Biochemical Engineering and Optimization of the \textit{glmS} riboswitch for use as a synthetic genetic device.

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Abstract

Synthetic biology is a rapidly emerging field focused on engineering biochemical systems and cellular functions for a variety of applications, including therapies for the treatment of infectious diseases and cancer, as well as tactics for vaccine development, microbiome engineering, cell therapy, and regenerative medicine. Many of the advances so far have involved engineering synthetic constructs for use in bacteria, but it is critical that synthetic biology tools be designed for use in mammalian systems. Riboswitches offer a unique set of “devices” for achieving synthetic gene regulation. This report involves investigating the possibility of controlling mammalian gene expression via engineered insertion of the bacterial \textit{glmS} riboswitch.

Introduction

Riboswitches are segments of RNA found in the 5’ untranslated region (UTRs) of mRNA. Through interaction with small molecules called metabolites, riboswitches are able to exert control over nearby genes involved in synthesis of the cognate metabolite. Each riboswitch consists of two universal, fundamental components: an aptamer domain and an expression platform. The aptamer domain consists of approximately 70-170 nucleotides and is highly conserved even among distantly related organisms. This RNA domain binds a specific
metabolite, inducing a structural change.\textsuperscript{1} The structural change is manifested in the expression platform, which exerts genetic control over essential metabolic genes via regulation of transcription termination, translation initiation, or RNA processing.\textsuperscript{2}

The \textit{glmS} riboswitch is structurally and functionally unique among riboswitches in that it is also a ribozyme. Upon binding to its cognate metabolite, Glucosamine-6-Phosphate (GlcN6P), the riboswitch undergoes catalyzed self-cleavage, with GlcN6P serving as a coenzyme. This cleaved mRNA is then targeted for destruction, effectively down-regulating the expression of nearby genes involved in GlcN6P biosynthesis.\textsuperscript{3}

Previous work in bacteria has shown that riboswitches are modular and can be inserted upstream of reporter genes to control gene expression.\textsuperscript{4} The focus of this report is to describe results and future work on the first synthetic genetic circuit created using the \textbf{bacterial} \textit{glmS} riboswitch to control \textbf{mammalian} gene expression.

The ability to control mammalian gene expression with drug-like small molecules that bind to the bacterial riboswitch is very powerful. With this type of synthetic circuit, mammalian gene expression could be controlled by an exogenous ligand introduced at an exact time. Insertion of a genetic switch, such as the \textit{glmS} riboswitch, into a mammal’s genome allows researchers and clinicians to control mammalian gene expression via the activation and deactivation of particular genes of interest. Given the genetic basis for a number of human ailments, this technology could have a powerful impact not only in the treatment of infectious pathogens, but also in cancer therapy, gene therapy, and a number of other biomedical applications.
Materials and Methods

Plasmid Preparation:

Plasmids were prepared by genetically engineering the *glmS* riboswitch into a PMIR-GLO plasmid harboring genes for both firefly and renilla luciferase. DNA encoding the *glmS* riboswitch was inserted into the 3’ UTR of firefly luciferase only (See Figure 1).

Thus, successful operation of the *glmS* riboswitch as a synthetic genetic switch should result in a decrease in the amount of light produced by firefly luciferase relative to renilla luciferase.

Control plasmids were generated using site-directed mutagenesis. One construct introduced a mutation at guanine residue 32 to an adenine (termed G32A). Another involved mutating the guanine residue at position 33 to an adenine (termed G33A). The guanines at positions 32 and 33 are instrumental in the catalysis mechanism of the *glmS* riboswitch, and thus these mutations decrease riboswitch function and serve as negative controls. Finally, a third control involved three subsequent mutations to generate a
riboswitch construct (termed AAA) shown to undergo self-cleavage in the absence of GlcN6P, thus providing another type of control.

**Redesigning Plasmid**

The plasmid was redesigned to contain a segment of DNA between the $glmS$ riboswitch and the firefly luciferase gene (See Figure 2).

This was hypothesized to give the riboswitch more freedom to fold into its complex three-dimensional form. The DNA chosen to accomplish this was the twister gene, which encodes a self-cleaving RNA catalyst. Two different plasmid constructs were created, one containing wild-type twister and another containing a mutated form of twister. The mutant twister gene was effectively “junk” DNA that allowed us to test the hypothesis that more freedom to fold would improve the function of the $glmS$ riboswitch in a synthetic circuit. The wild-type twister, however, allowed us to test the ability of another catalytic RNA to potentially serve as a “device” for building an artificial genetic circuit.
Cell Culture, Transfection, and Lysing

Before transfection, HEK 293 cells were trypsinized, counted by a hemocytometer, and plated into 24-well plates so as to be 50% confluent at time of transfection. Cells were transfected with the plasmids described above using Lipofectamine-2000. Cells were grown in the presence or absence of GlcN (which is facilely converted to GlcN6P upon uptake by cells) or an artificial analog, serinol. Cells were lysed with Passive Lysis Buffer. Lysates were stored at -20°C until assayed by a Dual Luciferase Assay (DLA).

Dual Luciferase Assays

Dual Luciferase Assays were performed (using Promega kit) and results were obtained on a Modulus Microplate Luminometer with dual injectors (Turner Biosystems). The luminometer calculated the firefly/renilla luminescence ratio, which was used to measure downregulation of the engineered glmS construct in response to GlcN or serinol.

In-Vitro Cleavage Assays:

Wild-type or mutant glmS was exposed to either GlcN6P or serinol in order to determine the ability of these ligands to actuate cleavage in either the wild-type or mutant construct. After the appropriate amount of time, the reactions were stopped with 10 µL of dye containing urea and EDTA. To visualize the results of the time trials, the reactions were run out on a denaturing polyacrylamide gel and exposed to a PhosphorImager screen. The gel image was obtained using a PhosphorImager.
Results and Discussion

As evident, there was no statistically significant downregulation of the wildtype construct in response to GlcN. This could be due to some of the assumptions we made about GlcN—such that it is: easily taken up by cells, facilely converted into GlcN6P, and not being used metabolically. Thus, we repeated the experiment using serinol as a ligand instead. Serinol has previously been shown actuate cleavage in the glmS riboswitch and we hypothesize that, because of its smaller molecular structure, it will be able to more easily cross the cell membrane.

Figure 3: Firefly:Renilla Luciferase Expression levels of four engineered glmS constructs in the presence or absence of GlcN.
Here, we see an unexpected downregulation of the mutant control in response to serinol. This prompted a serious question: what effect does serinol have on mutant glmS function? These mutants were designed as negative controls for the natural riboswitch-metabolite interaction. Serinol has previously been shown to support self-cleavage of the wild-type glmS riboswitch. It is possible that serinol promotes cleavage in the mutant constructs. Thus, to test whether or not serinol promoted cleavage in the mutant constructs, in-vitro cleavage assays were performed.

Figure 4: Firefly:Renilla Luciferase Expression of four engineered glmS constructs in the presence or absence of serinol, a GlcN6P analog.

Figure 5: Cleavage assay results for WT and G32A glmS in 10 mM serinol.
Results indicated that the mutant construct G33A, which had significant downregulation \textit{in vivo}, did not cleave appreciably in response to serinol.

We hypothesize that the reason the \textit{glmS} riboswitch was unable to effectively serve as a synthetic genetic device was due to the nature of the plasmid design. We believe the original plasmid design did not allow for the \textit{glmS} to fold into its final, function three-dimensional form, due to the riboswitch’s close proximity to the firefly luciferase gene. Thus, we repeated the Dual Luciferase Assays using plasmids containing either wild-type or mutant twister gene inserted between the firefly luciferase gene and the \textit{glmS} riboswitch. We used the original plasmid as a control, and measured luciferase production in the presence or absence of either GlcN or serinol.

\textbf{Figure 6:} Cleavage assay results for all \textit{glmS} constructs in 100 mM serinol
As apparent, the presence of the twister gene dramatically reduced the expression of firefly luciferase in this synthetic genetic circuit. Additionally, in the construct containing the mutated twister gene, there was a statistically significant downregulation of luciferase production in the
presence of serinol. This indicates that additional space between the riboswitch and the luciferase gene is potentially aiding in the proper folding of the *glmS* riboswitch and its ability to operate in a synthetic circuit. This information has important implications for optimization techniques of synthetic circuits utilizing the *glmS* riboswitch, and also elucidates a potentially promising new device for engineering artificial genetic circuits: twister RNA.

**Future Work**

Future work will involve repeating the above experiments to further confirm the validity of the results presented here. Additionally, given the substantial ability of twister RNA to downregulate gene expression in this circuit, future work will focus on creating a synthetic genetic switch utilizing twister. Briefly, we will explore the ability to artificially evolve twister to become responsive to a ligand of choice, allowing us to control gene expression via introduction of an exogenous ligand. These results, if positive, could be used to expand the “toolbox” of “devices” available to researchers as they design synthetic genetic circuits.

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