Bacterial Logic Devices Reveal Unexpected Behavior of Frameshift Suppressor tRNAs

Eric M. Sawyer1,2, Cody Barta2, Romina Clemente1, Michel Conn2, Clif Davis2, Catherine Doyle1, Mary Gearing1, Olivia Ho-Shing1, Alyndria Mooney1,3, Jerrad Morton1, Shamita Punjabi1, Ashley Schnoor1, Siya Sun1, Shashank Suresh5, Bryce Szczepanik1, D. Leland Taylor1, Annie Temmink2, William Vernon2, A. Malcolm Campbell1, Laurie J. Heyer2, Jeffrey L. Poet1 and Todd T. Eckdahl2*

1Department of Biology, Davidson College, Davidson, USA
2Department of Biology, Missouri Western State University, St. Joseph, USA
3Department of Biology, University of Arkansas at Pine Bluff, Pine Bluff, USA
4Department of Computer Science, Math and Physics, Missouri Western State University, St. Joseph, USA
5Department of Mathematics, Davidson College, Davidson, USA

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*Correspondence and requests for materials should be addressed to T.T.E. (eckdahl@missouriwestern.edu).

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SYNOPSIS

Introduction: We investigated frameshift suppressor tRNAs previously reported to use five-base anticodon-codon interactions in order to provide a collection of frameshift suppressor tRNAs to the synthetic biology community and to develop modular frameshift suppressor logic devices for use in synthetic biology applications.

Results and Discussion: We adapted eleven previously described frameshift suppressor tRNAs to the BioBrick cloning format, and built three genetic logic circuits to detect frameshift suppression. The three circuits employed three different mechanisms: direct frameshift suppression of reporter gene mutations, frameshift suppression leading to positive feedback via quorum sensing, and enzymatic amplification of frameshift suppression signals. In the course of testing frameshift suppressor logic, we uncovered unexpected behavior in the frameshift suppressor tRNAs. The results led us to posit a four-base binding hypothesis for the frameshift suppressor tRNA interactions with mRNA as an alternative to the published five-base binding model.

Conclusion and Prospects: The published five-base anticodon/codon rule explained only 17 of the 58 frameshift suppression experiments we conducted. Our deduced four-base binding rule successfully explained 56 out of our 58 frameshift suppression results. In the process of applying biological knowledge about frameshift suppressor tRNAs to the engineering application of frameshift suppressor logic, we discovered new biological knowledge. This knowledge leads to a redesign of the original engineering application and encourages new ones. Our study reinforces the concept that synthetic biology is often a winding path from science to engineering and back again; scientific investigations spark engineering applications, the implementation of which suggests new scientific investigations.

Key Words: tRNA; frameshift suppression; DNA-based logic gates; synthetic biology
INTRODUCTION

In the flow of information during gene expression, transfer RNAs (tRNAs) play a crucial role as the molecular adapters that translate the language of nucleic acids into the language of proteins. Understanding the interaction between a tRNA anticodon and its cognate three-base mRNA codon begins with a basic model in which the two RNA molecules interact in an antiparallel fashion according to the Watson/Crick base pairing rules. Observations about genetic code redundancy led to the development of the wobble hypothesis as an improvement to the basic model. Because the difference among alternative codons for a given amino acid is usually in the third (3’) position, the base pairing rules were expanded by the wobble hypothesis for this position. Post-transcriptional modification of tRNA anticodons can result in wobble base pairing. Modification of the tRNA anticodon loop in positions outside of the anticodon itself can also influence interactions with mRNA codons.

Normal protein production can be altered by nonsense mutations and frameshift mutations. Nonsense mutations alter an amino acid-encoding mRNA codon into one of the three stop codons. Suppression of nonsense mutations occurs when one of the three termination suppressor tRNAs, called amber, ochre, and opal, encounters a premature stop codon during translation. Nonsense suppressor tRNAs have anticodons that interact with one of the stop codons, thereby suppressing premature termination of translation and allowing protein synthesis to continue. Nonsense suppressor tRNAs have been identified in many types of cells, including human cells and bacterial cells. The biological cost to the cell is occasional read-through translation past stop codons in wild-type mRNAs. Frameshift mutations occur when nucleotides are inserted into or deleted from a coding sequence, disrupting the translational reading frame. The ability of bacterial cells to suppress a frameshift mutation using compensatory mutations in tRNAs is extensively documented. Magliery and Anderson used a selection protocol to discover four-base frameshift suppressor tRNAs that function in Escherichia coli. They found efficient and specific tRNAs that bind to the codons AGGA, UAGA, CCCU, and CUAG. The most efficient suppressors were those that used only Watson-Crick base pairing. Anderson et al. also used a combinatorial selection method to identify five-base frameshift suppressor tRNAs. They concluded that Watson-Crick base pairing was responsible for the five-base codon/anticodon interactions as well.

Biological engineers have constructed DNA-based systems to perform Boolean logic using several mechanisms. Li et al. used the quorum sensing system of Pseudomonas bacteria to produce a cellular AND logic gate. Riboswitches can execute Boolean logic in response to small molecules and regulate gene expression. Allosteric riboswitches and ribozymes are able to implement logic networks in bacteria and yeast. Construction of hybrid synthetic promoters produced genetic AND gates in bacteria. Populations of yeast cells function as cellular consortia that implement logical computations. DNA-encoded logic using nonsense suppressor tRNAs as inputs generated an XOR logical device. To date, no one has used frameshift suppressor tRNAs to perform cellular Boolean logic.

We proposed to expand the engineering options for cellular Boolean logic gates through the use of frameshift suppressor tRNAs in a modular programming language for bacterial computing. Frameshift suppressor tRNAs could be considered inputs for the system and their presence or absence could be controlled by regulation of promoters chosen by the engineers. Frameshift suppressor tRNA inputs would be evaluated by frameshift mutations in engineered mRNAs as processors of logical computation. Successful suppression of the frameshifted mRNA resulting in correct translation of a protein of interest would be dependent on codon/anticodon interactions. The simplest example of a frameshift suppressor logical processor is a five-base mutation in the coding sequence of a gene. If the cognate frameshift suppressor tRNA functions as predicted, translation occurs and a functional protein is produced. Incorporation of a mutation that has two five-base codons separated by a single base would result in an OR logic gate, as either one of the two cognate frameshift suppressor tRNAs would be sufficient to allow translation of the mRNA into a functional protein.

In this case, interaction of a five-base frameshift suppressor tRNA with the first five-base codon could lead to translation with two normal tRNAs of the one base spacer plus the other five base codon. Alternatively, translation of the first five-base codon plus the one base spacer by two normal tRNAs could be followed by interaction of the second five-base codon with a five-base suppressor tRNA. A mutation that has two five-base codons separated by zero or three bases would function as an AND gate, since there must be two five-base codon/anticodon interactions for successful translation. Linking two or more of these logic gates together with the appropriate spacing could result in more complicated logical clause combinations.

We had two purposes for investigating frameshift suppressor tRNAs. Our first purpose was to facilitate widespread availability and use of the previously described five-base frameshift suppressor tRNAs. We designed and built a collection of eleven frameshift suppressor tRNAs using synthetic biology standards of assembly. We also designed and assembled three genetic circuits to measure frameshift suppression. The three circuits employ three different mechanisms: direct frameshift suppression of reporter genes, frameshift suppression leading to positive feedback by quorum sensing, and enzymatic amplification of frameshift suppression signals. Our second purpose was to...
develop a system of frameshift suppressor Boolean logic that could be used in a variety of synthetic biology applications. In the course of developing frameshift suppressor logic, we uncovered unexpected behavior of the frameshift suppressor tRNAs. Our results led us to develop four-nucleotide binding hypotheses as alternatives to the originally published five-nucleotide binding model for the suppressor tRNA interactions with mRNA.

RESULTS AND DISCUSSION

Direct suppression devices

In support of our goals to investigate frameshift suppression and develop frameshift suppression logic, we cloned eleven of the five-base frameshift suppressor tRNA genes discovered by Anderson et al.13. Table 1 lists the tRNA genes, named for their five-base mRNA binding sites, their part numbers from the Registry of Standard Biological Parts14, the base sequence of their anticodon loops, their predicted mRNA codon binding sites, and the four-base binding sites that we deduced from our results, as described below.

In order to measure the function of five-base frameshift suppressor tRNAs, we designed and tested two direct suppression measurement devices, shown in Figure 1A. The designs include a RFP reporter gene containing one of four five-base frameshift mutations immediately following the start codon for RFP (Frameshift Suppressor Leader; FSL), as listed in Table 2. Each construct contains a frameshift suppressor tRNA that is either upstream or downstream of the FSL-RFP and cognate to the frameshift mutation in the RFP. Cellular production of frameshift suppressor tRNAs were regulated by either a pTet or a pBAD promoter. We built constructs including the four frameshift suppressor tRNAs CGGUC, CCACU, CCAUC (9), and CCAUC.

Figure 1B shows the fluorescence output of the direct suppression devices after growth in liquid culture. The relative fluorescence values are normalized against the negative control.

![Image](https://www.interdisciplinarybiocentral.org/fig1.png)

Figure 1. Devices for direct measurement of frameshift suppression. (A) The top diagram is of the genetic circuit used for K199093 and K199095 and the bottom diagram is for K199056 and K199057. (B) Normalized RFP fluorescence of bacteria grown in liquid culture graphed for the following transformants: K32 negative = K199032 (pBAD-CCAUC tRNA), K93 CGGUC = K199093 (CGGTC RFP mutation and CGGUC tRNA), K95 CCACU = K199095 (CCACT RFP mutation and CCACU tRNA), K56 CCAUC (9) = K199056 (CCATC RFP mutation and CCAUC (9) tRNA), K57 CCAUC = K199057 (CCATC RFP mutation and CCAUC tRNA).

Table 1. Frameshift suppressor tRNAs

<table>
<thead>
<tr>
<th>tRNA</th>
<th>Registry number</th>
<th>Anticodon Loop</th>
<th>5-base binding site</th>
<th>4-base binding site</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGGUC</td>
<td>K199028</td>
<td>UU GACG AC</td>
<td>CGGUC</td>
<td>GGCU</td>
</tr>
<tr>
<td>CCAUC</td>
<td>K199002</td>
<td>CU AGUG AC</td>
<td>CCAU</td>
<td>CCAU</td>
</tr>
<tr>
<td>CCAC (9)</td>
<td>K199007</td>
<td>GU GAUG GA</td>
<td>CCAUC</td>
<td>none</td>
</tr>
<tr>
<td>CCAUC</td>
<td>K199008</td>
<td>UUU GAUG AG</td>
<td>CCAUC</td>
<td>none</td>
</tr>
<tr>
<td>CAUGU</td>
<td>K199001</td>
<td>IU ACGU AC</td>
<td>CLAGU</td>
<td>CLAGU</td>
</tr>
<tr>
<td>CCAAU (a)</td>
<td>K199047</td>
<td>CU AUUUG AC</td>
<td>CCAAU</td>
<td>CGAG</td>
</tr>
<tr>
<td>AGGAC (b)</td>
<td>K199014</td>
<td>GU GUCCU AA</td>
<td>AGGAC</td>
<td>GGCAG</td>
</tr>
<tr>
<td>CUACC (c)</td>
<td>K199045</td>
<td>GU GGUG GA</td>
<td>CLACC</td>
<td>CLAC</td>
</tr>
<tr>
<td>CCACC (d)</td>
<td>K199048</td>
<td>IU GGUG GA</td>
<td>CCAAC</td>
<td>CCAG</td>
</tr>
<tr>
<td>CUAGC (e)</td>
<td>K199016</td>
<td>CU CCGU AA</td>
<td>CUAGC</td>
<td>none</td>
</tr>
<tr>
<td>CUAUC (f)</td>
<td>K199046</td>
<td>IU AGUG AU</td>
<td>CUAU</td>
<td>UACU</td>
</tr>
</tbody>
</table>

Frameshift suppressor tRNAs cloned and used in the study are listed with their Registry of Standard Biological Parts numbers, the base sequences of their anticodon loops, their expected mRNA codon binding sites according to the five-base codon/anticodon model, and their binding sites hypothesized on the basis of four-base codon/anticodon interactions. Bases from five-base binding sites used in hypothesized four-base binding sites are underlined. Bases in hypothized binding sites that are proposed to form non-Watson-Crick base pairs are underlined.

Table 2. Frameshift suppressor leader mutations

<table>
<thead>
<tr>
<th>Registry number</th>
<th>Gene</th>
<th>Frameshift Suppressor Leader (FSL) mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>K199093 (K93)</td>
<td>RFP</td>
<td>CGG TC</td>
</tr>
<tr>
<td>K199095 (K95)</td>
<td>RFP</td>
<td>CCA CT</td>
</tr>
<tr>
<td>K199056 (K56)</td>
<td>RFP</td>
<td>CCA TC</td>
</tr>
<tr>
<td>K199057 (K57)</td>
<td>RFP</td>
<td>CCA TC</td>
</tr>
<tr>
<td>J100015 (J15)</td>
<td>Lux</td>
<td>CCA CTG GTA GT</td>
</tr>
<tr>
<td>J100016 (J16)</td>
<td>Lux</td>
<td>CCA CTG GTA GT</td>
</tr>
<tr>
<td>J110056 (J56)</td>
<td>T7 RFP</td>
<td>CTA GCG CTC GCT CAC GTA GGA C</td>
</tr>
<tr>
<td>J110057 (J57)</td>
<td>T7 RFP</td>
<td>CTA GCG CTC GCT CAC GTA GGA C</td>
</tr>
<tr>
<td>J110058 (J58)</td>
<td>T7 RFP</td>
<td>CTA GCG CTC GCT CAC GTA GGA C</td>
</tr>
<tr>
<td>J110059 (J59)</td>
<td>T7 RFP</td>
<td>CTA GCG CTC GCT CAC GTA GGA C</td>
</tr>
</tbody>
</table>

Frameshift suppressor Leader (FSL) mutations are listed for RFP, Lux, and T7 RFP gene constructs used in this study. Registry of Standard Biological Parts numbers are also listed. In-frame stop codons are underlined.
K32, which is a construct with a frameshift suppressor tRNA driven by pBAD but no RFP reporter gene. The CGGUC and CCACU codon constructs have the frameshift suppressor tRNA downstream of the reporter, and show no increase in fluorescence compared with the negative control. The CCAUC (9) frameshift suppressor tRNA has nine nucleotides in the anticodon loop instead of ten, in the CCAUC tRNA. The CCAUC (9) tRNA was upstream of the RFP reporter gene in the direct suppression measurement device and showed no measurable fluorescence compared with the negative control. The increase in RFP expression can be interpreted in terms of frameshift suppression involving a five-base codon/anticodon interaction. Successful but weak detection of frameshift suppression caused us to redesign the detection system in a way that would amplify rare frameshift suppression events.

**LuxI positive feedback device**

We sought to amplify the frameshift suppression signal by adapting a positive feedback loop containing the LuxI quorum sensing system. The LuxI enzyme synthesizes the autoinducer 3OC6, which binds with the LuxR regulatory protein. LuxR bound to 3OC6 activates transcription from the pLux promoter, which in turn drives more transcription of the LuxI gene. As shown in Figure 2A, we incorporated a FSL mutation immediately following the start codon of the LuxI gene and cloned the new device into a high copy plasmid. If the mutation is suppressed by frameshift suppressor tRNAs, carried on a low copy plasmid, functional LuxI enzyme would be produced. Functional LuxI would produce 3OC6, which in turn would bind with LuxR to activate the pLux promoter, producing more LuxI and GFP mRNAs. Each frameshift suppression event results in the production of LuxI enzyme capable of producing many autoinducer molecules, leading to more transcription from the pLux promoter of not only the LuxI gene but also the GFP reporter gene. The positive feedback of LuxI and GFP production would amplify frameshift suppression signal output. This would occur at the cellular population level because of the quorum sensing communication system.

To develop frameshift suppressor logic and investigate the

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**Figure 2.** LuxI positive feedback device for frameshift suppression measurement. (A) The top diagram is for J10001 and the bottom diagram is of the genetic circuit used for K199180, J10015, and J10016. Suppression of Frameshift Suppressor Leader (FSL) mutations by frameshift suppressor tRNA(s) leads to LuxI expression and positive feedback. (B) Normalized RFP fluorescence of bacteria grown in liquid culture graphed for the indicated transformants and cotransformants. K180/1A3 = K199180 (wild type LuxI) in pSB1A3, J11/2K3 = J10011 in pSB2K3 (pLpp + CCACU, CUAGU, and CGGUC tRNAs), J15/1A3 = J100015 (CCACT OR CTAGT mutation in LuxI) in pSB1A3, J16/1A3 = J100016 (CCACT OR CTAGT AND CCAGC OR CCAGC mutation in LuxI) in pSB1A3. (C) Normalized RFP fluorescence of bacteria grown in liquid culture versus solid medium graphed for J16/1A3 + J11/2K3 cotransformants. (D) Bacteria transformed with either K180 (wild type LuxI) or J15 (frameshift mutation in LuxI cultured on solid medium). Pictures of the two plates were taken at the three indicated time points.
function of frameshift suppressor tRNAs, we built two LuxI positive feedback devices (Table 2). J15 has a FSL mutation that includes a binding site for the CCACU tRNA and the CUAGU tRNA. The FSL is designed to encode a logical clause of the form (CCACU OR CUAGU), which would be true if either the CCACU tRNA input or the CUAGU tRNA input were present. Construct J16 has an FSL designed to test the possibility of encoding the more complex logical clause of (CCACU OR CUAGU) AND (CGGUC OR CCACU). Suppression of the J16 FSL mutation is expected to occur with binding of either the CCACU tRNA or the CUAGU tRNA and a second binding event by either the CGGUC tRNA or the CCACU tRNA. Figure 2B shows the GFP fluorescence in liquid culture of these two LuxI positive feedback devices. The positive control is K180, which contains a wild type LuxI gene as part of the lower circuit shown in Figure 2A. It does not need suppression to activate the positive feedback mechanism and produce large amounts of GFP. As expected by the introduction of FSL mutations, both J15 and J16 constructs produced very little GFP in this liquid culture experiment. Cotransformation of J15 with construct J11, which encoded three tRNAs (CCACU, CUAGU, and CGGUC), resulted in higher GFP expression than even the positive control K180 device. The results for J15 can be interpreted in terms of a suppression event with binding by a five-base anticodon from either the CCACU tRNA or the CUAGU tRNA. In other words, the logical clause encoded in J15 was found to be true in the presence of the three input tRNAs. We measured a lower level of GFP expression in bacteria cotransformed with constructs J16 and J11. The reduced GFP fluorescence could be interpreted as being caused by two five-base suppression events which would be less common than single suppression events. It appears that the logical clause encoded in the FSL of J16 was found to be true, by successful double suppression in the presence of the three tRNA inputs from J11.

In the course of our LuxI positive feedback investigations, we observed an unexpected difference in the expression of the GFP reporter in liquid culture versus solid media plates. Figure 2C shows the results of comparing the fluorescence of the J16 construct in liquid culture to that on solid media plates. Colonies grown on plates were suspended in liquid LB media for fluorescence measurements. The fluorescence from the plated bacteria was significantly higher than that of bacteria grown only in liquid culture. The increase in fluorescence by bacteria grown on plates also extended to our negative control constructs even in the absence of frameshift suppressor tRNAs. Specifically, the frameshifted J15 and J16 devices showed equal GFP expression to the K180 positive control that lacked a frameshift mutation. In Figure 2D, bacteria containing K180 with the wild type LuxI are compared with bacteria transformed with J15, which has a FSL mutation in the LuxI gene. The J15 bacteria show a greater increase in green fluorescence over time than the K180 bacteria. These solid medium results contradict the liquid culture fluorescence data presented in Figure 2B. We suggest that an explanation for the discrepancy may lie in the difference in efficiency between quorum sensing on solid medium where bacteria are in direct physical contact with each other versus liquid culture where they are not. On solid medium, local concentrations of 3OC6 autoinducer produced by leaky transcription of LuxI and GFP or endogenous E. coli autoinducers might reach high enough levels to initiate positive feedback and cause significant GFP expression in the absence of frameshift suppression. In response to the unexpected solid medium results of the LuxI positive feedback design, we designed an alternative mechanism for amplification of frameshift suppression events.

**T7 RNA polymerase amplification device**

Our alternative design for amplification of the frameshift suppression signal was based on the viral T7 RNA Polymerase (RNAP; Figure 3A). The FSL was engineered to follow the start codon for T7 RNAP cloned on a low copy number plasmid. Production of functional T7 RNAP would lead to transcription from a T7 promoter (pT7) upstream of a RFP reporter gene, carried on a high copy number plasmid. The high copy plasmid also contained either three frameshift suppressor tRNA genes.

![Figure 3. T7 RNAP amplification device for frameshift suppression measurement.](image-url)
or one frameshift suppressor tRNA, as shown in Figure 3A. Figure 3B shows the T7 RNAP positive control after cotransformation of a construct containing the wild type T7 RNAP gene with a RFP reporter construct. The negative control experiment with bacteria transformed only with the RFP reporter construct regulated by pT7 did not show RFP expression. We introduced FSL mutations into the coding sequence for T7 RNAP which prevented the production of functional T7 RNAP, resulting in no RFP signal. The positive and negative controls of T7 RNAP frameshift suppression amplification system functioned as intended so we were ready to use suppressor tRNAs for logical clause interrogation.

We built four T7 RNAP mutants (Table 2) to test frameshift suppressor logic and investigate the function of frameshift suppressor tRNAs. Each of the constructs (J56, J57, J58, and J59) has an FSL that encodes a logical clause in the form (tRNA1 OR tRNA2) AND (tRNA3 or tRNA4), as detailed in Table 2. The tRNA binding sites are spaced in such a way that two suppression events resulting from two five-base codon/anticodon interactions are required for suppression to occur. We also assembled RFP reporter constructs with seven combinations of three tRNAs (labeled as a-f in Table 1). Figure 4A shows the cotransformation results with these seven reporter constructs and four T7 RNAP FSL mutants. Thirteen of the twenty-eight cotransformations resulted in RFP expression levels comparable to the positive control. The data are consistent with an initial hypothesis of five-base codon/anticodon interactions is inconsistent with over half of the experiments (15 out of 28).

To investigate these unexpected results, we constructed a series of six T7 RNAP FSL mutants that each contained one of the five-base frameshift mutations cognate to the six frameshift suppressor tRNAs used for the experiments in Figure 4. We assembled six reporter constructs, each with pT7-regulated RFP and one of the six frameshift suppressor tRNA genes. We cotransformed all 36 combinations of the six reporter constructs and the six T7 RNAP FSL mutants. The five-base codon/anticodon interaction hypothesis predicts that each of the six T7 FSL mutants would be suppressed by its cognate frameshift suppressor tRNA, leading to RFP expression. However, none of the 36 cotransformations showed RFP expression (data not shown). We also cotransformed the T7 RNAP mutants containing single tRNA binding sites with the seven RFP reporter constructs containing combinations of three tRNAs (data not shown). Although the five-base codon/anticodon interaction hypothesis predicts that half of these 42 cotransformations would result in RFP expression, none of them did. Despite this unexpected outcome, these results were consistent with the 36 cotransformation experiments using single tRNA reporter constructs. These results from 78 cotransformations are inconsistent with our direct suppression measurements where a five-base FSL mutation was suppressed by its cognate frameshift suppressor tRNA (see Figure 1B). The contradictory results caused us to doubt whether the results in Figure 4 could be accurately explained using five-base codon/anticodon interactions.

We derived additional insight into the unexpected behavior of the frameshift suppressor tRNAs by performing cotransformations with 24 combinations of six single tRNA reporters and four mutant T7 RNAP constructs containing 22-nucleotide FSL mutations of the form (tRNA1 OR tRNA2) AND (tRNA3 or tRNA4) (Figure 5A). Predictions based on five-base codon/anticodon suppression events are indicated in Figure 5A, with a + sign over conditions that were predicted to show suppression (RFP expression) and a – sign over those that were not (no RFP expression). Predictions from the hypothesis that match the data have a green +/- and those that do not have a red +/- . The hypothesis of five-base codon/anticodon interactions is inconsistent with 7 of the 24 experiments. Once again, the data con-
Deduced rules for suppressor tRNA binding

We considered several hypotheses to explain the unexpected results. Figure 6 shows suppressor tRNAs AGGAC and CUACC with the J58 T7 RNAP mRNA according to the five-base binding hypothesis. The same two tRNA and mRNA combinations are also shown interacting according to an alternative four-base binding hypothesis that we developed to explain our results. The new four-base anticodon begins with the first base of the original five-base anticodon for the AGGAC tRNA and with the second base of the original five-base anticodon for the CUACC tRNA. As listed in Table 1, we used the four-base binding hypothesis to explain the behavior of five of the six frameshift suppressor tRNAs used in the T7 RNAP cotransformations. The four-base anticodon begins with the first base of the original five-base anticodon for tRNAs AGGAC and CUACC, and with the second base for the CCAAU, CUACC, and CCACC tRNAs. The remaining tRNA, CUAAC, is hypothesized to have no binding site in the mutations tested. We invoked a wobble rule of a G in the first, or 5’ most, position of the anticodon for the CCAAU tRNA. The FSL mutations listed in Table 2 for J56, J57, J58, and J59 and our newly deduced four-base interaction hypothesis reveal that only one four-base suppression event would be necessary and sufficient for translation to continue past the FSL mutation. Two of the FSL mutations, J56 and J57, contain in-frame stop codons, so the suppression event must occur upstream of those codons. We used our experimentally deduced four-base interaction hypothesis to generate Figure 5B, which has a + sign when the rules call for suppression (RFP produced) and a − sign when they do not (no RFP produced). The deduced four-base codon/anticodon interaction model is consistent with all 24 experimental results, as indicated by a green color for all of the +/- signs. The four-base interaction rules were applied to the 28 results in Figure 4A where 7 RFP reporter constructs containing 3 tRNAs each were cotransformed with 4 mutant T7 RNAP constructs containing 22-nucleotide FSL mutations. The 28 results are reproduced in Figure 4B overlaid with + and − signs as predictions from the four-base interaction rules. The deduced rules accurately predict each of the twenty-eight experimental outcomes, as indicated by the green color for all of the +/- signs. The four-base interaction rules also provide an alternative explanation for the results of one of our LuxI positive feedback experiments (see Figure 2B). J16 suppression could occur with a single four-base suppression event by use of CCAC interaction with the CCAAU tRNA, a CUAG interaction with the CUAAC tRNA, or GGUC interaction with the CGGUC tRNA. The reinterpretations of data from Figure 2B using four-base interactions are included in Table 1.

Figure 5. Frameshift suppression by single tRNA constructs measured with T7 RNAP amplification device. Bacteria cotransformed with RFP reporter constructs containing the single tRNAs listed vertically and T7 RNAP mutant constructs listed horizontally were grown on solid medium. Images are overlaid with a + sign for combinations predicted to show suppression (RFP production) by the five-base (Part A) or four-base (Part B) codon/anticodon interaction hypothesis and a − sign for those not predicted to show suppression (no RFP production). Each +/- sign is colored green if the prediction from the hypothesis matches the experimental result and red if it does not.

Figure 6. Comparison of 5-base and 4-base binding models. Anticodon loops from the AGGAC and CUACC frameshift suppressor tRNAs (red) are shown interacting with J19058 T7 RNAP mRNA (blue) according to the 5-base and 4-base binding models.
CONCLUSION AND PROSPECTS

Dual functionality of frameshift suppressor tRNAs

The results of our experiments led us to hypothesize that the frameshift suppressor tRNAs originally described as using five-base anticodon/codon interactions in a selection system were primarily using four-base interactions in our devices. An explanation for this difference in behavior can be found by comparison of the two experimental approaches. In the system used by Anderson et al., five-base frameshift mutations were introduced in place of a codon that encodes a serine in the catalytic site for β-lactamase. This design incurred strong selection for antibiotic resistance, since failure to produce five-base frameshift suppression results in cell death. This allowed rare five-base frameshift suppression events by tRNAs to contribute to the phenotype of ampicillin resistance. In our direct suppression measurement experiments, we saw frameshift suppression in only one out of four frameshift suppressor tRNAs. This is likely because we were using a RFP reporter instead of antibiotic resistance, with no selective pressure on five-base frameshift suppressions. As a result, rare five-base frameshift suppression events failed to produce detectable levels of RFP reporter protein. Although we attempted to amplify the signal of frameshift suppression in our LuxI positive feedback and T7 RNAP amplification systems, our results showed that even the amplified systems are not sensitive enough to detect rare five-base binding by the frameshift suppressor tRNAs. Our devices did not have as much sensitivity to rare suppression events as the antibiotic resistance selection system used by Anderson et al. Nonetheless, we did measure suppression in experiments where the frameshift suppressor tRNAs could use four-base anticodon/codon interactions, and we proposed binding hypotheses to explain the results. Our analysis led to the hypothesis that these frameshift suppressor tRNAs possess a dual functionality, and are capable of binding with either four-base or five-base anticodon/codon interactions. In circumstances where five-base binding is required, such as the β-lactamase selection experiments, rare five-base interactions can be detected. In experiments where four-base binding can allow for frameshift suppression, such as our LuxI and T7 RNAP experiments, four-base interactions appear to be more common than five-base interactions.

The mechanism of frameshift suppression

Several published mechanisms can contribute to our interpretation of the unexpected behaviors of frameshift suppressor tRNAs in our study. For example, Magliery et al. also used four-base codon reporter libraries to identify “shifty” sites in mRNA were +1 frameshifting occurs with the highest frequency. This +1 shift mechanism could be used to explain our frameshift suppression results involving two five-base FSL mutations in the J15 LuxI construct, or for many of the frameshift suppressions measured in the T7 RNAP amplification system. If a shifty site occurred in the LuxI mRNA anywhere before a stop codon, suppression could occur in the J15 LuxI device by use of a four-base frameshift suppression instead of five-base suppression. In the T7 RNAP experiments, shifty sites could completely eliminate the need to propose frameshift suppression. An alternative hypothesis derives from mutations in an E. coli valine tRNA that increase the frequency at which the tRNA can “hop” from a cognate codon in the mRNA to a downstream site. The valine tRNA mutations were anticodon loop insertions, substitutions in the anticodon stem, and a deletion in the variable loop. In our LuxI and T7 RNAP experiments, if frameshift suppressor tRNAs were able to bind using a four-base codon/anticodon interaction and were also able to hop one base, then a five-base mutation could be suppressed. Alternatively, tRNA hopping by normal three-base anticodon tRNAs could result in successful translation without frameshift suppression. Yet another alternative hypothesis could be constructed from the observation that increased levels of wild type glycine tRNA capable of binding to GGG codons in E. coli increased the level of +1 frameshifting, with near cognate GGA codons read as GA. Especially since frameshift suppressor tRNAs are expressed at high levels in our experiments, this two-out-of-three hopping mechanism could explain our unexpected frameshift suppression results. Finally, another alternative hypothesis could come from a study showing that partial inactivation of a protein that functions as a translation termination factor in yeast led to nonsense suppression and frameshift suppression. Perhaps such an interaction between termination factors and frameshift suppressor tRNAs allowed frameshift suppression to occur in our bacterial experiments.

Applications of frameshift suppressor logic

Improved understanding of the function of frameshift suppressor tRNAs could lead to the development of a modular frameshift suppressor logic system. Such a system would be adaptable to the purpose of encoding logic gates such as OR and AND, as well as more complicated logical clauses. Using tRNAs for logic gates would in turn benefit the field of biological computing, in which solutions to mathematical problems are found by living systems. Frasemshift suppressor logic could be used to program bacteria to process environmental inputs and respond with changes in gene expression that are appropriate for biosensing or bioremediation. Metabolic engineering could benefit from the ability to control populations of bacteria that cooperate by processing inputs with logic to carry out metabolism. Frasemshift suppressor logic could also be used in eukaryotic cells, greatly expanding its range of applications. Many medical applications could derive from the ability to program bacteria to process environmental inputs and respond with changes in gene expression that are appropriate for biosensing or bioremediation.
Synthetic biology bridges engineering and science
In the course of the current study and others’\textsuperscript{14-15}, we have found that research in synthetic biology can be thought of as a winding path that travels back and forth through engineering and science, as depicted in Figure 7. The path usually begins within the realm of science as knowledge of the natural world leads to an engineering application. Synthetic biologists apply the engineering principles of standardized parts and assembly and levels of abstraction to the design and construction of systems for particular applications. Once the system is built, synthetic biologists measure its function. If a system behaves as expected, investigators develop additional engineering applications by incorporating the system as a whole into other systems, or by using its component devices or parts in other systems. If the system does not work as expected, synthetic biologists use what they measured to generate new questions that could lead to basic scientific investigations of the natural system upon which it was based. The subsequent research moves synthetic biologists from an engineering approach to a biological research approach. Follow-up questions could result in experiments that generate new knowledge. There is also a spin-off effect into other basic research questions and experiments that generate more scientific investigations. Better understanding of the underlying biological mechanisms can then lead to improved engineering applications as the synthetic biologist transitions again from scientist to engineer. The winding back and forth between engineering and science is neither predictable nor alternating.

MATERIALS AND METHODS

Bacterial growth
Bacteria were incubated at 37°C in LB medium prepared by dissolving 10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl in 800 mL water, adjusting the pH to 7.5 with NaOH, and adjusting the final volume to 1 L. Plates were prepared by addition of 15 g agar. After sterilization and cooling, ampicillin was added to final concentrations of 50 µg/mL. For cotransformations, chloramphenicol was also added to a final concentration of 35 µg/mL.

BioBrick assembly
All DNA parts and sub-parts produced for this project were generated using the standard BioBrick assembly protocol\textsuperscript{16}, and registered on the Registry of Standard Biological Parts\textsuperscript{21}. The Zymo Research Zyppy Plasmid Miniprep Kit (cat. D4020) was used according to the manufacturer’s instructions to extract plasmid DNA. BioBrick parts were digested with restriction enzymes, EcoRI, XbaI, SpeI and PstI to produce ‘sticky ends’ that were used to combine BioBrick parts. Digested DNA was purified using agarose gel electrophoresis followed by gel purification with the Zymoclean Gel DNA Recovery Kit (cat. D4002). BioBrick parts with matching ‘sticky ends’ were ligated for 5 minutes at room temperature using Promega T4 ligase (cat. M1801) and 2X Rapid Ligation Buffer (cat. C6711). Plasmid DNA was transformed into Zymo Research JM109 Z-Compe-
tent E. coli cells (cat. T3005). Cotransformations were conducted with 40 ng of each plasmid, outgrowth in SOC medium for 1 hour, and plating on solid medium containing both ampicillin and chloramphenicol. Colonies containing plasmids after ligation and transformation were screened by PCR using Promega GoTaq Green Master Mix (cat. M7123) with the appropriate primers and template DNA. All final constructs were sequenced for verification.

**Construction of basic parts**

All eleven 143 base pair frameshift suppressor tRNAs used in the study were derived from the study by Anderson et al.\(^\text{13}\). Oligonucleotides that overlap for 20 base pairs were designed for the 5’ and 3’ halves of a given tRNA sequence and were synthesized by Integrated DNA Technologies or Eurofins MWG\textregistered\Operon. The oligonucleotide for the 5’ half of the tRNA included the standard 22 nt BioBrick prefix, consisting of three different restriction sites, EcoRI, NotI and XbaI, plus four bases (GCAT) to facilitate EcoRI digestion at the 5’ end. The oligonucleotide for the 3’ half of the tRNA included the restriction sites Spel, NotI and PstI to form the standard 21 nt BioBrick suffix. The four bases GCAT were added to the 5’ of the primer to facilitate PstI digestion of the 3’ end. The two oligonucleotides were used for primer-dimer PCR amplification and the resulting 194 bp PCR product was purified with the Zymo Research DNA Clean & Concentrator (cat. D4003). Digestion of the PCR product with EcoRI and PstI was followed by agarose gel electrophoresis and purification of the 180 bp fragment. Digested tRNA gene fragments were ligated into pSB1A2 digested with EcoRI and PstI and the products were transformed into JM109 competent cells. The resulting frameshift suppressor tRNA parts were sequence verified and entered in the Registry of Standard Biological Parts with the part numbers listed in Table 1. The anticodon sequence of BBa_K199007 is correctly listed is Table 1 and in the Registry of Standard Biological Parts as part numbers BBa_J199056, BBa_J199057, BBa_J199058, BBa_J199059, and are listed in Table 2. Along with BBa_J119009, these parts are entered in the Registry as basic parts because they were synthesized as single units with a standard prefix and suffix, but they could be considered to be composite parts because they include a promoter, an RBS, and a coding sequence.

**Construction of composite parts**

Constructs used for the direct measurements of frameshift suppression reported in Figure 1A were assembled in the high copy number vector pSB1A2 using the basic parts described above and existing Registry parts. The negative control BBa_K199032 was built by ligation of BBa_J13453 (pBAD) to BBa_K199008 (CCAUC tRNA). BBa_K199093 was constructed from BBa_K199068 (pLacI + RBS + CGGTC-RFP) and BBa_S04281 (pTetR+CGGUC tRNA). BBa_K199095 was constructed from BBa_K199066 (pLacI + RBS + CCATC-RFP) and BBa_S04279 (pTetR+ CCAUC tRNA). BBa_K199065 was constructed from BBa_K199031 (pBAD + CCAUC (9) tRNA) and BBa_K199065 (pLacI + RBS + CCATC-RFP). BBa_K199057 was constructed for introduction of frameshift mutations into LuxI, we designed basic part BBa_K199130, which includes unique sites for “eight-cutter” restriction enzymes AsISI and Ascl immediately after the ATG start codon for LuxI. Part BBa_K199130 was synthesized by Eurofins MWG\textregistered\Operon and codon optimized for E. coli. Oligonucleotides were designed with sticky ends for AsISI and Ascl flanking a given frameshift mutation. The oligos were mixed with 1× annealing buffer [100 mM NaCl; 10 mM Tris-HCl, pH 7.4], so that the final concentration of each oligo was 5 \(\mu\)M. This solution was boiled for 10 minutes and allowed to slowly cool to room temperature. The annealed oligos were ligated into a BBa_K199130 digested with AsISI and Ascl, and transformed into JM109 cells. The LuxI frameshift mutation parts were sequence verified and entered in the Registry of Standard Biological Parts as part numbers BBa_J100013 (used to construct BBa_J100015) and BBa_J100014 (used to construct BBa_J100016).

Frameshift mutations were introduced into the T7 RNA Polymerase gene using the same strategy employed for the LuxI gene described above. Part BBa_J119009 was synthesized by Life Technologies GeneArt and includes a pLacI promoter, B0030 RBS, and the T7 RNAP gene with unique sites for AsISI and Ascl immediately after the ATG start codon. Oligonucleotides were designed with sticky ends for AsISI and Ascl flanking a given frameshift mutation. The oligonucleotides were annealed and cloned into BBa_J119099 carried on a low copy number pSB4C5 plasmid. The resulting T7 RNAP frameshift mutation parts were sequence verified and entered in the Registry of Standard Biological Parts as part numbers BBa_J119056, BBa_J119057, BBa_J119058, BBa_J119059, and are listed in Table 2. Along with BBa_J119009, these parts are entered in the Registry as basic parts because they were synthesized as single units with a standard prefix and suffix, but they could be considered to be composite parts because they include a promoter, an RBS, and a coding sequence.
from BBa_K199032 (pBAD + CCAUC tRNA) and BBa_K199065 (pLacI + RBS + CCATC-RFP).

The LuxI positive feedback constructs illustrated in Figure 2A were cloned from existing parts into the high copy number plasmid pSB1A2 as follows. BBa_S04424 was assembled from BBa_B0015 (double transcription terminator), BBa_S0500 (pLux + RBS B0034), and BBa_K199130 (wild type LuxI). BBa_K199175 was assembled from BBa_E5500 (RBS B0030 + GFP), BBa_S04509 (pLacI + RBS B0034), BBa_C0062 (LuxR), and BBa_B0015 (double transcription terminator). BBa_K199180 was constructed from BBa_S04424 and BBa_K199175. BBa_J100015 and BBa_J100016 were assembled from the same parts as BBa_K199180, except the mutant LuxI genes BBa_J100013 and BBa_J100014 were used instead of the wild type LuxI gene. BBa_J100011 was assembled in the low copy number plasmid pSB2K3 with BBa_K199106 (pLpp promoter) in front of each of the three tRNA genes: BBa_K199002 (CCAUU tRNA), BBa_K199001 (CJJAGU tRNA), and BBa_K199028 (CGGUC tRNA).

Assembly of the RFP reporter constructs illustrated in Figure 3A began with the construction of BBa_K199124 (pT7 + RBS B0034 + RFP + pLacI). This construction intermediate was ligated to each of the six tRNAs CAAAU (a), AGGAC (b), CJJACC (c), CCACC (d), CJJAGC (e), and CJJACU (f) to produce RFP reporter constructs BBa_J119115, BBa_J119117, BBa_J119119, BBa_J119116, BBa_J119118, and BBa_J119120, respectively. BBa_K199124 was also ligated to combinations of three tRNAs to produce BBa_J119100 (tRNAs a,b,c), BBa_J119095 (tRNAs a,b,f), BBa_J119093 (tRNAs a,c,e), BBa_J119099 (tRNAs a,e,f), BBa_J119096 (tRNAs b,d,f), BBa_J119097 (tRNAs c,d,e), and BBa_J119098 (tRNAs d,e,f). Each of the reporter constructs was carried on the high copy number plasmid pSB1A2.

Measuring fluorescence

For measurement of fluorescence, 2 mL of liquid media was inoculated with the appropriate cells and incubated for 18 hours at 37°C with shaking. After incubation, 600 μL of each construct-media combination was transferred to a microwell plate in 200 μL triplicates. The microwell plate was analyzed using a fluorometer to obtain an absorbance reading and fluorescence intensity for each 200 μL sample. Each fluorescence intensity value was divided by its corresponding absorbance (595 nm) reading to account for the varying levels of growth in each culture tube. Triplicates were then averaged. To measure red fluorescence, the fluorometer was set at 540 nm for excitation and 600 nm for emission. To measure green fluorescence, the fluorometer was set at 485 nm for excitation and 528 nm for emission.

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