Chemistry 5.08 (Spring 2016)
Recitation #4
"Affinity purification of in vivo-assembled ribosomes for in vitro biochemical analysis"
Youngman, E. M.; Green, R. *Methods* **2005**, *36*, 305-312.

I. Background

Robust methods for obtaining mutant ribosomes are needed to facilitate *in vitro* studies. Studying ribosomes with particular mutations in the rRNA provides a means to learn more about the molecular-level details of ribosome function. Obtaining wild-type ribosomes is relatively straightforward. The ribosome is an abundant biomolecule and can be purified from an organism using standard biochemical techniques. How can mutant ribosomes be obtained and purified for in vitro use?

Prior to the Youngman and Green method that we will discuss today, other approaches were developed and employed. These approaches have inherent advantages and disadvantages, and include:

i. Employ an E. coli strain that cannot produce endogenous ribosomes and use this strain to express the mutant ribosome (from a plasmid) in vivo (work of Squires group, ref. 16 of the assigned reading)

Advantage: No contamination from endogenous ribosome
Disadvantage: Only mutants with viable phenotypes can be studied.

Note: the lethal phenotype is a significant problem for this approach. Consider that many of the mutations that are interesting to study would influence decoding and peptidyl transfer, processes that are essential for cell viability.

ii. Employ an in vitro reconstitution system using in vitro-transcribed rRNA

Advantage: Can prepare mutant ribosomes that cause a lethal phenotype
Disadvantage: Technical issues, activity issues, often poor yield (historically)

iii. Express the mutant ribosome in vivo and in a background of wild-type ribosomes, and assay the activity of the mixed population following purification (there is no way to separate the mutant ribosome from the wild-type ribosome in this approach)

Advantage: No lethal phenotype because the wild-type ribosome is there
Disadvantage: Assaying a mixed population, complicates analyses

Clearly, all of these methods have inherent strengths and limitations. One way to improve option (iii) is to find a way to separate the mutant and wild-type ribosomes. By separating mutant from wild-type, the mutant ribosomes can be studied in the absence of contaminating wild-type, which is preferable and will simplify the analyses. Youngman and Green addressed this problem by developing a separation strategy.

WANT: A method that

1. Allows for expression of mutant ribosomes (including lethal mutants) *in vivo* and with a background of wild-type ribosomes
2. Allows for separation of the mutant ribosome from the wild-type
3. Provides ribosomes that are active (high integrity)

SOLUTION: Develop an expression and purification procedure that allows for the mutant ribosome to be separated by using an **affinity tag.** This tag will allow for the mutant ribosome to be fished out from all other components of the cell, including the wild-type ribosome. The procedure developed in this work is an example of **affinity purification.**

POTENTIAL PITFALLS: Placement of the tag – the tag cannot interfere with ribosome activity

II. The Tagged Ribosome Purification Method

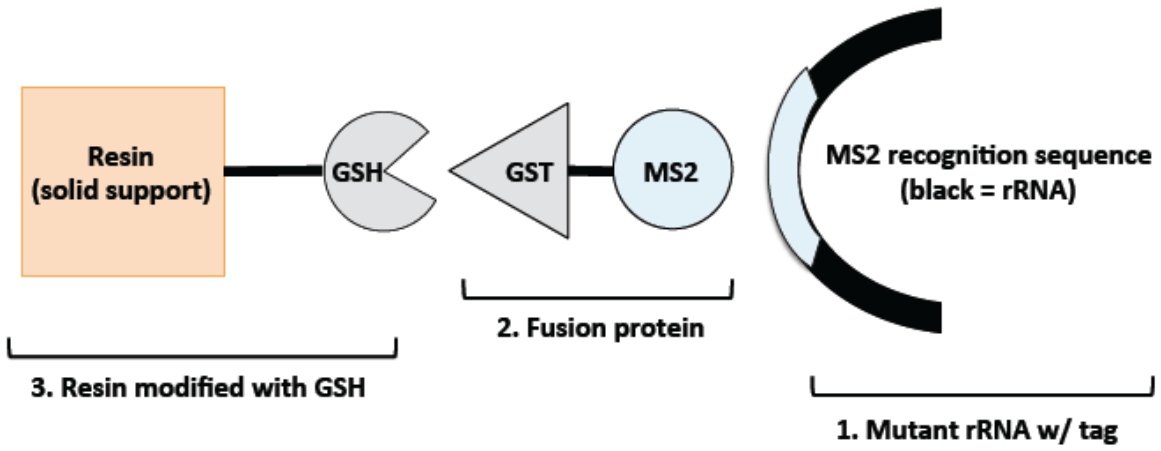
Youngman and Green employed two different ligand/receptor interactions for this method:

1. MS2 coat protein / MS2 RNA recognition sequence
2. Glutathione S-transferase (GST) / glutathione

There are three necessary components for this affinity purification strategy:

1. Mutant rRNA that has a tag. The tag is the MS2 RNA recognition sequence.
    a. Desired mutation in rRNA for biochemical study
    b. Modification of rRNA at another site such that it houses the tag
2. GST-MS2 fusion protein. This protein will bind to the tagged rRNA.
3. Solid support with attached glutathione (GSH).

Here is a diagram showing the components of this strategy:



The MS2 recognition sequence is a stem-loop structure. How did Youngman and Green decide where to put this tag?

The MS2 recognition tag:

1. Must be accessible such that the MS2 coat protein can bind to it. Thus, the tag must be on the surface of the ribosome.
2. The tag cannot interfere with ribosome function.

Placement of the tag:

1. The authors illustrate how to tag the 23S rRNA and 16S rRNA in this work. We will focus on tagging of the 23S.
2. First, the authors went to the literature and studied ribosome structures to see what has been done and what is already known.
3. Prior work showed that a tRNA can be inserted at the tip of helix 2791 of *E. coli* 23S rRNA → no negative impact on *E. coli* growth rate, tRNA protruded from 50S surface.
4. Youngman and Green therefore decided to put the tag at the tip of helix 2791.

To insert the tag into helix 2791 (domain VI) of the 23S rRNA, Youngman and Green first had to perform mutagenesis and insert the sequence 5'-ACTAGT-3' into the DNA encoding the 23S rRNA. This sequence is the recognition sequences for the restriction enzyme *Spe*I (*think:* why might they have selected this restriction site/enzyme?). Next, they used *Spe*I to cut the DNA at this site and then ligated the oligonucleotide sequence for the MS2 stem loop into the DNA. For this work, the researchers had to design the oligonucleotide sequence to have "sticky ends" such that it base pairs specifically with the *Spe*I restriction site. This technique is commonly used in molecular biology and we will not discuss the details in this recitation/course.

Figure 1A of the manuscript illustrates the results and is shown below. The AUU of the helix 2791 (green) is replaced by the MS2 stem loop (orange). The *Spe*I restriction site is shown in blue.

Figure 1A removed due to copyright restrictions. Please see E.M., R. Green. "Affinity purification of in vivo-assembled ribosomes for in vitro biochemical analysis." *Methods* 36(2005): 305-312.

The GST-MS2 fusion protein: This fusion protein had to be overexpressed and purified.

The GSH-modified resin: This resin is commercially available. Many proteins are overexpressed as GST-fusion proteins and the GSH-modified resin can be employed during purification. GST stands for glutathione-S-transferase and it binds to GSH with high affinity.

Youngman and Green expressed tagged ribosome from *E. coli*. After overexpression, the *E. coli* cells were lysed and the crude ribosomes were isolated using a sucrose cushion and centrifugation (the sucrose provides better separation of cell fractionation). To separate the tagged ribosomes from this crude mixture, first the GSH-modified resin, contained in a purification column, was treated with the GST-MS2 coat protein. To do so, the GST-MS2 fusion protein was loaded onto the column and a slow flow-rate was employed to allow GST-MS2 to bind to the column. The column was subsequently washed with buffer and then the crude ribosome preparation was loaded onto this column. The column was washed and the "flow through" eluted. In this step, the tagged ribosomes remain bound to the column because the MS2 protein binds to the MS2 stem-loop (the tag) and other components with low affinity for the GSH-modified resin elute. Finally, the tagged ribosomes were eluted from the column by adding an excess of GSH (*why?*). Note that the GST-MS2 protein remains attached to the tagged ribosome. Details for the purification are given in section 2.3 of the manuscript.

Youngman and Green first performed these experiments and ribosome purifications with a tagged ribosome that lacks any additional mutations. *Why?* Because first it is necessary to establish the purification method and determine whether the method provides ribosomes with comparable activity to the wild-type. By first studying the "native" ribosome harboring the MS2 tag, the researchers can determine whether the tag itself or purification strategy has any negative consequences (e.g. poor subunit association, reduced activity) that would need to be taken into account when studying mutant ribosomes or even necessitate revision of the method. *Note:* In general, affinity tags can have many unintended consequences. They may alter the activity of an enzyme, protein solubility, ability of a protein to oligomerize, etc. His tags will coordinate metal ions.

III. Analysis of the Tagged Ribosome - Assays

Youngman and Green performed a series of experiments to analyze the ribosomes obtained from this purification strategy:

1. Purity
2. Subunit integrity
3. Activity

1. Purity

Ribosome purity was analyzed by **primer extension analysis** (not responsible for details of this method). Note that the chromatograms for the affinity column purification also provide some insight into purity, but these chromatograms are not conclusive because multiple species can co-elute from the column. The data from the purity assays are shown in **Figure 2A.**

Chromatogram from affinity column purification **Gel from primer extension analysis**
(Note: why is A260 being monitored?)

Figure 2A removed due to copyright restrictions. Please see E.M., R. Green. "Affinity purification of in vivo-assembled ribosomes for in vitro biochemical analysis." *Methods* 36(2005): 305-312.

2. Subunit integrity

Next, experiments were performed to assess the overall structural integrity of the purified tagged ribosomes. The ribosomes were placed in low-magnesium buffer to cause dissociation of the 70S ribosome (recall magnesium is important for the association of 30S and 50S subunits), and the subunits were analyzed by using a 10-40% sucrose gradient and analytical ultracentrifugation. The results from this study are shown in **Figure 3.**

Panel A: Native untagged ribosome. The trace is obtained from the A280 (absorbance at 280 nm) of the sucrose gradient after centrifugation.

Figure 3 removed due to copyright restrictions. Please see E.M., R. Green. "Affinity purification of in vivo-assembled ribosomes for in vitro biochemical analysis." *Methods* 36(2005): 305-312.

Panel B: Purified 23S-tagged ribosome. The trace is obtained from the A280 (absorbance at 280 nm) of the sucrose gradient after centrifugation.

Note: the same concentration of ribosome was used for both the native and tagged samples.

What do we observe in these data?

1. Shift in 50S with MS2 tag – why?
2. Depletion of 30S with MS2 tag – why? Solution to this depletion?

3. Activity Assays

Youngman and Green performed three sets of assays to ascertain the activity of the tagged ribosome:

1. Peptide bond formation assays
2. RF1-mediated peptide release assays
3. EF-G translocation assays (we will not discuss these assays in recitation)

In each case, the activity of the tagged ribosome was compared to that of the native ribosome.

Note: in these assays, Youngman and Green added extra 30S subunit to the MS2-tagged ribosomes in order to make up for the depletion of 30S observed from the sucrose gradient experiments.

Peptide bond formation assays: Peptide bond formation between fMet-Phe-tRNA^{Phe} (P site; ^{35}S-label on the fMet) and puromycin (A site) was monitored using a rapid mixing device. The set-up of this assay is similar to the one we discussed in class for studying the effect of EF-P on translation (application of the translation inhibitor puromycin for biochemical analysis) and also similar to the ones discussed in recitation #3. TLC analysis was used to monitor for formation of the tripeptide fMet-Phe-Pmn (Pmn = puromycin).

Here is a depiction of the assay set up (taken from *Cell* **2004**, *117*, 589-599).

Assay set up diagram removed due to copyright restrictions. Please see Youngman, E.M., et al. "The Active Site of the Ribosome Is Composed of Two Layers of Conserved Nucleotides with Distinct Roles in Peptide Bond Formation and Peptide Release." *Cell* 117, no. 5(2004): 589-599.

Note the components in each syringe (why are they all there?). “S100” is the post-ribosomal supernatant. It is the supernatant obtained after the *E. coli* cells are lysed and the mitochondria and ribosomes are removed by centrifugation. (Why is this S100 mixture used?). The mRNA is not listed, but it is drawn in the box cartoon of the 70S in syringe 1. Hence, it is in syringe 1.

Here are the results from the experiment. These results are given in **Figure 4** of the manuscript.

Figure 4A removed due to copyright restrictions. Please see E.M., R. Green. "Affinity purification of in vivo-assembled ribosomes for in vitro biochemical analysis." *Methods* 36(2005): 305-312.

Formation of fMet-Phe-Pmn (peptidyl transfer assay)

Data fit to single exponentials to get rate constants.

Closed circles = native ribosome (14.6 s^{-1})

Open circles = tagged ribosome (14.4 s^{-1})

RF1-mediated peptide release assays: In these assays, release of fMet from fMet-$tRNA^{fMet}$ (initiator tRNA) was monitored. The fMet harbored a ^{35}S radiolabel.

These assays were set up in a similar manner to the peptide bond formation assays. Syringe 1 contained the mixture shown in the cartoon above (fMet-$tRNA^{fMet}$ instead of the dipeptide version). Syringe 2 contained RF-1. For RF1 release, a mRNA with a Met-UAA was employed to put the UAA stop codon in the A site.

Here are the results from the experiment. These results are given in **Figure 4** of the manuscript.

Figure 4B removed due to copyright restrictions. Please see E.M., R. Green. "Affinity purification of in vivo-assembled ribosomes for in vitro biochemical analysis." *Methods* 36(2005): 305-312.

RF1-mediated release of ^{35}S-labeled fMet.

Data fit to single exponentials to get rate constants.

Closed circles = native ribosome (0.5 s^{-1})

Open circles = tagged ribosome (0.4 s^{-1})

IV. Summary

Youngman and Green demonstrated a new affinity purification method for obtaining ribosomes and that this method has utility. Overall, the activity assays reveal that the MS2-tagged ribosomes show similar behavior to the untagged wild-type ribosome.

The authors performed many assays in this work. By doing so, they were able to verify their results. They also addressed various problems they encountered along the way with the purification (e.g. issues with GST-MS2 fusion protein) in the manuscript text. These details are helpful to future researchers wanting to use the methods described in this work.

Was this approach employed to help us learn more about the ribosome? Yes! In a separate paper by Green and co-workers, this purification approach was used to obtain four mutant ribosomes for *in vitro* biochemical analyses. Conserved nucleotides in the 50S that reside near the CCA end of the peptidyl/aminoacyl-tRNA were mutated and the subsequent biochemical analyses demonstrated that these residues are important for peptide release.

5.08J Biological Chemistry II

Spring 2016