Monitoring ribosomal frameshifting as a platform to screen anti-riboswitch drug candidates

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CHAPTER NINETEEN

Monitoring Ribosomal Frameshifting as a Platform to Screen Anti-Riboswitch Drug Candidates

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Abstract

Riboswitches are regions within mRNAs that can regulate downstream expression of genes through metabolite-induced alteration of their secondary structures. Due to the significant association of bacterial essential or virulence genes, bacterial riboswitches have become promising targets for development of putative antibacterial drugs. However, most of the screening systems to date are based on *in vitro* or bacterial systems, lacking the possibility to preobserve the adverse effects to the host's translation machinery. This chapter describes a novel screening method based on monitoring the riboswitch-induced −1 ribosomal frameshifting (−1 FS) efficiency in a mammalian cell-free lysate system using $preQ_1$ class-I ($preQ_1$-I) riboswitches as model target.

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1. INTRODUCTION

Riboswitches are gene regulation elements generally located in the 5′-untranslated regions (5′-UTR) of bacterial mRNAs to control gene expression by forming two mutually exclusive structures elicited upon binding of small metabolites (Roth & Breaker, 2009). Specifically, the ligand-binding domain (aptamer) acts as a molecular sensor, switching the secondary structures along with following variable sequences (expression platform) when the metabolite exceeds a threshold level, resulting in gene regulation at the transcription or translation level. Although riboswitches are found in all three domains of life, the majority of examples are identified across bacterial species. These bacterial riboswitches are mainly located upstream of the biosynthesis or transporter genes of corresponding metabolites, offering the possibility to develop antibiotics against bacterial diseases. Since the selectivity of riboswitches is entirely programmed in the metabolite-sensing domain, great efforts have been made to determine the high-resolution metabolite-bound RNA structures in order to facilitate anti-riboswitch drug designs (Serganov & Patel, 2012). Interestingly, among these available riboswitches aptamer structures, some adopt hairpin-type (H-type) pseudoknot structures that display a wide range of highly specific functions in a variety of biological processes (Brierley, Pennell, & Gilbert, 2007), such as −1 FS, highlighting the convergent role of this important structural motif.

−1 FS is a translational recoding mechanism by which translating ribosomes slip one nucleotide (nt) into the 5′-direction (−1 reading frame) on the mRNA and generate an alternative protein (Farabaugh, 2000). It is well known that two *cis*-acting RNA elements are the main signals to induce −1 FS: (i) a heptameric nucleotide sequence called the slippery sequence, where the ribosome changes reading frame with consensus X XXZ ZZN (where X and Z are any nucleotide, N≠Z, and spaces denote the initial reading frame) and (ii) a stimulatory RNA structure, such as a hairpin or a pseudoknot, downstream of the slip site. The length of the spacer between slip site and downstream structure, generally 6–9 nts, is also crucial for efficient −1 FS. The appropriate spacer length presumably serves to fine-tune the tension generated by the downstream RNA structure, thereby eliciting the appropriate fraction of frameshifting.

Due to the high similarity between H-type pseudoknotted riboswitch aptamers [preQ1-I from *Fusobacterium nucleatum* (*F. nucleatum*) (Roth et al., 2007) as the described example] and frameshift-inducing pseudoknot

structures, we introduced a novel method to investigate ligand-binding ability by monitoring the −1 FS efficiency (Fig.1; Yu, Luo, Iwata-Reuyl, & Olsthoorn, 2013). This method can be used to study other ligand-sensing riboswitches and has been successfully applied to a class II *S*-adenosyl-L-methionine riboswitch as well (C.H. Yu & R.C.L. Olsthoorn, unpublished data).

The method described here offers advantages: (i) the possibility for high-throughput screening when fused to adequate reporters, (ii) simultaneously monitoring potential adverse effects on the eukaryotic translation machinery by using a rabbit reticulocyte cell-free lysate, and (iii) the riboswitch aptamers are embedded within a large mRNA and thereby better mimic bacterial polycistronic mRNAs.

2. MATERIALS

2.1. Nucleobases

1. Nucleobases of adenine (Ade) (A8626), guanine (Gua) (G11950), 2,6-diaminopurine (DAPu) (247847), xathine (Xan) (X0626), hypoxanthine (Hpx) (H9377), and 2,4-diaminopyrimidine (DAPy) (4682131) were from Sigma-Aldrich. $PreQ_1$ compound was a generous gift from Dr. Iwata-Reuyl, Portland State University, Oregon, USA
2. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich)
3. Potassium hydroxide (KOH) (Sigma-Aldrich)

2.2. Plasmid DNA template

1. The frameshifting reporter (pSF208) (Olsthoorn, Reumerman, Hilbers, Pleij, & Heus, 2010) contains an abridged influenza virus A/PR8/34 PB1 gene with inserted U_3A_3C slippery sequence followed by *Spe*I and *Nco*I sites for cloning $preQ_1$ riboswitch aptamers through complementary oligonucleotides (Note 1).
2. Restriction enzymes *Spe*I and *Nco*I (Thermo Scientific).
3. Synthesized pair of stabilized *F. nucleatum* $preQ_1$ riboswitch aptamer (stab-Fnu) oligonucleotides: 5′-CTAGTTGACGCGGTGCTAGCAA AACCCGCGTTAAACAAACTAGACTTCATG-3′ (Note 2) (Sigma-Aldrich).
4. Gel extraction spin column (Machery-Nagel).
5. T4 DNA Ligase (Thermo Scientific).
6. *E. coli* XL-1 Blue competent cells.

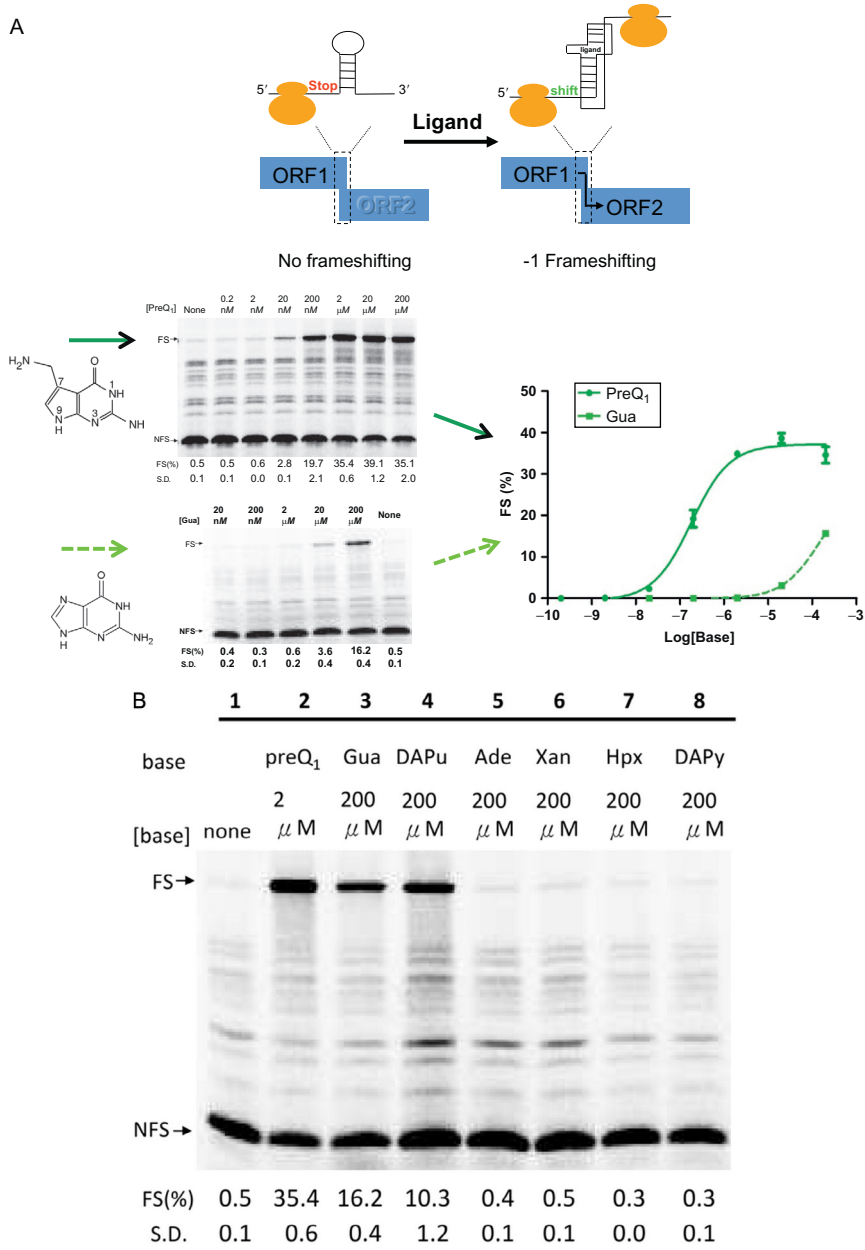

Figure 1 (A) Conceptual illustration of the evaluation of ligand-preQ$_1$-I aptamer binding affinities by monitoring −1 FS efficiency. The cartoon describes the principle of ligand-induced −1 FS. Given examples are two preQ$_1$ aptamer ligands: preQ$_1$ (optimal) and guanine (Gua, suboptimal). −1 FS efficiencies induced by either preQ1 or guanine at the indicated concentrations are quantified by the appearance of −1 FS protein products (FS) on the SDS-PAGE (see Note 11 for details). The dose (various ligand concentration, *X*-axis)-responsive (−1 FS efficiency, *Y*-axis) curves can be drawn to compare the ligand-binding affinity. (B) The SDS-PAGE shows the level of −1 FS efficiency induced by various preQ1 analogs at the indicated concentrations.

2.3. *In vitro* transcription

1. *BamH*I for template linearization (Thermo Scientific).
2. RiboMAX Large-Scale RNA Production System-SP6 (Promega).
3. Phenol–chloroform–isoamyl alcohol (25:24:1).
4. Micro-Bio-Spin P-6 gel column, tris buffer (Bio-Rad).

2.4. Cell-free translation

1. Rabbit reticulocyte lysate (RRL) System, nuclease Treated (Promega).
2. EasyTag L-[^{35}S]-Methionine, >1000 Ci/mmol (PerkinElmer).
3. 2 × Laemmli sample buffer [120 m*M* Tris–HCl (pH 6.8), 20% glycerol, 4% sodium dodecyl sulfate (SDS), 0.02% bromophenol] stored at room temperature.
4. 2-Mercaptoethanol (Sigma-Aldrich).
5. 30% acrylamide/bis solution (29:1) (Bio-Rad).
6. Ammonium persulfate.
7. Tetramethylethylenediamine (Bio-Rad).
8. SDS polyacrylamide gel (SDS-PAGE).
   - **8.1.** stacking gel (5% acrylamide/bis, 0.13 *M* Tris–HCl pH 6.8, 0.1% SDS).
   - **8.2.** separation gel (13% acrylamide/bis, 0.375 *M* Tris–HCl pH 8.8, 0.1% SDS).
9. SDS-PAGE running buffer (25 m*M* Tris–HCl pH 8.3, 250 m*M* glycine, 0.1% SDS).
10. Whatman filter paper (3 mm).
11. Saran wrap.

2.5. Equipment

1. Spectrophotometer [e.g., Varian Cary 5000 UV–vis-NIR (Agilent)].
2. NanoDrop 1000 (Thermo Scientific).
3. Gel Dryer [e.g., Model 583 (Bio-Rad)].
4. PhosphorImager screen (Molecular Dynamics)
5. PhosphorImage scanner [Personal Molecular Imager FX System (Bio-Rad) or Typhoon 9400 (GE Healthcare)].
6. Quantity-One software (Bio-Rad demo version suffices).
7. SDS-PAGE gel running system (e.g., Mini-Protean 3 Bio-Rad).
8. Electrophoretic power supply.
9. Waterbath.
10. Heating block.

11. Micropipettes.
12. Freezer.
13. Eppendorf centrifuge.

3. METHODS

3.1. Preparation of nucleobases stock

1. Dissolve $PreQ_1$, Xan, Hpx, and DAPy at 200 m*M* in DMSO as stocks, and store at −20 °C (Note 3).
2. Dissolve Gua and Ade at 200 m*M* in 0.15 N KOH (Note 4).
3. Dissolve DAPu at 200 m*M* in RNase-free water.
4. Check suitably diluted ($0.1 < \text{absorption} < 1$) nucleobase samples by spectrophotometer and verify the concentration by Beer's law.
5. For working solution, dilute all above nucleobase stocks to 2 m*M* by RNase-free water, followed by adding 1 μl into translation reaction mixtures (Step 1 in Section 3.4) in total volume of 10 μl. Serial dilutions are applied to make desired concentrations of $preQ_1$ solutions.

3.2. Preparation of DNA template for *in vitro* transcription

1. Digest the parental pSF208 by *Spe*I and *Nco*I at 37 °C for 1 h.
2. In the meantime, mix stab-Fnu oligonucleotides pair at final concentration of 10 μ*M* of each strand, followed by heating up the mixtures to 95 °C for 1 min. Then, the mixtures were allowed to cool to room temperature for annealing.
3. Purify the digested pSF208 plasmid by gel extraction spin column after migration on 1% agarose gel.
4. Ligate the *Spe*I–*Nco*I digested pSF208 with annealed stab-Fnu fragments at room temperature for 30 min followed by transformation into *E. coli* XL-1 blue cells.
5. Isolate plasmids from selected clones and verify the sequence by automated dideoxy sequencing with chain terminator dyes.

3.3. *In vitro* transcription

1. Linearize 10 μg of stab-Fnu as determined by NanoDrop by *BamH*I to create the template for run-off transcription.
2. Purify the linearized plasmid by phenol–chloroform extraction with successive ethanol precipitation or by one of the commercially available spin columns.

3. Dissolve the linearized plasmid in RNAse-free water to make a working solution of 500 ng/μl.
4. Transcribe the DNA template with the RiboMAX SP6 Kit according to the manufacturer's protocol. Typically, incubate 10 μl of the reaction solution containing 2 μl of the plasmids at 37 °C for 2 h (Note 5).
5. Add 0.5 μl DNase RQ1 to the transcription mixtures with further incubation at 37 °C for 15 min.
6. Check the quality and quantity of the transcribed mRNA by comparing the migration and brightness with the control RNA with similar length and known quantify after ethidium bromide-stained agarose gel electrophoresis (Notes 6 and 7).
7. Dilute the mRNA to 15 ng/μl as working solution for *in vitro* translation.
8. Aliquot the mRNA to small volumes and store them at −20 °C.

3.4. Cell-free translation

1. Label eppendorf tubes and add 1 μl of designated nucleobases working solution into reaction tubes and leave them on ice.
2. Prepare a translation reaction master mix on ice by adding 4 μl nuclease-treated RRL, 2 μl mRNA working solution, 0.25 μl amino acids mix without methionine, 0.25 μl ^{35}S-methionine, and 2.5 μl RNase-free water for each sample.
3. Add 9 μl translation mix into each nucleobase-containing tube (Note 12).
4. Mix the solution briefly and incubate in a 28 °C water bath for 1 h.

3.5. Monitoring −1 FS

1. Stop the translation reaction by adding 10 μl cold 2× Laemmli sample buffer followed by immediately boiling the sample for 3 min.
2. Separate the translation products by 13% SDS-poly acrylamide gel electrophoresis (SDS-PAGE) at a constant voltage of 200 V.
3. Stop running when bromophenol blue dye front reaches the bottom of the separating gel (Note 8).
4. Dry the gel on 3 mm Whatman paper (cover the gel with Saran wrap) by gel dryer, applying PAGE gel program cycle at 80 °C for 2 h.
5. Expose the dried gel (remove Saran wrap) to a phosphorimager screen (Note 9).
6. Scan the phosphorimager screen at 50 or 100 μm resolution.
7. Quantify the in-frame and frameshifted bands by Quantity-One (Note 10).
8. Calculate the −1 FS efficiency induced by designated ligands (Note 11).

4. NOTES

1. For potential high-throughput screening, the −1 FS cassette, including slippery sequence, spacer, and target riboswitch aptamer, can be inserted into suitable reporter (for example, dual-luciferase reporter).
2. The stabilized version of the *F. nucleatum* aptamer is created by mutating the A–U pair to G–C pair within the first stem region. This mutant, without losing ligand specificity, provides a better platform for ligand-binding assays due to its higher sensitivity to preQ1 compound. Although this way of improving aptamer-ligand-binding may be applied to other targets, the orientation of the G–C pair should be determined experimentally. We observed that a G–C flipped mutant shows lower $preQ_1$ binding, probably due to a nonoptimal stem-loop tertiary interaction. The spacer length, i.e., the sequence between slippery sequence and riboswitch aptamer, is another tunable factor for optimal −1 FS efficiency.
3. These compounds are quite stable in DMSO. The working solution (Step 5 in Section 3.1) can be stored at 4 °C for several months without noticeable decay.
4. Gua and Ade stocks should be kept at −20 °C for no longer than two weeks. Significant decay is observed afterward. Alternatively, stocks can be made freshly.
5. Since noncapped and nonpolyadenylated mRNAs can be translated in RRL, we do not add cap analog into our transcription mixtures, and also no polyA-tail is transcribed cotranscriptionally or added post-transcriptionally. As a consequence, some non-specific translational products with negligible intensity are observed on gel due to occasional usage of alternative start codons.
6. To prepare control mRNA, the DNase-treated transcription mixtures are first extracted by phenol–chloroform solution, followed by purification of the mRNA through P-6 gel filtration column to remove DNA and unincorporated ribonucleotides. Then, the eluted control mRNA is quantified by NanoDrop.
7. Using nonpurified mRNAs can better mimic the molecular crowding conditions as well as the complexity in the cytoplasm.
8. Since the gel will be dried on the filter paper without staining, it is not necessary to fix the gel to prevent diffusion. We did fix the gels by 45%

methanol, 45% water, 10% glacial acetic acid, and find no noticeable difference to the unfixed ones.

9. In our experimental condition, a 16-h exposure time (overnight) is enough to obtain clear signals.
10. Typically, the intensities of target bands are determined by using the function of "Volume Rect Tool" in Quantity-One. For background subtraction, an identical rectangular volume is selected immediately above the corresponding target band area as the defined background.
11. The −1 FS efficiency representing the riboswitch aptamer-ligand affinity is calculated by: [intensity of the −1 FS band (FS)] − (intensity of the background of the −1 FS band)/{[intensity of the in-frame band (NFS)] − (intensity of the background of the in-frame band) + [intensity of the −1 FS band (FS)] − (intensity of the background of the −1 FS band)} × 100, after correcting for the number of incorporated methionines.
12. To save on RRL, the reaction can also be carried out in 5 μl volumes but this requires good pipetting skills.

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