Stimulation of -1 programmed ribosomal frameshifting by a metabolite-responsive RNA pseudoknot

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ABSTRACT

Specific recognition of metabolites by functional RNA motifs within mRNAs has emerged as a crucial regulatory strategy for feedback control of biochemical reactions. Such riboswitches have been demonstrated to regulate different gene expression processes, including transcriptional termination and translational initiation in prokaryotic cells, as well as splicing in eukaryotic cells. The regulatory process is usually mediated by modulating the accessibility of specific sequence information of the expression platforms via metabolite-induced RNA conformational rearrangement. In eukaryotic systems, viral and the more limited number of cellular decoding -1 programmed ribosomal frameshifting (PRF) are commonly promoted by a 3' mRNA pseudoknot. In addition, such -1 PRF is generally constitutive rather than being regulatory, and usually results in a fixed ratio of products. We report here an RNA pseudoknot capable of stimulating -1 PRF whose efficiency can be tuned in response to the concentration of *S*-adenosylhomocysteine (SAH), and the improvement of its frameshifting efficiency by RNA engineering. In addition to providing an alternative approach for small-molecule regulation of gene expression in eukaryotic cells, such a metabolite-responsive pseudoknot suggests a plausible mechanism for metabolite-driven translational regulation of gene expression in eukaryotic systems.

Keywords: -1 ribosomal frameshifting; riboswitch; pseudoknot

INTRODUCTION

The -1 programmed ribosomal frameshifting (PRF) is a translational regulation mechanism adopted by a variety of viruses to synthesize two or more proteins at a fixed ratio from the same start codon (Gesteland and Atkins 1996). Examples of -1 PRF characterized in the cellular genes of eukaryotic cells are also reported (Manktelow et al. 2005; Wills et al. 2006; Jacobs et al. 2007). Efficient eukaryotic -1 PRF requires two RNA elements (Chamorro et al. 1992). The first element is a hepta-nucleotide slippery site sequence of X XXY YYZ, where the recoding occurs. Sequence analysis indicates X can be any three identical nucleotides, whereas Y represents three A's or U's, and Z is A, U, or C for efficient -1 PRF in eukaryotic systems (Farabaugh 1996). The second element is a stimulator RNA structure, located 5–7 nucleotides (nt) downstream from the slippery site. This downstream RNA stimulator is usually

a hairpin (H)-type RNA pseudoknot in which nucleotides from a hairpin loop form base pairs with a single-stranded region outside of the hairpin (Giedroc et al. 2000).

Metabolite-responsive RNA elements are distributed widely within messenger RNAs (mRNAs). They are most frequently identified in the 5' untranslated regions (UTRs) of bacterial mRNAs (Barrick and Breaker 2007; Weinberg et al. 2007), and their characterization within eukaryotic genomes has also been reported (Sudarsan et al. 2003; Cheah et al. 2007). Such riboswitches participate in a variety of regulatory gene expression processes, ranging from transcription termination to translation initiation and splicing (Mandal and Breaker 2004; Nudler and Mironov 2004; Henkin 2008). Most riboswitch-mediated regulatory processes involve accessibility of crucial gene expression sequences, which are masked or exposed by the metabolite-induced conformational change of the riboswitches (Grundy and Henkin 2006). Distinct RNA motifs are adopted to build the metabolite-responsive sensors (Serganov and Patel 2007). Interestingly, the H-type pseudoknot has been used to build the riboswitches of several metabolites, including *S*-adenosylmethionine (SAM), *S*-adenosylhomocysteine (SAH), and pre-queuosine₁ (PreQ₁) (Corbino et al. 2005; Meyer et al. 2008; Wang et al. 2008).

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Recently, we have demonstrated that the pseudoknot derived from human telomerase RNA, hTPK (Theimer et al. 2005), can serve as a stimulator to induce –1 PRF when it is placed downstream from a slippery sequence (Chou and Chang 2010). More importantly, the frameshifting efficiency of hTPK can be modulated by manipulating base-triple interactions flanking the helical junction of this pseudoknot (Chen et al. 2009; Chou and Chang 2010). Interestingly, the structure of an SAM-bound SAM-II riboswitch pseudoknot (SAMII-PK) was shown to adopt structural features similar to those of hTPK (Gilbert et al. 2008). Furthermore, the solved structures of ligand-bound SAMII-PK and PreQ₁-I riboswitch indicated that they both possess ligand-induced base-triple interaction networks surrounding the helical junctions of the pseudoknots (Gilbert et al. 2008; Kang et al. 2009; Klein et al. 2009; Spitale et al. 2009). Realizing that the metabolite-responsive riboswitch pseudoknots provide an opportunity to build a metabolite-responsive –1 PRF stimulator, we examined the –1 PRF stimulation activities of several riboswitch pseudoknots. We describe here the finding that the pseudoknot derived from an SAH riboswitch can induce –1 PRF of a reporter gene in an SAH-dependent way. Furthermore, we also demonstrate that this SAH-dependent –1 PRF activity can be further improved by RNA engineering. In addition to providing an *in trans* approach for the regulation of –1 PRF (Kollmus et al. 1996), this discovery means that the intracellular metabolite concentration could be a direct factor in the regulation of –1 PRF activity within the cells. Finally, our finding suggests that –1 PRF has the potential to serve as a gene expression platform for a regulatory riboswitch.

RESULTS

The 68 meth RNA can stimulate –1 PRF in response to SAH in vitro

The SAH riboswitch is distributed widely in the genes involving SAM metabolism in bacteria and is proposed to regulate biochemical pathways involving SAM recycling (Wang et al. 2008). Sequence alignment and secondary structure prediction of potential SAH riboswitch sequences suggested that SAH riboswitch contains a three-stemmed pseudoknot core (Fig. 1A) with an optional fourth stem (Wang

et al. 2008). The pseudoknot is formed by the complementary base pairing between two single-stranded regions from an internal loop and the 3'-portion of the riboswitch (P4 in Fig. 1A). We chose the three-stemmed 68 meth RNA to evaluate its –1 PRF activity because the –1 PRF pseudoknot stimulator identified in SARS corona virus (SARS-PK) also contains three stems (Baranov et al. 2005; Plant et al. 2005; Su et al. 2005). In addition, the well-characterized ligand-binding specificity of the 68 meth RNA will facilitate further analysis if it does have –1 PRF stimulation activity (Wang et al. 2008).

We cloned the 68 meth RNA into the p2Luc-based –1 PRF reporter containing a UUUAAC slippery sequence and a spacer of 7 nt (Fig. 1A). Control constructs, with sequences mutated to destroy the slippery site or the 0 frame stop codon, were also prepared (see Supplemental Fig. 1A). SAH-related ligands (Fig. 1B) were then examined for their effects on the frameshifting efficiencies of these –1 PRF reporters in vitro. As shown in Figure 1C, the –1 PRF efficiency of the 68 meth RNA-containing –1 PRF reporter construct was less than 0.4% without the addition of SAH.

FIGURE 1. The SAH-dependent –1 PRF stimulated by the 68 meth RNA in vitro. (A) Predicted secondary structure of the core three-stemmed pseudoknot in the 68 meth RNA and the chemical formula of SAH-related ligands. The nucleotides residing in predicted duplex and single-stranded regions are in boldface and in plain type, respectively, and the sequences corresponding to the slippery site and the spacer are underlined and in gray, respectively. The numbering of nucleotides and stems (P1/P2/P4) follows previous designation by Wang et al. (2008), whereas the single-stranded regions are labeled from L1 to L4. The optional fourth stem is not shown for clarity. (B) The chemical formulas of SAH and its related derivatives. (C,D) Twelve percent SDS-PAGE analysis of the –1 PRF assays of 68 meth RNA in the presence of different amounts of SAH or its related derivatives. The translated proteins corresponding to the 0 frame and –1 frame products are labeled as indicated. Please note that the intensities of bands labeled with *1 and *2 can respond to SAH variation and are presumably the shortened –1 frame translated products. Both bands are also evident among the products from the control construct that lacks the 0 frame stop codon (the 68 meth TL in Supplemental Fig. 1A). In contrast, the intensity of the band labeled with *3 does not respond to SAH variation and also appears in the products from the control construct lacking the slippery site (68 meth Sm in Supplemental Fig. 1A).

However, the -1 PRF efficiency of the same reporter increased 10-fold to around 4% upon the addition of 100 μM of SAH. In contrast, the addition of either precursor (SAM) (Fig. 1C) or hydrolysis products (adenosine/homocysteine) of SAH (Fig. 1D) did not induce comparable -1 PRF activity as SAH (all below 0.5%). Interestingly, the total amount of translated proteins decreased when the concentration of the added SAM exceeds 100 μM (Fig. 1C). This suggests that certain activities responsible for protein translation within the reticulocyte lysate may be affected by SAM concentration. Due to the existence of extra translated protein products between 0 frame and -1 frame products shown in the Figure 1C, a reporter with a premature stop codon introduced into the reading frame of firefly luciferase was also constructed to further verify the -1 frameshifted products (Supplemental Fig. 1B). Finally, we also examined an RNA (PSahcY P3m RNA) derived from the four-stemmed pseudoknot of SAH riboswitch in *P. syringae* (PSahcY RNA) (Wang et al. 2008), and found that its SAH-dependent -1 PRF activity is much weaker than that of 68 methH RNA (Supplemental Fig. 1A). As PSahcY RNA was also shown to bind with SAH (Wang et al. 2008), it implicates the extra fourth stem in PSahcY RNA in hindering -1 PRF stimulation. However, deleting the P3 region of PSahcY RNA to form a three-stemmed pseudoknot (PSahcY P3d RNA) did not enhance its SAH-dependent -1 PRF activity to the level of the 68 methH RNA (Fig. 4B, see below). Therefore, the SAH-dependent -1 PRF activity of the 68 methH RNA is very likely to be caused by specific features within this unusual pseudoknot.

The full SAH-dependent -1 PRF activity of 68 methH RNA requires all three predicted duplex regions

To link the observed SAH-dependent -1 PRF activity to components within the 68 methH RNA, mutations were introduced into the three predicted stems of the 68 methH RNA to disrupt the potential base pairs (Fig. 2A). The SAH dependency of -1 PRF activity of these mutants was then evaluated. As can be seen in Figure 2B, the disruption of three potential base pairs in the P1 stem (P1UGG) impaired the SAH-dependent -1 PRF efficiency dramatically (below 0.5%). Similarly, the -1 PRF efficiency of the mutant with partially disrupted P4 stem (P4AGA) was also reduced compared with that of the wild-type construct under the same SAH concentration (below 1%). In contrast, disruption of the two base pairs in the bottom of the P2 stem (P2AA) led to a constitutive -1 PRF activity with

FIGURE 2. Mutagenic analysis of base-pairing formation requirement in all three predicted stem regions for the SAH-dependent -1 PRF activity of 68 methH RNA. (A) Illustration of mutant constructs for manipulation of the base-pairing scheme. For each mutant, the nucleotide identities before and after mutation are boxed and linked by an arrow. (B) Results of 12% SDS PAGE analysis of frameshifting efficiency for constructs of different base-pairing disruption mutants in the presence of SAH. (C) Results of 12% SDS PAGE analysis of frameshifting efficiency for different stem restoration constructs in the presence of SAH. The concentration of SAH and the translated proteins corresponding to the 0 frame and -1 frame products are labeled as indicated.

(Fig. 2B) or without (data not shown) the addition of SAH. However, the SAH-dependent -1 PRF activity was restored for each compensatory mutant that restores the base-pairing interactions within individual stems (Fig. 2C). As disruption of the two base pairs in the bottom of P2 stem in PSahcY RNA had no negative effect on its riboswitch activity (Wang et al. 2008), it is thus interesting to see the SAH-independent -1 PRF activity of P2AA mutant. Perhaps, an alternative base-pairing scheme in the L3/P2 junction of the P2AA mutant may lock the mutant into a conformation capable of stimulating -1 PRF activity. Together, mutagenesis studies suggest that the three potential stems in the 68 methH RNA may contribute differently to its SAH-dependent -1 PRF activity.

The SAH-free and -bound 68 methH RNAs adopt distinct conformations

To better understand the molecular basis of the observed SAH-dependent -1 PRF stimulated by the 68 methH RNA, we applied enzymatic mapping on both free and SAH-bound 68 methH RNAs. Ribonuclease V1 and T2, the probes for duplex/stacked conformations and single-stranded regions, respectively, were used to track the distribution of duplex- and single-stranded regions of the 68 methH RNA under different SAH concentrations. As can be seen in Figure 3A,B, the distributions of cleavage pattern by ribonuclease V1 and T2, for both free and SAH-bound 68 methH RNAs, were in agreement with the formation of the three predicted stems in both conditions. However, the intensities of cleavage products clearly changed in several regions of the 68 methH RNA when the concentration of added SAH was increased gradually from 0 to 300 μM . For

FIGURE 3. Results of free and SAH-bound 68 meth RNAs mapped by enzymatic probing. (A) Electrophoretic analysis of the 5'-portion of 68 meth RNA probing data. Please note that the vertically tilted elongated band on the upper-left corner of the gel image was caused by stucked radioactivity within a cut on the phosphorimager plate. (B) Electrophoretic analysis of the 3'-portion of 68 meth RNA probing data. The enzymatic cleavage reactions, with different conditions as indicated on top of the panel, were resolved in 20% (for 5'-portion) or 12% (for 3'-portion) sequencing gel. The treatment of RNase T2 or V1 was performed in the presence of different SAH concentrations (0, 0.1 nM, 0.25 nM, 5 nM, 100 nM, 2 μ M, 40 μ M, and 300 μ M), and the abbreviations for other conditions are as follows: C, nontreated control; A, ribonuclease A treatment; Alk, alkaline treated ladder; and T1, ribonuclease T1 treatment. In addition, the assigned residues and the corresponding stem/loop regions are listed in the center of the gel. (C) Summary of the V1/T2 cleavage patterns of SAH-free 68 meth RNA and SAH-induced cleavage pattern change. The extent of enzymatic cleavage of the SAH-free 68 meth RNA is defined as major, medium, and minor cuts, with rhombuses representing RNase T2 cleavage and filled triangles representing RNase V1 cleavage. The regions with major conformational rearrangement in P4 and P1/L4 junction, supported by coupled V1 and T2 cleavage pattern variation, are boxed and in boldface, respectively.

example, variation of the T2 cleavage pattern was observed in the P1/L4 junction and L4 upon SAH treatment, whereas the regions corresponding to P1 and P4 stems showed variation of V1 cleavage in response to SAH increment. In

addition, the V1 cleavage intensities of residues located in the two ends of the P2 stem responded to the SAH treatment in the opposite ways (Fig. 3A). Together, the broad SAH-dependent variation of nuclease cleavage pattern in

the predicted stem regions is consistent with the conformational change of the 68 meth RNA upon the binding of SAH.

Further analysis revealed simultaneous V1 cleavage weakening and T2 cleavage strengthening upon SAH treatment for two consecutive adenines bridging the P1/L4 junction (A₅₀A₅₁). In contrast, the enhancement of V1 cleavage and the reduction of T2 cleavage were observed simultaneously for both 5' and 3' portions of P4 in response to the increment of SAH (summarized in Fig. 3C). It thus suggests an SAH-driven conformational rearrangement for pseudoknot formation. Interestingly, previous local structure-dependent spontaneous RNA cleavage study (in-line probing) (Soukup and Breaker 1999) of the 68 meth RNA suggested that binding of SAH stabilizes the P4 helix and locks the 3' nucleotides into a more restricted conformation, suggesting the formation of an SAH-binding pocket (Wang et al. 2008). Therefore, conformational rearrangement driven by SAH binding may convert the 68 meth RNA into a better –1 PRF stimulator.

Adenosine-2', 3'-dialdehyde can further enhance SAH-dependent –1 PRF activity stimulated by the 68 meth RNA in vitro and in vivo

Based on these in vitro studies, we decided to investigate the SAH-dependent –1 PRF stimulating activity of the 68 meth RNA in vivo. As SAH may maintain a static equilibrium within the cells, the manipulation of intracellular SAH concentration will be crucial for the detection of SAH-induced –1 PRF in vivo. Unfortunately, SAH cannot penetrate the cell membrane (Ueland 1982) and thus the addition of SAH cannot increase the intracellular SAH concentration (Hermes et al. 2004). However, it was reported that adenosine-2', 3'-dialdehyde can enter the cell to inhibit the SAH degradation activity of SAH hydrolase and thus can lead to the accumulation of intracellular SAH (Hermes et al. 2004; Wang et al. 2008). Because the reticulocyte lysate used for in vitro –1 PRF assay may also contain SAH hydrolase activity, we therefore tested if the SAH-dependent –1 PRF activity of 68 meth RNA in vitro can be affected by the addition of adenosine-2', 3'-dialdehyde. As can be seen in Figure 4A, the –1 PRF activity of 68 meth RNA, in the presence of 10 μ M SAH, was changes from $1.0 \pm 0.2\%$ to $5.2 \pm 1.1\%$ when the concentration of adenosine-2', 3'-dialdehyde was added from 0 to 5 μ M. In addition, luciferase activity in vitro was also measured to calculate the in vitro –1 PRF efficiency independently. A similar trend was observed although the increment of frameshifting efficiency was less dramatic (Fig. 4A). Therefore, it is very likely that the enhancement of the SAH-dependent –1 PRF activity of the 68 meth RNA is caused by accumulation of SAH due to the inhibition of SAH hydrolase activity in the reticulocyte lysate.

FIGURE 4. Adenosine-2', 3'-dialdehyde can further enhance SAH-dependent –1 PRF activity stimulated by the 68 meth RNA in vitro and in vivo. (A) The frameshifting efficiency calculated by 35 S content and dual-luciferase assays in vitro (represented by filled black and gray bar, respectively) with different amounts of adenosine-2', 3'-dialdehyde. Please note the higher standard deviation for frameshifting efficiency calculated by the measurement of 35 S content of the translated proteins. (B) The in vivo frameshifting efficiency from reporter constructs encoding 68 meth RNA, PSahcY P3m RNA, and PSahcY P3d RNA with different amounts of adenosine-2', 3'-dialdehyde as indicated. They were calculated from the results of the dual-luciferase assay and are the average of at least three repeated experiments.

Although the SAM recycling network, including the exact SAM/SAH concentration and the SAH-degrading enzyme activity, was not characterized within the reticulocyte lysate, the in vitro experiments above clearly demonstrate that the 68 meth RNA can stimulate –1 PRF activity with the addition of exogenous SAH (Fig. 1C). In addition, the inhibitor that blocks the activity for SAH hydrolase further enhanced the –1 PRF efficiency calculated from two different measurement approaches in vitro (by translated protein content or expressed enzyme activity) (Fig. 4A). Based on these results, we transfected a 68 meth RNA-containing –1 PRF reporter gene into the HEK-293T cells, and then treated the cells with adenosine-2', 3'-dialdehyde to see the effect on in vivo –1 PRF activity. As shown in Figure 4B, the –1 PRF efficiency of these cells increased from $1 \pm 0.09\%$ to $4.39 \pm 0.18\%$ (with adenosine-2', 3'-dialdehyde from 0 to 25 μ M). This efficiency is indeed close to the reported value of the –1 PRF efficiency of HIV in vivo (Hung et al. 1998; Dulude et al. 2002). In contrast, cells harboring the construct encoding PSahcY P3m or

PSahcY P3d RNA possessed only minor –1 PRF efficiency increment when 25 μ M of adenosine-2', 3'-dialdehyde was added (Fig. 4B). Together, these data argue that the –1 PRF stimulation activity of the 68 metH RNA can be modulated in a dosage-dependent manner by the inhibitor of SAH hydrolase in vivo.

The improvement of the SAH-dependent –1 PRF efficiency by RNA engineering

Two strategies were used to explore the possibility of further enhancing the observed SAH-dependent –1 PRF stimulation activity of the 68 metH RNA. As the length of spacer between slippery site and stimulator were reported to affect the –1 PRF efficiency (Kollmus et al. 1994), the spacer length in reporter containing 68 metH RNA was changed from 7 to 4, 5, or 8 nt, respectively (Fig. 5A). As shown in Figure 5B, variation in the length of the spacer did affect the SAH-dependent –1 PRF efficiency. However, none of the changes in spacer length improved the SAH-dependent –1 PRF efficiency further (Fig. 5C).

We then asked if the stimulator itself can be engineered to enhance the SAH-dependent –1 PRF activity. Analysis of the enzymatic probing data has indicated that the nuclease V1 accessibilities of residues in the P2 stem are affected differently in the presence of SAH (Fig. 3A).

Furthermore, the SAH dependency of –1 PRF activity was lost in mutant with partially disrupted P2 stem (P2AA in Fig. 2B). As the number of base pairs in the extra third stem of SARS-PK (corresponding to the P2 of 68 metH RNA) has been demonstrated to affect the –1 PRF efficiency (Baranov et al. 2005; Plant et al. 2005; Su et al. 2005), we mutated the three consecutive Us in the 5'-portion of P2 in 68 metH RNA to CCG, and thus creating a mutant (P2CCG) with six GC base pairs in P2 (Fig. 6A). As can be seen in Figure 6B, the P2CCG mutant stimulated stronger –1 PRF activity than the 68 metH RNA under the same SAH concentration in vitro. In the presence of 25 μ M of adenosine-2', 3'-dialdehyde, the –1 PRF efficiency was further improved to $7.23 \pm 0.22\%$ under 100 μ M of SAH in vitro (Fig. 6C). Furthermore, the in vivo –1 PRF assay of reporter construct containing P2CCG mutant revealed an almost 20% of relative efficiency increment compared with that of the 68 metH RNA-containing reporter in the presence of 25 μ M of adenosine-2', 3'-dialdehyde (Fig. 6D). Finally, two more mutants (P2UACG and C26G/G35C) were constructed by mutagenesis on the P2 of P2CCG mutant. Alternative GC base pairs (C26G/G35C) or a stable UACG loop (P2UACG) was introduced into the terminal end of P2 to further stabilize the stem (Fig. 7A). The result shown in Figure 7B indicated that both mutants have higher SAH-dependent –1 PRF efficiency than that of the P2CCG mutant. Therefore, the stability of P2 stem may play an interesting role in the modulation of the SAH-dependent –1 PRF stimulation activity of the 68 metH RNA.

DISCUSSION

The improvement of the SAH-dependent –1 PRF activity by P2 stem engineering and its mechanism

The SAH-dependent conformational changes of metH 68 RNA, revealed by enzymatic mapping, are consistent with previous in-line probing data (Wang et al. 2008). Together, they suggest an SAH-induced stabilization of pseudo-knot conformation, which can be coupled to the stimulation of –1 PRF. Structural information of the 68 metH RNA is crucial for understanding the mechanism of its SAH-responsive –1 PRF stimulation activity and improving its activity by RNA engineering. However, no high-resolution structure of the SAH-bound SAH riboswitch is available. Fortunately, the enzymatic probing

FIGURE 5. Manipulation of the length of a spacer can affect the –1 PRF efficiency. (A) Illustration of –1 PRF constructs with the length of their spacers manipulated. The nucleotides corresponding to the spacer are boxed for each mutant. The two nucleotides, inserted in the 3'-end of the stimulator to correct the shifted reading frames for the 5- and 8-nt-spacer mutants, are underlined. (B) Results of 12% SDS PAGE analysis of the –1 PRF assay for constructs with different spacer length in the presence of SAH. The concentration of SAH and the translated proteins corresponding to the 0 frame and –1 frame products are labeled as indicated. (C) The relative –1 PRF efficiency of different spacer mutants compared with that of the 7-nt-spacer construct.

FIGURE 6. Improvement of -1 PRF efficiency by P2 stem engineering. (A) Illustration of the P2CCG mutant. The nucleotides and base pairs changed before and after mutation are boxed and linked by an arrow. (B) Results of 12% SDS PAGE analysis of the -1 PRF assay for the wild-type and P2CCG mutant constructs in the presence of SAH. (C) The comparison of frameshifting efficiency, calculated by ^{35}S content in vitro, between the wild-type and P2CCG mutant constructs with differences in SAH (0 and 100 μM) and adenosine-2', 3'-dialdehyde (0 and 25 μM) concentrations. (D) The relative -1 PRF activity between the wild-type and P2CCG mutant constructs under different adenosine-2', 3'-dialdehyde concentrations. Please note that the frameshifting efficiency was calculated by dual-luciferase assay in vivo with the in vivo frameshifting efficiency of the wild-type construct treated as 1 for comparison.

analysis of free and SAH-bound 68 meth RNAs (Fig. 3) revealed valuable information, such as the differential V1 cleavage pattern changes within the P2 stem of 68 meth RNA under SAH treatment. Furthermore, base-pairing disruption mutations on the bottom of P2 stem created mutant (P2AA) with constitutive SAH-independent -1 PRF activity (Fig. 2B). Therefore, the P2 stem seems to play an interesting role in the SAH-dependent -1 PRF activity of the 68 meth RNA. This hypothesis is further supported by the finding that RNA engineered mutants designed to stabilize the P2 stem further enhance the SAH-dependent -1 PRF efficiency. As single-molecule analysis has revealed that the -1 PRF efficiency of a pseudoknot stimulator can be correlated with its mechanical stability (Chen et al. 2009), it will be interesting to see if the P2 stem also affects the mechanical stability of the 68 meth RNA. In the future, the high-resolution structures of free and SAH-bound 68 meth RNAs will help understanding the mechanism and the role of P2 stem in this intriguing SAH-dependent -1 PRF activity and

will provide insight for its further improvement.

The significance of SAH-dependent -1 PRF activity on the regulation of -1 PRF efficiency in vivo

Efficient -1 PRF requires RNA elements such as slippery sequences and downstream stimulators. However, evidences for stimulation of -1 PRF by designed RNA or DNA in *trans* have been presented recently (Howard et al. 2004; Olsthoorn et al. 2004; Plant and Dinman 2005; Chou and Chang 2010). In this work, we describe the finding of -1 PRF induced in *trans* by a specific cellular metabolite, although the induced frameshifting efficiency is not dramatic. However, this activity is significant and can be further improved by RNA engineering to the level of -1 PRF activity identified in several viruses (Kollmus et al. 1994; Hung et al. 1998; Dulude et al. 2002). It has been documented that the $+1$ PRF of antizyme can respond to the concentration of intracellular polyamine (Rom and Kahana 1994; Matsufuji et al. 1995). However,

the sensor for polyamine is not a pseudoknot and may involve part of the translational machinery (Petros et al. 2005; Ivanov and Atkins 2007). Moreover, our finding raises the possibility that a specific metabolite, by binding directly to an RNA sensor and converting it into an efficient stimulator, can be an important cellular factor for modulating -1 PRF activity in vivo. An open question here is if there is a natural counterpart of SAH-dependent

FIGURE 7. P2 stem stabilization and its effect on SAH-dependent -1 PRF activity. (A) Illustration of the P2UACG and C26G/G35C P2 stem mutants. The nucleotides and base pairs changed before and after mutation are boxed and linked by an arrow. (B) The relative -1 PRF activity between the P2CCG and the other two P2 stem mutants in the presence of 100 μM SAH. Please note that the frameshifting efficiency was calculated by ^{35}S content in vitro with the in vitro frameshifting efficiency of the P2CCG construct treated as 1 for comparison.

–1 PRF module in the genomes of eukaryotic cells. As bioinformatics tools for the identification of –1 PRF module and SAH riboswitch motif within a genome are both available, it will be very interesting to combine both approaches to address this issue (Jacobs et al. 2007; Weinberg et al. 2007).

MATERIALS AND METHODS

Construction of reporter genes and mutagenesis

The p2luc reporter was a kind gift from Professor John Atkins at the University of Utah (Grentzmann et al. 1998). Oligonucleotides containing the slippery sequence (TTTAAAC), spacer (GGGTAAC), and the coding sequences for the 68 meth RNA were chemically synthesized. They were amplified by forward and reverse primers containing SalI and BamHI restriction sites, respectively, and ligated into the SalI and BamHI sites of restriction enzymes treated with the p2luc reporter. Base-pairing disruption and restoration mutants were constructed using the QuikChange mutagenesis kit (Stratgene) according to the manufacturer's instructions. The identities of all cloned and mutated genes were confirmed by DNA sequencing analysis.

RNA synthesis and enzymatic structure probing

RNA transcripts were generated by in vitro transcription using T7 RNA polymerase. The purified RNAs of desired length were then dephosphorylated by shrimp alkaline phosphatase (USB), 5'-end labeled with [γ - 32 P] ATP using T4 polynucleotide kinase (NEB), and then separated by a 20% sequencing gel for recovery. All the RNase protection experiments were performed with 50,000–70,000 cpm of 5'-end labeled RNA for each reaction in the presence of RNase cleavage buffer (30 mM Tris-HCl at pH 7.5; 3 mM EDTA; 200 mM NaCl, and 100 mM LiCl), except that 10 mM MgCl₂ is included for RNase V1 experiments. The final RNA concentration was estimated to be 100–150 nM. Before the addition of probing enzymes, the RNAs were denatured by heating at 65°C for 5 min, followed by slow cooling to 30°C, and were then incubated with different amounts of SAH for 5 min. Finally, 0.04 U of RNase T2 (USB) or 0.16 mU of RNase V1 (Amersham Pharmacia) was added to each reaction to digest the RNAs at 30°C for 10 min. The alkaline-treated RNA ladders were obtained by incubation of labeled RNA in the RNA cleavage buffer at 100°C for 2 min, and parallel RNA sequencing products were obtained by the treatment of unfolded RNA with RNases T1 or A. They were used as markers for the assignment of guanines and pyrimidines, respectively. The reactions were terminated by addition of a gel loading dye, and the cleavage products were resolved by a denaturing gel, and visualized by phosphorimager.

In vitro –1 PRF assay

The capped reporter mRNAs were prepared by the mMESSAGE mMACHINE high-yield capped RNA transcription kit (Ambion) by following the manufacturer's instructions. Reticulocyte lysate (Progema) was used to generate the shifted and nonshifted protein products. In each assay, a total of 5 μ L reaction containing

250 ng of capped reporter mRNA, 2.5 μ L of reticulocyte lysate, and 0.2 μ L of 10 μ Ci/ μ L 35 S-labeled methionine (NEN) was incubated at 30°C for 1.5 h. The samples were then resolved by 12% SDS polyacrylamide gels, and exposed to a phosphorimager screen for quantification after drying. The reported –1 PRF efficiency was calculated, by dividing the counts of the shifted product by the sum of the counts for both shifted and nonshifted products, with calibration of the methionine content in each protein. This was reported as the average of at least three experiments.

Mammalian cell culture and luciferase assay

Human embryonic kidney HEK-293T cells were cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum. One day before the transfection, 0.5–2 $\times 10^5$ HEK-293T cells per well were plated in a 24-well culture plate with 500 μ L growth medium without antibiotics. Transfection was carried out by adding the mixture of 0.8 μ g DNA and Lipofectamine 2000 (Invitrogen) into each well, according to the manufacturer's instructions. Adenosine-2', 3'-dialdehyde was added 6 h after transfection, and the cells were further incubated for 18–40 h before being processed for assay. All the in vivo experiments were repeated three times with four to six assays for each reaction. Luciferase activity measurements for both in vitro reticulocyte lysate and in vivo transfected 293T cell lysates were performed using the Dual Luciferase reporter assay (Promega) according to the manufacturer's instructions on a CHAMELEON multilabel plate reader (HIDEX).

SUPPLEMENTAL MATERIAL

Supplemental material can be found at <http://www.rnajournal.org>.

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