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In vitro selection of RNA aptamer against *Escherichia coli* release factor 1

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Abstract—A pool of 84-nt RNAs containing a randomized sequence of 50 nt was selected against gel-immobilized *Escherichia coli* release factor 1 (RF-1) responsible for translation termination at amber (UAG) stop codon. The strongest aptamer (class II-1) obtained from 43 clones bound to RF-1, but not to UAA/UGA-targeting RF-2, with $K_d = 30 \pm 6$ nM (SPR). A couple of unpaired hairpin domains in the aptamer were suggested as the sites of attachment of RF-1. By binding to and hence inhibiting the action of RF-1 specifically or bio-orthogonally, aptamer class II-1 enhanced the amber suppression efficiency in the presence of an anticodon-adjusted (CUA) suppressor tRNA without practically damaging the protein translation machinery of the cell-free extract of *E. coli*, as confirmed by the translation of amber-mutated ($gfp^{amber141}$ or $gfp^{amber178}$) and wild-type (gfp^{wild}) genes of GFP.

There has been much interest in the site-specific incorporation of unnatural amino acids into proteins.[1] This technique provides us with a new route for engineering protein activity by expanding natural diversity. Amber codon (UAG) suppression[2,3] using chemically misacylated $tRNA_{CUA}$[4] in an *Escherichia coli* (*E. coli*) cell-free translation system has been used widely to incorporate a variety of unnatural amino acids and analogues at desired positions. However, it works effectively only for relatively hydrophobic amino acids; less hydrophobic ones often result in low incorporation efficiency.[2] This is partly explained by competition with release factor 1 (RF-1), an essential cofactor which is responsible for translation termination at stop codons UAA and UAG. This has been clearly demonstrated by experiments using an S-30 protein translation system prepared from an XAC–RF strain that expresses diminished RF-1.[5b] In this system, the nonsense suppression efficiency was improved for almost all the hydrophobic and hydrophilic amino acids tested. The reconstituted *E. coli* translation system has also allowed the preparation of an RF-1-diminished translation solution, leading to improved nonsense suppression.[6]

Another potential approach to effective amber codon suppression in the *E. coli* system involves the use of binders/inhibitors of RF-1. Sprinzl et al. isolated a polyclonal antibody against *Thermus thermophilus* RF-1.[7] The antibody also bound to *E. coli* RF-1 and highly enhanced the efficiency of amber codon suppression by the suppressor $tRNA^{Ser}_{CUA}$ in an *E. coli* cell-free translation system.[7] Although the antibody-based system achieved enhanced amber codon suppression, RNA-based binders/inhibitors, that is, aptamers, have the advantages of easy handling and preparation, and can be expressed even in cells.

To date, some RNA aptamers have been isolated as eRF1[8]- or *T. thermophilus* RF-1[9]-specific binders. However, to the best of our knowledge, there has been no successful selection of RNA aptamers directed against *E. coli* RF-1 or their practical application to improving amber suppression in an *E. coli* translation system. The aim of this work was to isolate RNA-based inhibitors/binders specific for *E. coli* RF-1. We report an in vitro-selected RNA aptamer that specifically binds to RF-1 with a dissociation constant in the low nanomolar range. The RNA aptamer binds

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tightly to and bio-orthogonally inhibits the activity of *E. coli* RF-1, enhancing the efficiency of amber suppression in an *E. coli* cell-free system.

RNA aptamers directed against N-terminally His_6-tagged RF-1 were selected from an RNA pool with a randomized sequence of 50 nt (total 84 nt in length, approximately $\sim 10^{15}$ different initial RNA molecules).[10,11] The RNA pool was mixed with *E. coli* RF-1 immobilized on Ni–NTA agarose and washed several times. The remaining RNA–RF-1 complexes were detached from the agarose with an elution buffer containing 250 mM imidazole. The eluted RNA–RF-1 complexes were then subjected to filter-binding selection using nitrocellulose membrane to further remove the false-positive aptamers. The recovered RNA was amplified by RT-PCR and the RNA pool transcribed from the resulting RT-PCR products was used for the next round of selection. Forty-three clones were isolated from the pool after the 11th round of selection and sequenced. Twenty-six of the 43 isolated clones could be classified into three families (classes I, II, and III in Fig. 1a). A rough estimation of the binding affinity using RF-1-immobilizing agarose revealed that all three families were capable of binding to *E. coli* RF-1. Aptamer class II showed the strongest binding among the families (data not shown). Aptamer class II-1, which was the most abundant clone in class II, was chosen for further investigation.

The precise binding affinity for RF-1 of aptamer class II-1 was assessed by SPR analysis. Biotin-$d(A)_{11}$ was attached to the 3′ end of the aptamer using T4 DNA ligase to generate a class II-1-bio aptamer (class II-1-bio in Fig. 1c),[12] which was immobilized on a streptavidin-coated SPR sensor chip. Injection of various concentrations of *E. coli* RF-1 (10–100 nM) over the chip resulted in distinct association and the following dissociation curves (Fig. 1b). The binding data were fitted to an equilibrium with a mass transport effect, with a dissociation constant of $K_d = 30 \pm 6$ nM. The binding specificity of aptamer class II-1 is rather high. When *E. coli* RF-2, which is responsible for translation termination at UAA and UGA stop codons, was injected onto the aptamer-immobilizing sensor, almost no binding response was observed, even at an RF-2 concentration of 100 nM (Fig. 1b). Further, the aptamer showed almost no inhibitory effect on other components of the protein translation machinery, at least at low micromolar concentrations (*vide infra*, Fig. 3). This explains the high discriminatory activity, that is the bio-orthogonality, of the aptamer in its interaction with *E. coli* RF-1.

The secondary structure of the class II-1 aptamer was predicted by calculation with the Mfold program (Fig. 1c)[13] and nuclease mapping with RNase A, which is specific for single-strand C or U (Fig. 1d). Enzymatic digestion of the 3′-Texas Red-labeled aptamer (class II-1—TexR in Fig. 1c) produced major cleavage bands in the predicted single-stranded region, at U13, U20, U23, U26, C38, U40, U43, U45, and C51, in marked contrast to the lack of or less efficient cleavage at the predicted double-stranded regions, at C5 or U53–56. This is in good agreement with the calculated secondary structure. The addition of RF-1 inhibited cleavage, especially at U20, U23, U40, and U43 (lanes 3–8 in Fig. 1d; these nucleotides are shown in the circle in Fig. 1c), suggesting that the class II-1 aptamer interacts with RF-1 at one or both of the unpaired hairpin domains.

We then evaluated an in situ deactivation of *E. coli* RF-1 using a co-existing RF-1-binding aptamer. The inhibitory activity of the aptamer was assessed by typical amber codon suppression experiments.[3] GFP-coding wild-type *gfp* (gfp^{wild}) and amber-mutated *gfp* (gfp^{amber}) genes under the control of the T7 promoter were prepared as templates for coupled transcription/translation. The transcribed $gfp^{amber141}$ and $gfp^{amber178}$ mRNAs have a UAG stop codon at position 141 and 178, respectively, numbering from the AUG start codon (Fig. 2a). Both mRNAs had an HA-tag sequence at the N-terminus with which to detect the translated full-length or truncated proteins. To terminate peptide elongation, a UAA stop codon was inserted that was recognized by RF-1 or RF-2. Run-off transcribed *E. coli* $tRNA^{Leu5}$ having an altered CUA anticodon ($tRNA^{Leu5}_{CUA}$) was used as an amber suppressor tRNA (Fig. 2b).

In vitro transcription/translation from the *gfp* genes was performed at 30 °C for 4 h, followed by SDS–PAGE analysis. The translated proteins were detected on Western blots probed with an anti-HA-tag antibody (Fig. 3). The amber-mutated $gfp^{amber141}$ gene yielded a truncated protein, but no detectable full-length GFP (data not shown). We first checked the bio-orthogonality of the aptamer class II-1 against the other components of the *E. coli* translation machinery. The bio-orthogonality of aptamer–RF-1 interaction is of vital importance for its practical application in enhanced nonsense suppression. As shown in Figure 3, the presence of aptamer (2, 4, or 6 μM) caused no apparent decrease in the yield of full-length GFP from gfp^{wild} mRNA (lanes 7–9 vs. lane 6), whose translation can be terminated by RF-2 even under the RF-1-deactivated conditions. This indicates that the selected class II-1 aptamer is highly specific for *E. coli* RF-1 and can co-exist, at least at low micromolar concentrations, with this prokaryotic translation system without disrupting translation activity.

In the presence of 0.6 μg of suppressor (in a 10 μL translation solution), < 20% of full-length GFP was produced from $gfp^{amber141}$ mRNA (lane 16 in Fig. 3). The addition of class II-1 aptamer under otherwise identical conditions resulted in the enhancement of suppression efficiency (lanes 16–19), which reached ~40% in the presence of 6 μM aptamer. These yields are relative to that (100%) of wild-type GFP obtained from gfp^{wild} under otherwise identical conditions (lane 15) and are based on band intensities in reference to the in-gel calibration set (lanes 10–15).

In situ deactivation of RF-1 activity was further confirmed by fluorescence-based assay. Because the anticodon sequence of $tRNA^{Leu5}$ is thought to be not involved in the critical domain recognized by the corresponding endogenous leucyl tRNA synthetase (LeuRS),

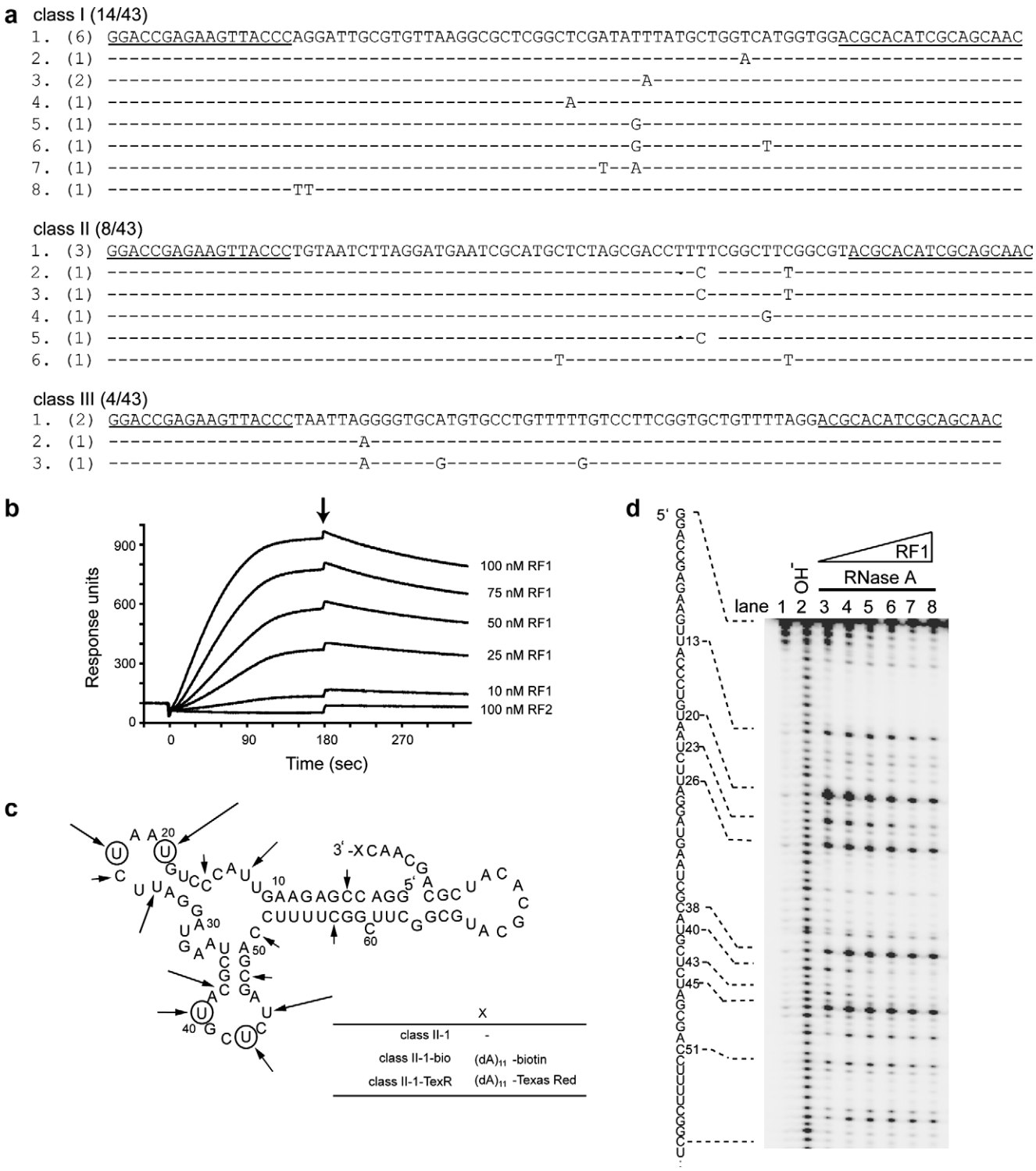

Figure 1. (a) Sequences of aptamer classes I–III. The numbers in parentheses indicate the sum of the individual sequences among 43 clones. Bars represent unchanged nucleotides. Primer sites are underlined. (b) Overlay of SPR sensorgrams showing the specific affinity of class II-1 aptamer for *E. coli* RF-1. Various concentrations of *E. coli* RF-1 (10–100 nM) or *E. coli* RF-2 (100 nM) were injected (total 60 μL, 180 s) at a flow rate of 20 μL/min over a sensor chip SA precoated with class II-1-bio aptamer. The arrow indicates the end of injection. (c) Predicted secondary structure of aptamer class II-1, generated by the Mfold program,[13] and sequences of the modified aptamers class II-1—bio and class II-1—TexR. Positions digested by RNase A in the absence of RF-1 are indicated by arrows. The lengths of the arrows correlate with the band intensities observed by PAGE analysis. Open circles indicate the positions at which the enzymatic digestion was inhibited by the presence of RF-1. (d) Enzymatic digestion of 3′-Texas Red-labeled class II-1 aptamer (class II-1—TexR) by RNase A in the absence or presence of *E. coli* RF-1. Lane 1, undigested control; lane 2, alkaline hydrolysate (OH^-); lanes 3–8, RNase-A-digested aptamer in the presence of 0, 0.65, 1.3, 2.6, 6.5, and 13 μM *E. coli* RF-1, respectively.

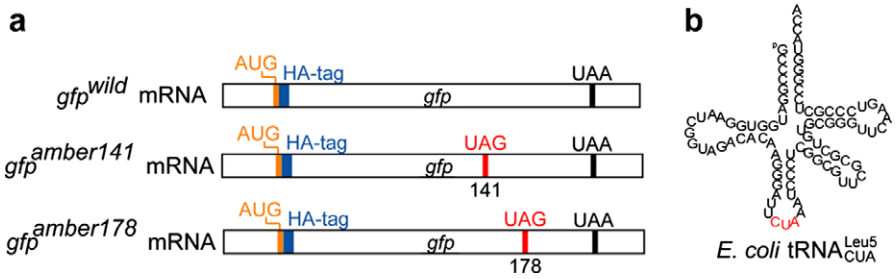

Figure 2. (a) mRNA templates used for in vitro translation experiments. AUG start codon, HA-tag site, UAG stop codon, and UAA stop codon are colored orange, blue, red, and black, respectively. (b) Sequence of amber suppressor *E. coli* $tRNA^{Leu5}_{CUA}$.

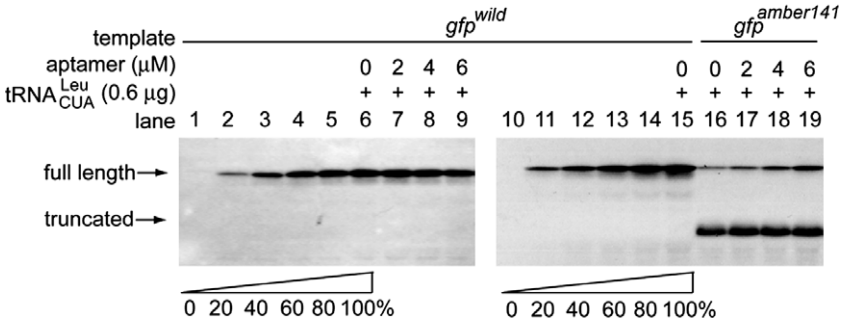

Figure 3. Western blot analysis of HA-tagged GFP translated (30 °C for 4 h) from gfp^{wild} (lanes 1–15) or $gfp^{amber141}$ (lanes 16–19) mRNA in the presence of amber suppressor $tRNA^{Leu5}_{CUA}$ (0.6 μg) and various concentrations of aptamer class II-1 (0–6 μM in lanes 6–9 and 16–19). Lanes 1–6 and 10–15 represent an in-gel calibration set of variously diluted (0%, 20%, 40%, 60%, and 80%) solutions of GFP translated from gfp^{wild} mRNA (lanes 6 and 15) under aptamer-free conditions in the presence of suppressor $tRNA^{Leu5}_{CUA}$.

the anticodon-modified $tRNA^{Leu5}_{CUA}$ still acts as a substrate for LeuRS and could be catalytically in situ acylated with Leu,[14] and deliver the charged Leu at the amber codon. The original amino acid at position 178 is Leu, so that suppression thereof results in a production of wild-type GFP, enabling us to evaluate the suppression efficiency by comparing the fluorescence intensity derived from translated full-length GFP. After the in vitro transcription/translation from the amber-mutated $gfp^{amber178}$ gene in the absence or presence of suppressor $tRNA^{Leu5}_{CUA}$ (0.6 μg), fluorescence intensity was measured by Wallac 1420 instrument. As shown in Figure 4, the presence of aptamer class II-1 (finally 12 μM) increased the suppression efficiency (44% → 89%). These results thus show that the selected aptamer class II-1 acts as a bio-orthogonal inhibitor of *E. coli* RF-1 to enhance nonsense suppression efficiency.

We have shown an in vitro-selected RNA aptamer that specifically binds to *E. coli* RF-1 with a dissociation constant in the low nanomolar range. The orthogonality of the aptamer–RF-1 interaction is high enough to allow the selective inhibition of RF-1 without practically damaging the translation activity. The aptamer system described here is suitable for an application in nonsense-suppression-based technology, especially given its ease and simplicity. In addition, these RNA-based inhibitors/binders might also be applicable to in-cell use, where aptamers could be directly transcribed from plasmids. However, it should also be noted that, although the binding of RF-1-aptamer is strong and orthogonal, the deactivation of RF-1 by the RNA aptamer was not complete. Even at 6 μM of the aptamer (lane 19 in Fig. 3), which is 200 times over the K_d (30 nM), truncated GFP is still formed, suggesting that amber codon and aptamer are still competing for RF-1, the RF-1-aptamer complex still possesses a residual protein-termination activity, or the aptamer is easily deactivated by the action of RNases. The resin-immobi-

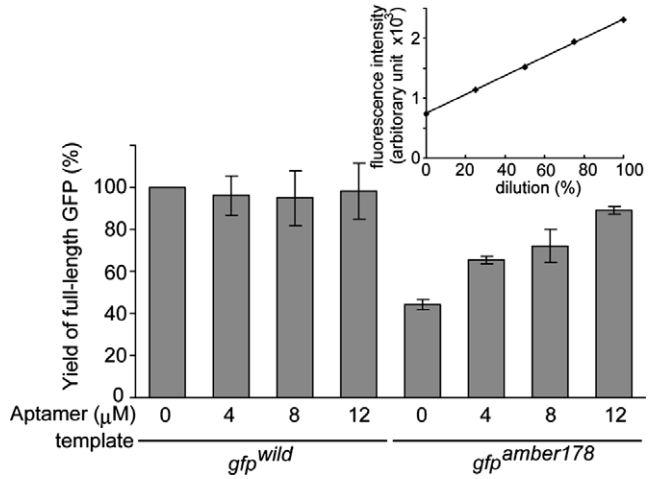

Figure 4. Relative yield of full-length GFP translated from gfp^{wild} or $gfp^{amber178}$ genes in the presence of suppressor $tRNA^{Leu5}_{CUA}$ (0.6 μg) and aptamer class II-1 (0–12 μM). These yields were determined by comparing the fluorescence intensities with those of variously diluted full-length GFP translated from gfp^{wild} gene. Inset shows a linearity of fluorescence intensity against amount (0%, 25%, 50%, 75%, and 100%) of GFP under the typical translation/assay conditions used here.

lized aptamer might overcome the limitation, since target in concern (RF-1 in this case) can be eliminated on the basis of its simple binding affinity for aptamer, regardless of the binding site or in situ inhibitory activity. Such improvement could allow an enhanced incorporation of unnatural substrates with much higher efficiencies.[5b] This work is now underway in our laboratory.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.12.013.

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