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Exploiting preQ$_1$ Riboswitches To Regulate Ribosomal Frameshifting

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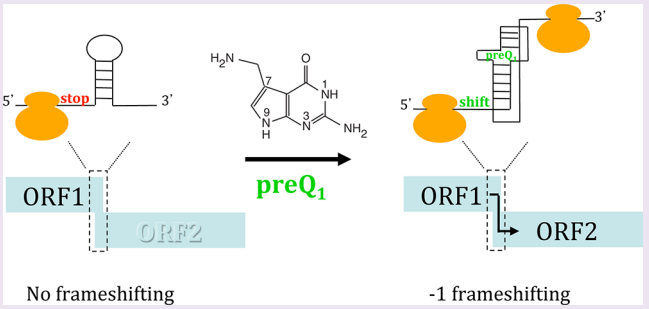
Supporting Information

ABSTRACT: Knowing the molecular details of the interaction between riboswitch aptamers and their corresponding metabolites is important to understand gene expression. Here we report on a novel *in vitro* assay to study preQ$_1$ riboswitch aptamers upon binding of 7-aminomethyl-7-deazaguanine (preQ$_1$). The assay is based on the ability of the preQ$_1$ aptamer to fold, upon ligand binding, into a pseudoknotted structure that is capable of stimulating −1 ribosomal frameshifting (−1 FS). Aptamers from three different species were found to induce between 7% and 20% of −1 FS in response to increasing preQ$_1$ levels, whereas preQ$_1$ analogues were 100−1000-fold less efficient. In depth mutational analysis of the *Fusobacterium nucleatum* aptamer recapitulates most of the structural details previously identified for preQ$_1$ aptamers from other bacteria by crystallography and/or NMR spectroscopy. In addition to providing insight into the role of individual nucleotides of the preQ$_1$ riboswitch aptamer in ligand binding, the presented system provides a valuable tool to screen small molecules against bacterial riboswitches in a eukaryotic background.



Standard translation of a mRNA proceeds by linear decoding of non-overlapping triplets but for functional reasons can sometimes be overruled to permit translation of an alternative reading frame, a process referred to as ribosomal frameshifting (reviewed in refs 1−3). Most reported examples involve −1 ribosomal frameshifting (−1 FS) where translating ribosomes slip one nucleotide (nt) into the 5′-direction (−1 reading frame) on the mRNA and generate an alternative protein. 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 slip site where the ribosome changes reading frame with consensus X XXY YYZ [where X, Y are any nucleotide, Z ≠ Y, and spaces denote the initial reading frame][4] and (ii) a stimulatory RNA structure, a hairpin or a pseudoknot, downstream of the slip site [reviewed in refs 5 and 6]. 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.[7,8] Although primarily found in mammalian, plant, and bacterial viruses and a number of transposons,[9,10] a growing body of evidence shows that −1 FS is involved in decoding bacterial, worm, and mammalian cellular genes.[11−15] Moreover, the propagation and infectivity of some clinically relevant RNA viruses, such as *Human immunodeficiency virus* type-I (HIV−I), are negatively affected by modulating −1 FS efficiency,[16] representing −1 FS as a promising therapeutic target to interfere with viral infections. Recently, ligands have been selected that are able to bind the *Severe acute respiratory syndrome coronavirus* (SARS-CoV) FS pseudoknot and inhibit −1 FS.[17]

Therefore, it is of importance to understand how the downstream stimulatory RNA structures affect −1 FS efficiency. It has been suggested by structural and single molecule studies that the mechanical strength of downstream structures correlates with −1 FS efficiency.[18−20] Intriguingly, for simple perfect hairpin structures and antisense oligonucleotide-forming duplexes, it has been shown that their ability to promote −1 FS positively correlates with the calculated Gibbs free energy,[21−23] while there is no clear correlation between stability and FS efficiency in frameshifter pseudoknots.[24] The latter may be due to the hard-to-predict loop-stem interactions within the pseudoknot frameshifters that play a critical role in enhancing mechanical strength and reducing brittleness of these structures.[25−30]

High-resolution structural data is required to specifically define the loop-stem interactions within RNA pseudoknots. In reported frameshifter pseudoknots, however, structural details are available only for those with a short stem 1 [≤6 base-pair (bp)].[6] Importantly, they all share a common structural feature, the presence of loop 3 (L3) and stem 1 (S1) (see Figure 1A for nomenclature of stems and loops of a pseudoknot in this study) tertiary interactions close to the helical junction of the stems. Furthermore, a recently defined hTPK-DU177 pseudoknot derived from human telomerase RNA was shown to rely on

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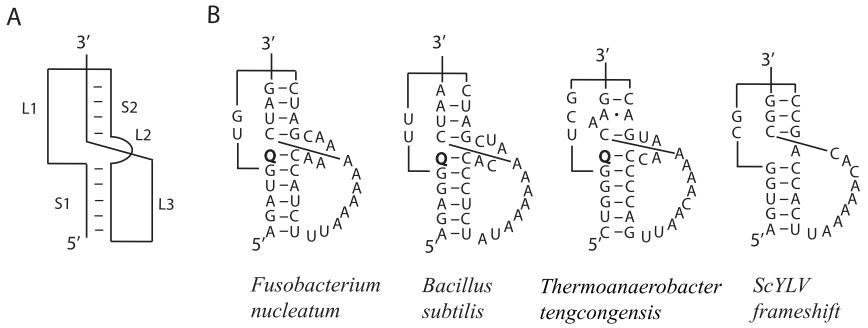

Figure 1. Structural comparison of preQ$_1$ riboswitch aptamers and a frameshifting pseudoknot. (A) Schematic representation of the secondary structure of hairpin (H)-type pseudoknots. The "S" denotes the stem region, and the "L" denotes the loop regions. (B) Secondary structures representation of preQ$_1$-bound aptamers from three indicated bacteria and the *Sugar cane yellow leaf virus* (ScYLV) P1-P2 frameshifting pseudoknot (ScYLV frameshift). Q stands for preQ$_1$.

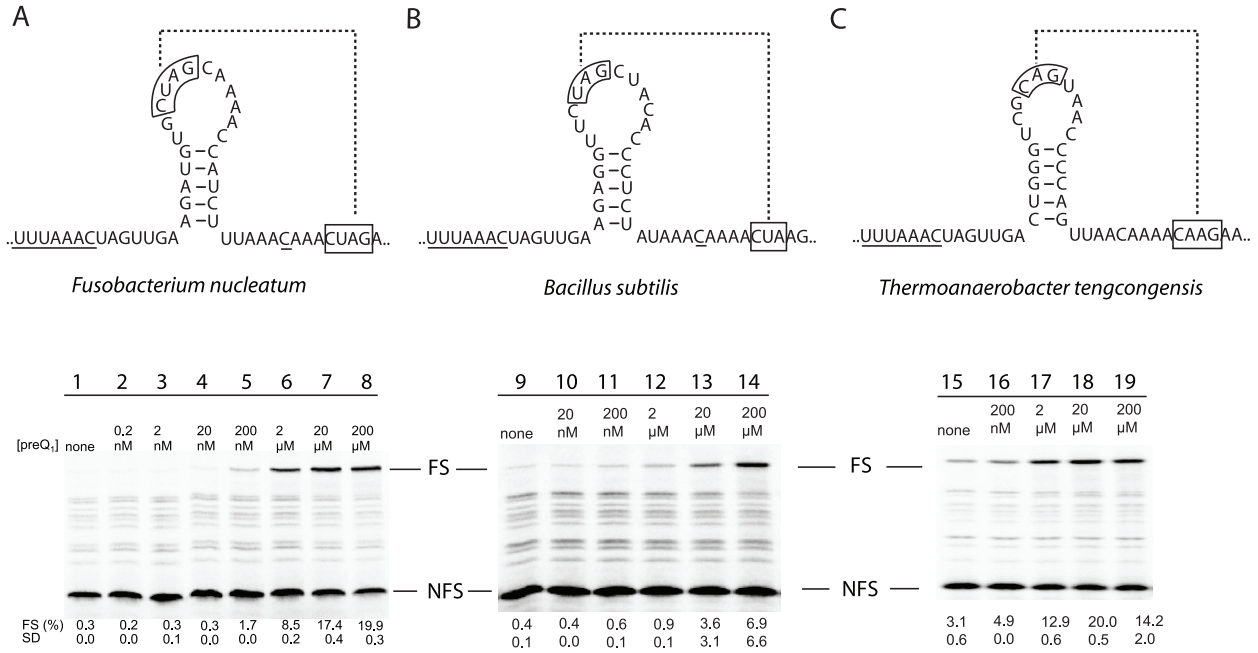

Figure 2. PreQ$_1$ riboswitch aptamers form H-type pseudoknots upon preQ$_1$ binding and induce −1 ribosomal frameshifting (−1 FS) in a concentration-dependent manner. (A−C) preQ$_1$ riboswitch aptamers from *Fusobacterium nucleatum* (Fnu), *Bacillus subtilis* (Bsu), and *Thermoanaerobacter tengcongensis* (Tte), respectively. Note that a C (underlined) was inserted in loop L3 of Fnu and Bsu aptamers to prevent ribosomes from encountering a stop codon 5 nts downstream. Bracketed sequences indicate the formation of base pairs in the presence of preQ$_1$. Slippery sequences are underlined. SDS-PAGE analysis of ^{35}S-methionine labeled translation products in the presence or absence of preQ$_1$ (0−200 μM) in rabbit reticulocyte lysates (RRL). −1 ribosomal frameshifting is monitored by appearance of the 65-kD product (FS). The non-shifted zero-frame product is indicated by NFS. Quantitative analysis of frameshifting efficiency [FS (%)] is described in Methods. Reported values of FS (%) and SD are from at least three independent experiments.

loop 1 (L1)-stem 2 (S2) triples near the helical junction to promote efficient −1 FS,[30] once more highlighting the crucial role of loop-helix interactions.

Riboswitches are gene regulation elements generally located in the 5′-untranslated regions (5′-UTR) of bacterial mRNAs to control gene expression by forming mutually exclusive structures elicited by environmental changes such as the binding of small metabolites or temperature change.[31] Among riboswitches with available structural information, three of them [*S*-adenosylmethionine class II (SAM-II), *S*-adenosylhomocysteine (SAH), and 7-aminomethyl-7-deazaguanine class I (preQ$_1$-I)] can adopt a pseudoknot conformation upon ligand binding.[32] Interestingly, the SAH riboswitch aptamer can induce SAH-dependent −1 FS [33] to a maximal efficiency of 4%. The relatively inefficient −1 FS may be due to the unusual pseudoknot topology of the SAH aptamer.[34] In contrast, the preQ$_1$-I and SAM-II riboswitch aptamers adopt the typical hairpin (H)-type pseudoknot structure, which is selected for most frameshifting signals. The preQ$_1$-I riboswitch controls a set of genes associated with preQ$_1$ biosynthesis.[35] PreQ$_1$ is a biosynthetic precursor of queuosine (Q), which is a hypermodified nucleotide found in the wobble position of GUN anticodons of tRNA^{Tyr}, tRNA^{His}, tRNA^{Asn}, and tRNA^{Asp} and is important for translational fidelity.[36] The preQ$_1$-I riboswitch is only 34 nt in size, and detailed structural information is available for two of them.[37−39]

In the present work, we demonstrate that several wild-type (wt) preQ$_1$ riboswitch aptamers[35] can also function as true ligand-responsive frameshifter pseudoknots. By stabilizing S1 of the preQ$_1$ aptamer of *Fusobacterium nucleatum* (Fnu), about

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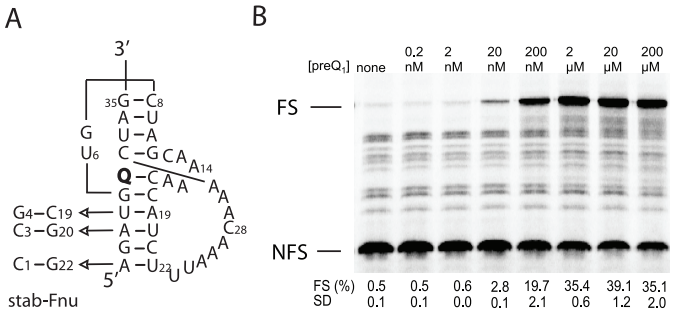

Figure 3. The stabilized version of Fnu (stab-Fnu) induces highly efficient −1 FS upon preQ$_1$ binding. stab-Fnu was constructed by replacing the three A-U and U-A pairs by C-G and G-C pairs in S1 of Fnu as shown. SDS-PAGE analysis of ^{35}S-methionine labeled translation products in RRL using stab-Fnu mRNA in the presence or absence of preQ$_1$. See legend to Figure 2 for more details.

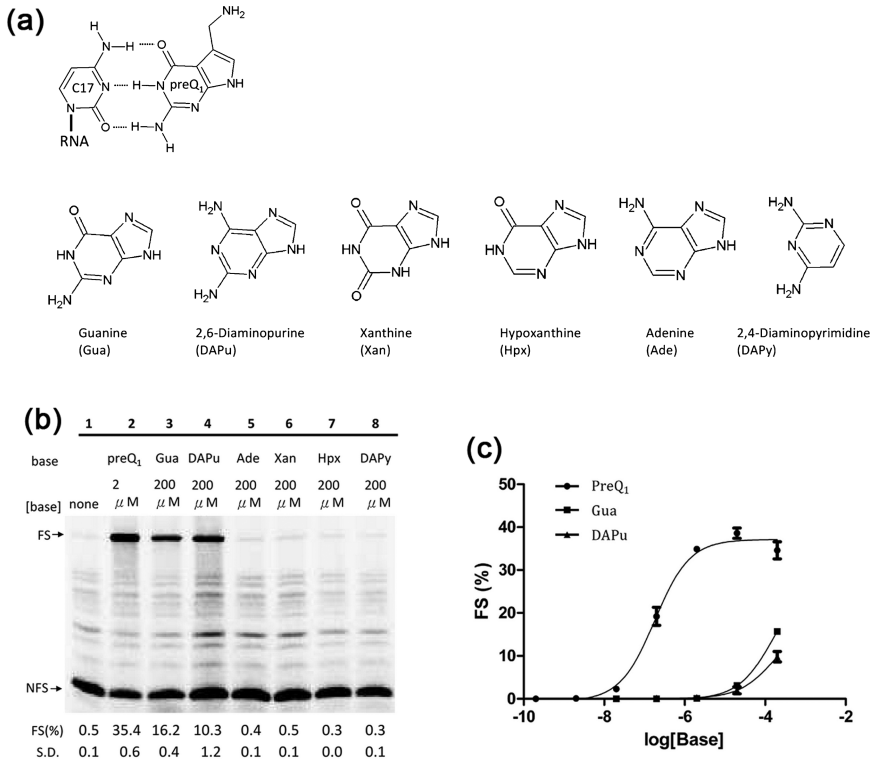

Figure 4. Observation of nucleobase discrimination by preQ$_1$ riboswitch aptamer using −1 FS as a reporter. (A) Schematic representation of Watson−Crick base pairing of preQ1 with C17, the determinant nucleotide of preQ1 riboswitch aptamers. The chemical structure of preQ$_1$-related compounds tested in FS assays is also presented. (B) SDS-PAGE analysis of ^{35}S-methionine labeled translation products in RRL using stab-Fnu mRNA in the absence or presence of preQ1 and preQ1-related nucleobases at indicated concentrations. See legend to Figure 2 for more details. (C) Graph showing the dose−response curves of −1 FS of stab-Fnu induced by various concentrations of preQ$_1$, guanine, or 2,6-diaminopurine.

40% of ribosomes shifted frame upon addition of 20 μM preQ$_1$. In depth mutational analysis of the Fnu aptamer recapitulated most of the structural details previously identified for preQ$_1$ aptamers from other bacteria by crystallography and/or NMR spectroscopy. The preQ$_1$ aptamer-based frameshifting system presented here may be used in high throughput screening of putative antibacterial drugs.

RESULTS

PreQ$_1$ Riboswitch Aptamers Can Stimulate −1 FS. The preQ$_1$ riboswitch aptamers that fold into H-type pseudoknot structures upon ligand binding are structurally similar to typical frameshifting pseudoknots.[6,40] The three preQ$_1$ aptamers used in this study are shown (Figure 1B) in comparison with the *Sugar cane yellow leaf virus* (ScYLV) frameshifter pseudoknot.[27]

Since it has been reported that upon binding of preQ$_1$ these aptamer pseudoknots are greatly stabilized, we asked whether these ligand-induced pseudoknots could promote FS as well. The preQ$_1$ aptamers were cloned into our frameshift reporter construct and subsequently assayed for −1 FS in a rabbit reticulocyte lysate expression system (see Methods). In the absence of preQ$_1$ the *Bacillus subtilis* (Bsu) and Fnu aptamers showed close to zero −1 FS (Figure 2, lanes 1, 9). However −1 FS increased steadily with increasing concentrations of added preQ$_1$, reaching 7% for the Bsu aptamer (Figure 2, lane 14) and 20% for the Fnu aptamer (Figure 2, lane 8) at the highest concentration of preQ$_1$ still compatible with efficient translation (200 μM). Interestingly, the *Thermoanaerobacter tencongensis* (Tte) aptamer exhibited 3.5% of −1 FS in the absence of preQ$_1$ (Figure 2, lane 15) and peaked at 20 μM with 20% (Figure 2,

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lane 18). This result is consistent with the finding that the Tte aptamer forms a pseudoknot structure in the absence of preQ$_1$.[39] In control assays (Supplementary Figure S1) the addition of preQ$_1$ did not affect FS induced by a non-preQ$_1$-binding 12-bp hairpin, while disruption of the S2 stem resulted in a non-frameshifting hairpin, indicating that the pseudoknot conformation induced by preQ$_1$ is responsible for the recorded FS.

Since the Fnu aptamer displayed both high FS efficiency and high preQ$_1$ sensitivity, we decided to perform additional studies with this aptamer. By substituting G-C for A-U bps in stem S1 of the Fnu aptamer (stab-Fnu), the FS efficiency could be enhanced to 39.1% allowing a better read-out in further analyses (Figure 3). The enhanced FS efficiency is in line with previous data on frameshifter pseudoknots showing that stability of S1 is a major determinant for −1 FS.[41]

We note that in order to preserve the reading frame after −1 FS, a C-residue was inserted into L3 of the Bsu and Fnu aptamers. Although the effect of the C in the corresponding position within the A-rich loop (corresponding to L3 in preQ$_1$ aptamer) of known frameshifter pseudoknots is ambiguous,[6,42,43] we demonstrated that the additional nucleotide in this position has a negligible effect on the preQ$_1$ aptamer in our frameshifting-based system since (1) substitution of C by G showed nearly identical FS activity as stab-Fnu (C28G, Supplementary Figure S2A), and (2) simultaneous substitution of A10-U33 by G10-C33 in S2 and removal of the inserted C (A10G+U33C+ΔC28, Supplementary Figure S2B) did induce similar levels of frameshifting as the stab-Fnu construct. These results demonstrate that the preQ$_1$-induced pseudoknot is an efficient frameshifter.

Ligand Recognition by the *F. nucleatum* preQ$_1$ Aptamer. The aptamer domain of a riboswitch is responsible for highly selective binding of the target ligand rather than suboptimal analogues to regulate gene expression. The binding affinity for their cognate ligands generally varies by over 2 orders of magnitude to avoid mis-regulation.[44] To demonstrate that this frameshifting-based assay system can be applied to study ligand selectivity of preQ$_1$ aptamers, several preQ$_1$ analogues (Figure 4A) were incubated with the stab-Fnu aptamer to assess their ability to induce frameshifting. As shown in Figure 4B, addition of 2 μM preQ$_1$ induced 35.4% of frameshifting (Figure 4B, lane 2), 2.2-fold higher than the level (16.2%) induced by 200 μM guanine (Figure 4B, lane 3), which has minor differences in chemical features at the 7 position (Figure 4A). 2,6-Diaminopurine, a preQ$_1$ analogue capable of inducing structural changes in the preQ$_1$ aptamer,[35] induced 10.3% frameshifting (Figure 4B, lane 4) when present at 200 μM. This 1.5-fold lower efficiency of 2,6-diaminopurine compared to guanine could be due to the fact that only the 2-amino proton can interact with C17 (Figure 4A). However, xanthine and hypoxanthine, both of which can form two hydrogen bonds with C17, did not promote frameshifting (Figure 4B, lanes 6 and 7), consistent with previous findings that the 2-amino group is crucial in ligand recognition and structure modulation.[35] Adenine, which cannot base pair with cytidine, did not result in frameshifting at a concentration of 200 μM (Figure 4B, lane 5), as expected. However, 2,4-diaminopyrimidine, although capable of forming identical hydrogen bonds with C17 as 2,6-diaminopurine, exhibited no significant frameshifting (Figure 4B, lane 8) even at a concentration of 200 μM, indicating that the purine moiety is important in molecular recognition and/or base stacking.

On the basis of the observation that the discriminator of purine riboswitches relies in part on Watson−Crick (WC) base pair formation for their ligand selection,[45,46] we substituted U17 for C17 to test the selectivity for adenine analogues. In a panel of test compounds (Supplementary Figure S3A), we found that 2,6-diaminopurine, which can base pair with U17 by three H-bonds, and preQ$_1$, which forms a non-canonical wobble pair with U17, were able to promote weak but significant frameshifting (Supplemetary Figure S3B). This result agrees in part with previous in-line probing analysis[35] showing that 2,6-diaminopurine but not preQ$_1$ can induce significant structural changes in the mutant C17U preQ$_1$ aptamer of Bsu. The inability to detect preQ$_1$ binding may be due to the lower concentration (1 μM) used in that study versus 200 μM in our assays. Our data, in combination with the results of the Bsu preQ$_1$ aptamer in ligands recognition, recapitulate the importance of 2-amino and 7-deaza-7-aminomethyl groups as well as interactions between the discriminator base and ligand in this type of compact aptamer.

To further compare our frameshifting-based assay in ligand binding to typical in-line probing assays, we plotted the frameshifting efficiency as a function of the concentration of preQ$_1$ along with two selected ligands to calculate their potency (EC_{50}) (Figure 4C). Although the concentrations of guanine and 2,6-diaminopurine could not be raised high enough to obtain saturating levels of FS, it is clear from Figure 4c that the EC_{50} of preQ$_1$ (180.7 nM) is at least 3 orders of magnitude lower than that of either guanine or 2,6-diaminopurine. Previous in-line probing analyses showed a 25-fold difference between preQ$_1$ and guanine (K_d of preQ$_1$ and guanine were determined as 20 and 500 nM, respectively[35]). This suggests that our assay system more closely resembles the natural riboswitch aptamer in discriminating between analogues with over 2 orders of magnitude in binding affinity, despite the fact that our assay primarily responds to the thermodynamic properties of the aptamer and lacks the kinetic properties of riboswitches needed for regulating transcription termination.[47]

Role of Stems of the *F. nucleatum* preQ$_1$ Aptamer in Frameshifting. It has been suggested that in frameshifting pseudoknots the lower stems (S1) play a major role in stalling elongating ribosomes while the upper stems (S2) may provide torsional restraints to resist ribosome unwinding.[48] Therefore, variations of residues in either stem that result in a change in ligand binding affinity or stability should affect frameshifting efficiency. We showed in the prior construct (stab-Fnu) that stabilizing S1 could increase FS about 2-fold. Disruption of 1 bp in S2 dramatically reduced the responsiveness to preQ$_1$ (U33C, Figure 5). The compensatory mutant (A10G+U33C, Figure 5) in which this base pair was restored again acted as a preQ$_1$-dependent frameshifter. Interestingly, this S2 stabilized mutant resulted in even higher levels of FS (47%.) In the absence of preQ$_1$ this construct showed already some FS (0.8%), suggesting that the S2 formed in the absence of the ligand. These results indicate that the overall stability and integrity of stems affect the ability to induce FS.

We next focused on residues that are located in stems that may be directly involved in the preQ$_1$-binding pocket. The C18U substitution (Figure 5), which results in the formation of a G5-U18 wobble base pair, was detrimental to FS (less than 1%) even though there is only one WC H-bond missing compared to G5-C18. Moreover, flipping the G5-C18 bp (G5C +C18G, Figure 5) was also detrimental to FS. These results indicate that the highly conserved G5-C18 is crucial for

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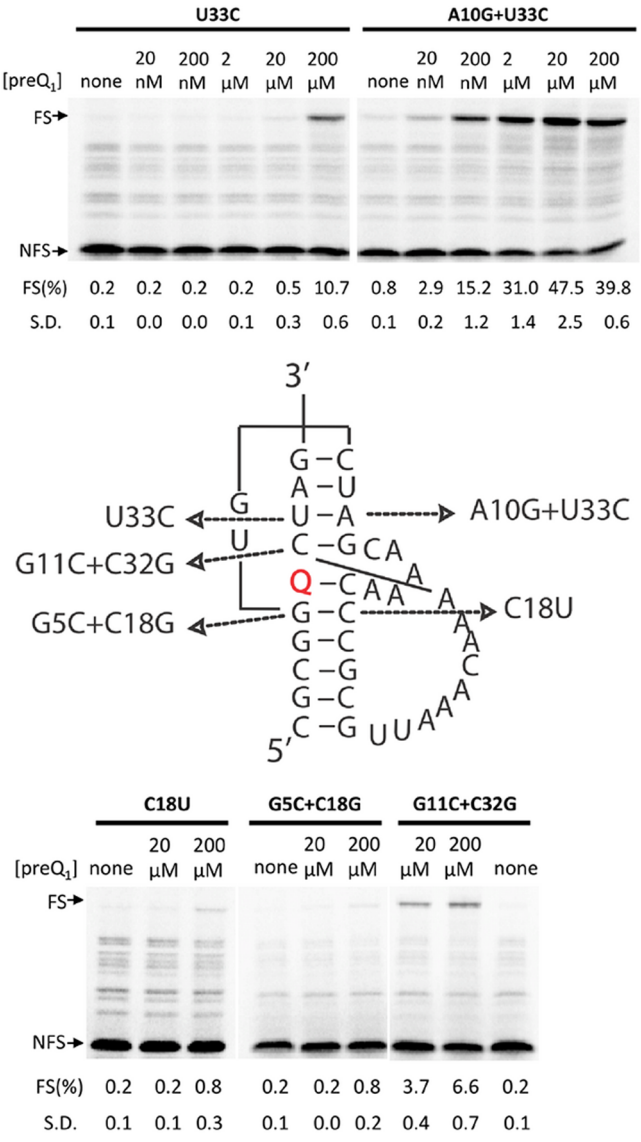

Figure 5. Effect of mutations in the stem regions on −1 FS. Mutations are indicated in the secondary structure representation. SDS-PAGE analysis of ^{35}S-methionine labeled translation products in RRL using mRNA of each mutant in the presence or absence of preQ$_1$. See legend to Figure 2 for more details.

formation of the preQ$_1$-binding pocket. The G11-C32 bp also showed greatly suppressed FS when flipped into C11-G32 (G11C+C32G, Figure 5), consistent with the analogous G11-C32 bp of Bsu aptamer being part of the preQ$_1$ binding pocket.[37,38]

We noticed that the conserved YUAR sequence, which forms the 5′ half of stem S2 in preQ$_1$ riboswitches, exhibits the so-called "U-turn" YUNR (Y = pyrimidine, R = purine) motif. This motif has been shown to provide a scaffold for rapid interaction with complementary RNA, forming a paradigm in antisense RNA/target recognition.[49] In preQ$_1$ riboswitches it may have evolved to rapidly interact with the downstream complementary sequence to facilitate pseudoknot formation upon ligand binding. To test this hypothesis, we made mutations in the CUAG sequence (and its complement to preserve S2 integrity) of the Fnu pseudoknot and studied their FS efficiency in response to preQ$_1$. The results, however, indicate that the conserved YUAR sequence in S2 does not function as a U-turn since CAUG and CAAG sequences were equally efficient as CUUG, which does have the YUNR motif (Supplementary Figure S4).

Effects of Loop Mutants on preQ1-Responsive Frameshifting. The two stems of the preQ$_1$ aptamer pseudoknot are separated by three loops (L1 to L3, Figure 1A). These loops strongly resemble the loops of frameshifter pseudoknots either in length or sequence except for the 6-nt L2, which is generally one or zero nucleotide in frameshifter pseudoknots.[6] Since it has been shown that the loop-stem tertiary interactions are crucial in stabilizing frameshifter pseudoknots,[25−30] we further investigated the contribution of these tertiary interactions within the preQ$_1$ aptamer to FS. The results are summarized in Figure 6.

In L1, the sixth nucleotide is found exclusively to be U in all class I type II preQ$_1$ aptamers. Moreover, in high resolution structures, U6 is involved in the quadruple interaction together with preQ$_1$, C17, and A31 to form part of the preQ$_1$ binding pocket.[37,38] We made a U6A substitution to disrupt the quadruple interaction and found its ability to induce frameshifting is dramatically impaired, confirming that the highly conserved U6 is a major component of the preQ$_1$-responsive pseudoknot.

The presence of a 6-nt loop between stems S1 and S2 in preQ$_1$ aptamers is rarely seen in frameshifter pseudoknots (see below) as such a loop would have a large destabilizing effect. Possibly in the ligand-bound form, reorganization of L2 compensates for the destabilizing effect. The only known frameshifter pseudoknot with a large L2 is found in the ovine *Visna-Maedi lentivirus* in which 7 nts (5′CGUCCGC3′) are located between two stems of 7 bp each.[50]It is reasonable to propose that the L2 plays some role in ligand recognition. To investigate the involvement of L2 in the pseudoknot structure in frameshifting, we first deleted all the nucleotides in L2 except C17, the preQ$_1$ binding nucleotide. This construct (ΔC12-A16), although still capable of inducing a substantial level of FS (7.8%), is not responsive to preQ$_1$, indicating the L2 is indeed important in trapping preQ$_1$ in the binding pocket. A16, which forms a base triple with G11-C32 in the Bsu aptamer[37] and is the only other highly conserved residue in L2, is also crucial in inducing FS as evidenced by the fact that the A16G substitution is more than 3-fold less efficient (11.5%) in FS compared to stab-Fnu at 200 μM preQ$_1$. Structural studies indicate that the other nucleotides in L2 are not involved in any interactions with preQ$_1$,[37,38] and our mutational analysis of these residues is consistent with that; mutations C12U, C12A, and A15C all displayed a similar level of frameshifting as stab-Fnu. The relatively high tolerance toward nucleotide changes in L2 also agrees with the low phylogenetic conservation of these nucleotides in preQ$_1$ aptamers.[35]

L3 of preQ$_1$ aptamers resembles L2 of frameshifter pseudoknots by its high number of adenosines. It has been shown in structural studies that some of the adenosines form interactions with bases in the minor groove of S1 exclusively via A-amino kissing motifs, in which the Watson−Crick edge of adenines are involved in the interaction, or via mixed A-amino kissing and A-minor motifs, in which the sugar edge of adenines is involved.[37,38,51] To investigate whether such interactions are important for the stability of the Fnu preQ$_1$ aptamer, mutations were made in this region and assayed for their FS efficiencies. As shown in Figure 6, the A31U and A30U nucleotide substitutions that were designed to disrupt base quadruples at the junction of the preQ$_1$ aptamer pseudoknot are both inactive

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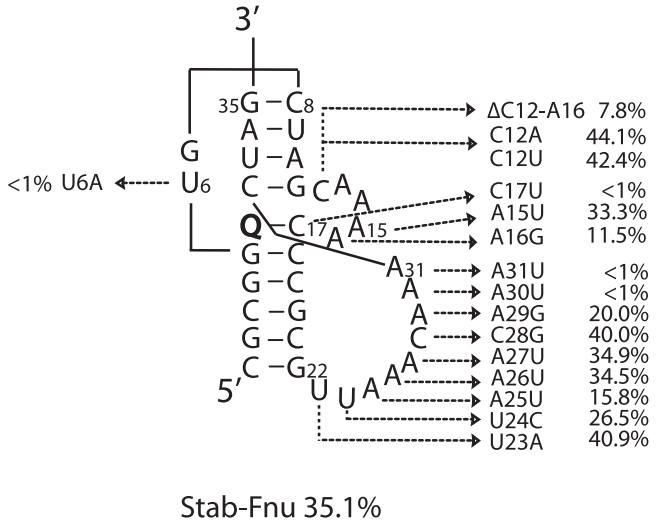

Figure 6. FS efficiency of loop mutants. Mutations are indicated in the secondary structure representation. The reported −1 FS efficiency is in the presence of 200 μM preQ$_1$.

in frameshifting (<1%). These data indicate that the role of these adenosines of the Fnu aptamer is very similar to that at the corresponding position of the Bsu aptamer for which similar A to U changes also dramatically reduce preQ$_1$ binding.[37] Changing other nucleotides in L3 showed less dramatic changes in FS efficiency suggesting that their individual contributions to the stability of the pseudoknot are less important (see Figure 6). Similar observations have been reported for the *Simian retrovirus* type-I (SRV-1) frameshifter pseudoknot where single changes in L3 did not strongly affect FS but the combined changes did.[28]

Conclusions. We have shown here that preQ$_1$ riboswitch aptamers from *B. subtilis*, *F. nucleatum*, and *T. tencongensis* can induce significant levels of −1 FS upon ligand binding. Structurally and functionally these aptamers resemble the small luteovirus frameshifter pseudoknots, and the levels of −1 FS are comparable: 20% for Fnu versus 22% for ScYLV (after correcting for the 1.5-fold more efficient UUUAAAC slippery sequence in our system; GGGAAAC is used by luteoviruses). In the *ScYLV* and *Beet western yellows luteovirus* (BWYV) pseudoknots[25] the structure is stabilized by interactions between nucleotides in L1 with S2, and L3 with S1 (preQ$_1$ aptamer nomenclature). However, the absence of a large L2 loop in luteovirus pseudoknots makes these pseudoknots more stable and frameshifting independent of ligand-binding.

It has been suggested that due to their relatively small size preQ$_1$ aptamers are less likely to be detected by automated searching methods and may comprise a substantial fraction of yet to be discovered riboswitches.[35] Pseudoknots like that of the Tte aptamer would be especially difficult to predict due to the presence of non-canonical base pairs in S2,[39] and it is conceivable that there exists an undiscovered frameshift mechanism exploiting riboswitch-like ligand-induced conformational changes to regulate gene expression. Algorithms aimed at detecting potential frameshifting elements involving this type of pseudoknot should take this into account.

In our frameshift reporter constructs, the preQ$_1$ aptamers are flanked by long strands of RNA but are nonetheless fully responsive to ligand addition. This may explain why we need a higher concentration of preQ$_1$ than expected (given a reported K_d of ∼280 nM[52] versus EC$_{50}$ of ∼2 μM for wt Fnu] to induce frameshifting, since alternative structures may form in this situation. However, these constructs may be closer to the natural situation than the small synthetic RNAs used in structural studies and binding assays. Moreover, frameshifting assays for detecting ligand-aptamer interactions, although not quantitative, show not only ligand-dependent but also reasonable sensitivity (between 20 and 200 nM) and a broad dynamic range (20 to 200 μM). Since the preQ$_1$ riboswitch is responsible for regulating the expression of genes involved in queuosine synthesis, which is essential for survival at least in stationary growth phase,[53] we think it is likely that we can utilize frameshift assays for selecting compounds that can bind to the preQ$_1$ aptamer and inhibit the growth of pathogens. Furthermore, using a eukaryotic cell-free translation system to monitor prokaryotic RNA−ligand interaction is an advantage for antibacterial drug discovery, since we can simultaneously monitor potential adverse effects on eukaryotic translation. Thus, using frameshift assays in analyzing preQ$_1$ aptamers may have great potential in high throughout selection of compounds with antibacterial activity.

METHODS

Frameshift Reporter Constructs and Oligonucleotides. The −1 FS was monitored by the SF reporter construct described earlier.[28] Mutants were constructed by ligating pairs of complementary oligonucleotides (Eurogentec and Sigma-Aldrich) into SpeI-NcoI digested SF reporter plasmids. Contructs were verified by automated dideoxy sequencing using chain terminator dyes (LGTC, Leiden, The Netherlands). A list of oligonucleotide sequences is available upon request.

Chemicals. PreQ$_1$ was synthesized and purified as described.[35] Adenine, uracil, guanine, hypoxanthine, xanthine, 2,6-diaminopurine, and 2,4-diaminopyrimidine were purchased from Sigma-Aldrich. Guanine was dissolved in 0.15 N KOH, 2,6-diaminopurine was dissolved in RNase-free water, and the other compounds were dissolved in DMSO.

***In Vitro* Transcription.** DNA templates were linearized by *Bam*HI (Fermentas) digestion and purified by successive phenol/chloroform extraction. SP6 polymerase directed transcription was carried out in a 50 μL reaction containing ∼2 μg linearized DNA, 1 mM NTPs, 40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 10 mM DTT, 6 mM $MgCl_2$, 2 mM spermidine, 6 units of RNase inhibitor (RNAguard, Pharmacia), and 15 units of SP6 polymerase (Promega). After an incubation period of 2 h at 37 °C, samples were taken and run on agarose gels to determine the quality and quantity of the transcripts. Appropriate

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dilutions of the reaction mix in sterilized water were directly used for *in vitro* translations.

***In Vitro* Translation.** Experiments were carried out in duplicate using ~30 ng of mRNA in the presence or absence of the indicated compounds (typically tested at a concentration between 0 and 200 μM). Reactions contained 4 μL of rabbit reticulocyte lysate (RRL, Promega), 0.25 μL of ^{35}S-methionine (Amersham, *in vitro* translation grade), 0.25 μL of 1 mM amino acids without methionine, and mRNA and water to a final volume of 10 μL. After incubation for 60 min at 28 °C, samples were boiled for 3 min in 10 μL of 2X Laemmli buffer followed and resolved on 13% SDS polyacrylamide gels. Gels were dried and exposed to phosphoimager screens (Biorad). After scanning (Molecular Imager FX, Biorad), band intensity of 0-frame and −1 frameshift products was quantified by Quantity One software (Biorad). Frameshift percentages were calculated as the amount of −1 frameshift product divided by the sum of 0 and −1 frame products, corrected for the number of methionines (10 in the 0-frame product and 28 in the fusion product), multiplied by 100.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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