecific interactions with other proteins in a crude extract may mask the desired activity. However, if upon assay of the proteomic expression library a signal is not detected, it may be necessary to optimize the assay by varying experimental conditions such as time, temperature or buffer conditions.

this case, the ORF located in plate 14 at position B12 is Trm5, the yeast protein which has been previously demonstrated by other methods to catalyze the methylation of G₃₇ to m¹G in tRNA (12).

Once the identity of an individual protein is known, standard biochemical techniques can be used to prove that this is the ORF responsible for the desired activity, and to rule out a requirement for additional yeast proteins for the desired activity. These include: purification of the individual protein to prove that it is associated with activity; cloning of the identified ORF into a vector for purification from *E. coli*, to determine if the protein alone is sufficient for catalytic activity; and analysis of the corresponding yeast deletion strain to determine if the protein is responsible for the activity in vivo.

Notably, neither Trm5, nor another tRNA methyltransferase, Trm10, (6) contains any previously known SAM-binding motifs; in fact neither of these proteins was identified in a search of *S. cerevisiae* proteins for putative methyltransferases (20). In the case of Trm5, the protein was only identified subsequent to the identification of another distantly related homolog of the *E. coli* protein known to catalyze m¹G₃₇ modification (12). Furthermore, in the case of Trm10, the protein is a member of a previously undescribed family of proteins that also bears no homology to any other known methyltransferases, and therefore it is highly unlikely that this protein could have been found by any method that relies on sequence comparison.

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Figure 1. Schematic representation of m¹G₃₇ methyltransferase assay with G37 *Leu tRNA substrate

m¹G₃₇ methyltransferase activity can be detected by using a substrate tRNA with a radiolabeled phosphate 3' of G37 (see section 3.1.2). The ribonuclease used for this assay is RNase T2, which yields 3'-phosphorylated nucleotides, but different RNases may be used for other labeled RNA molecules, depending on the position of the radiolabeled phosphate (see Note 2). The chromatography system used to resolve m¹Gp from Gp is an isobutyric acid: water: ammonium hydroxide system (66:33:1, v/v/v).

Figure 2. Assay for m¹G₃₇ methyltransferase activity with G₃₇*Leu tRNA

The m¹G₃₇ methyltransferase activity was performed as described (section 3.1.2) using decreasing concentrations of yeast crude extract as the source of m¹G₃₇ methyltransferase activity. Lane 1: no crude extract, Lane 2: 1 mg/mL total protein, Lane 3: 0.25 mg/mL total protein, Lane 4: 60 µg/mL total protein, Lane 5: 15 µg/mL total protein.

Figure 3. Overview of the biochemical genomics approach
The GST-ORF fusion protein library consists of 6144 strains of *S. cerevisiae*, each of which bears a plasmid that can be induced to express the protein product of one ORF. These strains are stored on 64 microtiter plates and grown in pools. The overexpressed proteins can be purified as 64 separate pools of 96 proteins each, and each pool assayed for a desired activity. Once that activity is found, sub pools of the rows and columns from the original plate can be constructed and assayed to pinpoint the ORF responsible for the activity, as shown here for Trm5 on plate 14.

Figure 4. Assay of the GST-ORF library to identify Trm5

A. Assay of the 64 initial pools with G37^{*}Leu tRNA. Lanes 1–64: each of the 64 pools of proteins (approximately 96 proteins per pool) assayed as described in section 3.1.2, with the positive m¹Gp signal observed in pool 14. Lane a: buffer only control. Lane b: yeast extract positive control (1 mg/ml total protein). B. Deconvolution of the positive signal to identify the individual ORF by assay of the individual columns (Lanes 1–12) of plate 14 with G37^{*}Leu tRNA. Lane a: buffer only control, Lane b: yeast crude extract control (1 mg/ml total protein), Lane P14: pool 14 control.