**INTRODUCTION**

Riboswitches are highly structured regions of RNA that make genetic decisions based on ligand occupancy. They have two principal components: a receptor domain, and a regulatory domain. The receptor domain, also called the aptamer domain, is a highly structured region that binds ligands with high specificity and selectivity (21925376, 14523920). The regulatory domain, or expression platform, is a dynamic region that creates a genetic output, through gene regulation, from the ligand input, of binding of the ligand to the receptor domain (20943759). While robust, functional riboswitches have been created, understanding of the principles underlying that function is still lacking (33259551).

Current knowledge about riboswitch design comes from studies surveying natural riboswitches (25015992, 36150954) or evolving novel aptamers through techniques like SELEX that often modify existing riboswitch scaffolds (36617976, 28092358). Limitations to these approaches are three-fold. First, the focus is placed on the aptamer domain, usually with the goal of evolving a novel sensor for a chosen effector ligand. Second, many of these analyses produce answers without providing any insight into the principals governing these sensors. And third, many of these aptamers are developed *in vitro,* potentially limiting their potential portability into other systems. This has created a lack of understanding both about overarching strategies to design nucleic acid-based sensors, and more specifically how to design and manipulate a regulatory domain.

A previous study by the Batey lab created a riboswitch modeled from the natural *pbuE* adenine responsive riboswitch found in *B. subtilis* aimed atmaximizing signal amplitude between the ON- and OFF-states of the switch (33259551). This starting scaffold is small, simple, and well characterized, making it ideal for more extensive modifications (24590258). The structure guided mutagenesis preformed yielded a modified and truncated switch that greatly outperformed the 5.6-fold switching of pbuE with a near 100-fold induction in cells. The overarching hypothesis guiding the modifications was that Watson-Crick (WC) base pairing in both the receptor and regulatory domains would create the best switch (33259551). Therefore, the switch became symmetrical, save the aptamer domain, and regions thought to be key to nucleation of the terminator helix (such as P4) were enriched with GC pairs. In addition to changing the primary sequence, the pre-aptamer sequence was shortened by 27 nucleotides to reduce misfolding and an AA mismatch was introduced in P3, proximal to L3, to promote strand invasion into the aptamer domain and destabilize the tertiary structure of the binding pocket. The mutagenic analysis, while yielding a high preforming variant did not aim elucidate the design principles behind this improved switching ability.

In this study, we aim to understand the design principles governing a transcriptional riboswitch’s ability to fold co-transcriptionally and attenuate gene expression. This study, through multiple genetic screens, offers insights into primary and secondary structural requirements for the processes of strand-invasion and nucleation that allow for the dynamic properties of this unique regulatory element. Through the analysis of regions in the terminator helix, WC pairing promotes efficient repression, though some positions seem to be more important than others, specifically positions near paired regions in the aptamer. When looking at paired regions or regions in competition between the aptamer and terminator helix, balance between the two paired regions must be tuned to optimize functionality. From these data it seems that the development of novel biosensors that use this scaffold can be easily designed with limited manipulation to key areas of the switch based on the parameters of the system.