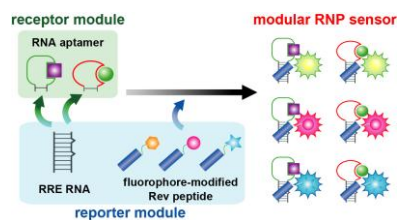# Graphical Abstract

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Facile conversion of RNA aptamers to modular fluorescent sensors with tunable detection wavelengths

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Shun Nakano[a,b], Eiji Nakata[a] and Takashi Morii[a,b,]*

Facile conversion of RNA aptamers to modular fluorescent sensors with tunable detection wavelengths

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ARTICLE INFO

ABSTRACT

A GTP aptamer was converted to a modular fluorescent GTP sensor by conjugation of RRE (Rev responsive element) RNA and successive complex formation with a fluorophore-modified Rev peptide. Structural changes associated with substrate binding in the RNA aptamer were successfully transduced into changes in fluorescence intensity because of the modular structure of ribonucleopeptides. A simple modular strategy involving conjugation of a fluorophore-modified ribonucleopeptide to the stem region of an RNA aptamer deduced from secondary structural information helps produce fluorescent sensors, which allow tuning of excitation and detection wavelengths through the replacement of the fluorophore at the N-terminal of the Rev peptide.

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The modular structure of biomacromolecules is the key feature that promotes them execute highly sophisticated biological functions such as signal transduction in cells.[1] Structural modules are embedded in monomeric proteins or in the RNA, in assemblies of protein dimers or multimers, and also in protein-RNA complexes so as to facilitate structural and functional communications between the modules. An assembly of RNA molecules and peptides, such as in the case of an RNP (ribonucleopeptide), is an ideal scaffold for the construction of a novel modular structure.[2] A complex of the Rev peptide and RRE (Rev responsive element) RNA, for which the three-dimensional structure and the interaction mode between the peptide and RNA have been established,[3] was used to construct an RNP library. Complex formation of randomized RNA sequences bearing the RRE RNA sequence and the Rev peptide affords the RNP library, which is then used for in vitro selection to obtain RNP receptors.[4] In a stepwise manner, RNP receptors for various target ligands thus selected are converted to RNP sensors by a simple modification of the Rev peptide with a fluorophore at its N-terminus. These RNP sensors exert fluorescence spectral changes upon binding to the substrate. This stepwise method takes advantage of the modular structure of RNP to generate fluorescent sensors with a variety of fluorescence emission wavelengths, while the modular RNA aptamer-based sensors reported by other groups used only a limited number of dyes.[5]

Recently, the substrate-binding mode of ATP-binding RNP was verified by structural analyses.[6] RNP was found to undergo a structural change upon binding to ATP. The results suggested that the structural change in RNP was transduced into a change in the fluorescence spectrum of the RNP sensor due to

environmental changes of the attached fluorophore. Because a number of RNA aptamers have been characterized or suggested to have changed their structures upon substrate binding, we reasoned that such a structural transition in an RNA aptamer when used as a receptor module would be transduced into a change in the fluorescence spectrum of the fluorophore-modified Rev-RRE complex by properly conjugating the fluorescent RNP so that it serves as a reporter module (Fig. 1a). If this is the case, it will be possible to convert the vast majority of RNA aptamers reported to date into modular fluorescent sensors.

As proof-of-principle, we report here a fluorescent GTP sensor with a modular RNP structure by using a GTP aptamer[7] as a receptor module and a fluorophore-labeled Rev/RRE RNA complex as a reporter module. The three-dimensional structure of the GTP aptamer[8] indicates that GTP binds to the aptamer through formation of G:G base pair, which is stacked by a G:G:G base triplet and a G:A base pair to stabilize the overall bent structure of the aptamer. The GTP aptamer is presumed to change its structure upon binding to GTP derivatives. The RNA component of the modular GTP-binding RNP (GTP-RRE) was constructed by overlapping A4:U38 present in the stem of the GTP aptamer with the A52:U66 base pair present in wild-type RRE RNA[9] (Fig. 1b). The A4:U38 base pair in the GTP aptamer does not participate in the formation of the GTP-binding pocket.[8] The stem region of the original GTP aptamer is retained as a junction to promote the folding of the GTP-binding site in the aptamer. GTP-RRE RNA thus constructed was complexed with the N-terminal 7-methoxycoumarin-modified Rev peptide (7mC-Rev)[2d-2f] to produce a modular fluorescent RNP GTP-RRE/7mC-Rev.
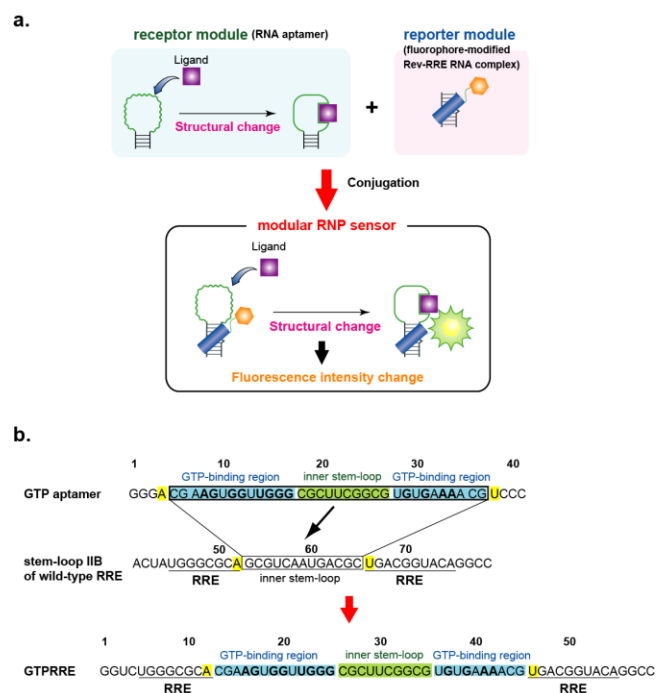
Figure 1. (a) A scheme represents the construction of a modular RNP fluorescent sensor by using an RNA aptamer and the fluorophore-labeled Rev/RRE complex. An induced fit-type aptamer is used for the ligand-binding module, and the fluorophore-labeled Rev/RRE complex is used for the reporter module. (b) Nucleotide sequences of the GTP aptamer, RRE RNA, and modular GTP-binding RNP (GTP-RRE RNA). The GTP aptamer fused to the RRE sequence (GTP-RRE) was complexed with 7-methoxycoumarin-modified Rev (7mC-Rev). The stem region of the GTP aptamer was made to overlap with the stem region of RRE (yellow). The GTP-binding region and internal stem-loop region originated from the initial library is marked in light blue and light green. The consensus sequence of the GTP aptamer is shown in bold face.

The change in the fluorescence intensity of the GTP-RRE/7mC-Rev complex was monitored at 390 nm by titration with an increasing GTP concentration (Fig. 2). The titration curve for complex formation between GTP and GTP-RRE/7mC-Rev afforded an equilibrium dissociation constant (K_D) that was much lower than 1 μM and a maximum change in the relative fluorescence intensity (I/I_0) of 2.0.[10] The high K_D value is consistent with the previously reported value for the GTP and aptamer complex.[7] In order to vary the excitation and emission wavelengths of the sensor, GTP-RRE RNA was complexed with 5-carboxyfluorescein- and NBD-modified Rev (5FAM-Rev and NBD-Rev) to produce GTP-RRE/5FAM-Rev and GTP-RRE/NBD-Rev, respectively. Titration of fluorescent RNP by GTP while monitoring the change in fluorescence intensity at 535 nm also revealed distinct fluorescence intensity changes (Fig. 2). The results demonstrate that modular RNP retains high affinity for the GTP aptamer and shows tunable excitation and the detection wavelengths.
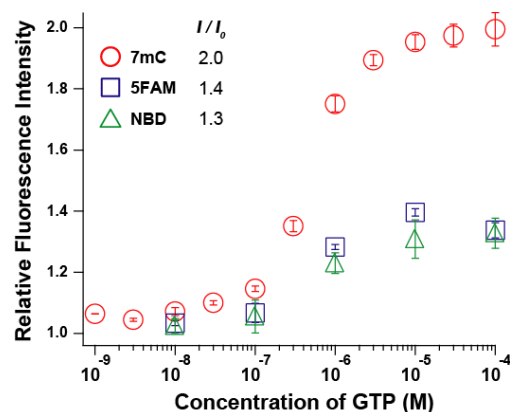

Figure 2. Saturation curves of GTP-RRE/7mC-Rev, GTP-RRE/5FAM-Rev and GTP-RRE/NBD-Rev in the presence of increasing concentrations of GTP in a buffer containing 10 mM potassium phosphate (pH 6.2), 200 mM KCl, 5 mM MgCl$_2$, 0.1 mM EDTA and 0.005% Tween 20 at 25 °C. The relative change in fluorescence intensity (I/I_0) was obtained from the fluorescence intensity in the absence (I_0) and presence (I) of the indicated concentration of GTP.

The solution structure of modular GTP-RRE/Rev was studied by in-line probing[11] in the absence and presence of the substrate guanosine (Guo) in order to investigate whether there was in fact a structural change in GTP-RRE/Rev during complex formation between modular RNP and Guo. The nucleotides that are able to achieve the in-line conformation because of structural freedom owing to the phosphodiester linkage or those that can retain the in-line conformation are prone to be hydrolyzed by the phosphodiester linkage.[11] In-line probing of the complex between GTP-RRE/Rev and Guo (Fig. 3) revealed strong cleavage bands at U18, U21, and U38 that were reported to form a bulge structure in the ligand-binding complex.[3] The loop region (U29-G32) of the inner stem-loop, which was introduced in the original RNA library for the selection of a GTP aptamer, was moderately cleaved in the presence of Guo. Further, the intensity of the bands corresponding to the nucleotides reported to form the GTP-binding pocket (G19, G20, U22—G25, U36, G37, and G39—A42) tended to decrease as the concentrations of Guo increased. These cleavage patterns in the ligand-bound state are all consistent with the structure deduced on the basis of NMR measurements.[8] The changes in the intensity of the cleavage bands clearly indicate the transition of a flexible GTP-RRE RNP structure to a well-folded structure of the complex of GTP-RRE RNP and Guo. Hence, the modular GTP-binding RNP exerts a change in fluorescence intensity in conjugation with the progressive structural changes that the receptor module undergoes upon binding to Guo.
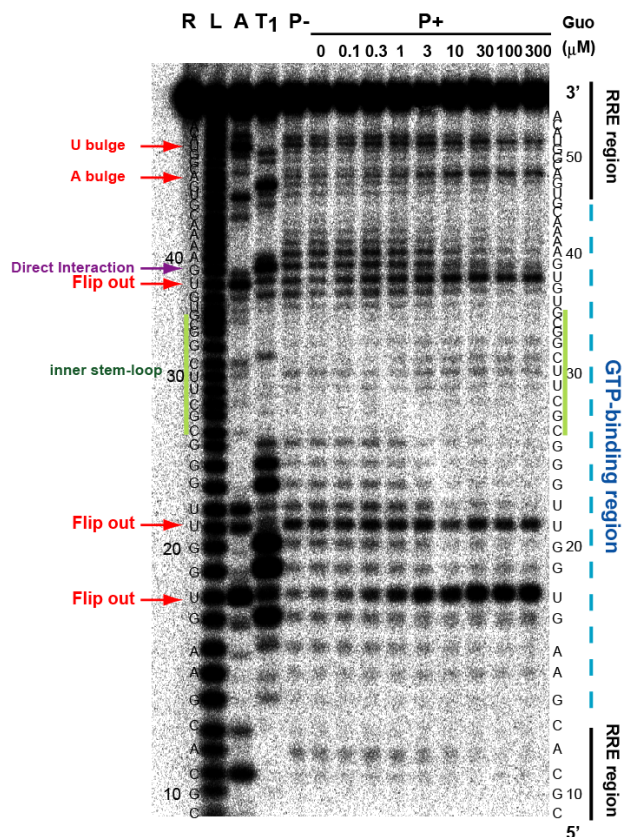
Figure 3. An autoradiogram shows the in-line probing of the GTP-RRE RNP-Guo complex incubated for 144 hours in a buffer containing 10 mM sodium cacodylate (pH 7.0), 100 mM NaCl, and 10 mM MgCl$_2$ at 25 °C. The nucleotide contributed to the direct interaction with Guo and flipped out as revealed by the previous three-dimensional structure analysis of the GTP aptamer,[8] shown in purple and red, respectively. R: intact RNA, L: RNA hydrolyzed on heating with Mg^{2+}, A: pyrimidine marker digested with RNase A, T1: G marker digested by RNase T1, P-: RNA only, P+: RNP. The concentration of Guo (μM) is indicated above each lane. GTP-RRE/Rev shows the ability to bind to Guo and GTP under conditions similar to those of the in-line probing experiments (Fig. S1).

The applicability of the design strategy of modular fluorescent RNP was further tested by using an ATP aptamer isolated by Sassanfar *et al.*[12] The structure of the ATP-binding region in the ATP aptamer changes from an unstructured form into a well-folded GNRA tetra loop upon binding to ATP.[13] The reported structure of an ATP aptamer is different from that of a GTP aptamer, and the former also contains a stem region that does not participate in ATP binding. In the case of the ATP aptamer, the A5:U32 base pair lying adjacent to the ATP-binding pocket in the aptamer[13] was made to overlap with A52:U66 of wild-type RRE RNA in order to construct ATP-RRE RNA. Thus, one base pair of the original ATP aptamer is retained to stabilize the ATP-binding site of the aptamer. The ATP-RRE/7mC-Rev complex also showed a change in fluorescence intensity on titration with ATP in a buffer containing 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM MgCl$_2$ and 0.005% Tween 20 at 4 °C, and provided a I/I_0 value of 2.6 and K_D lower than 1 μM[10] (Fig. S2). These results indicate that the modular RNP constructed from the induced fit-type aptamer and the fluorescent RRE/Rev module successfully transduces the ATP-binding event into measurable fluorescence signals. The role of the structural changes in the receptor module was verified by using an RNA aptamer that undergoes little structural change when forming a ligand-binding complex. The RNA component in ATP-binding RNP (An33 RNA) selected by us[6] from the RNP library shows a quite similar arrangement of the receptor module and the reporter module in the predicted secondary structure as observed in GTP-RRE and ATP-RRE designed here (Fig. S3a). The in-line probing of the

RNP complex (An33/Rev) formed between An33 RNA and the Rev peptide revealed that the intensity of cleaved bands both in the absence and presence of the substrate adenosine (Ado) showed quite similar patterns (Fig. S3b). This result indicates that An33/Rev undergoes little structural change upon binding to Ado. As expected, titration of the fluorescence intensity of An33/7mC-Rev by ATP showed little change in fluorescence intensity ($I/I_0 = 1.1$, $K_D = 11.2$ μM, Fig. S3c). Therefore, structural changes in the receptor module upon substrate binding are a prerequisite for effective communication between the receptor module and reporter module to achieve the modular arrangement of RNP studied here.

A simple modular strategy that conjugates a fluorophore-modified RNP to the stem region of an RNA aptamer based on secondary structural information can be used to construct fluorescent sensors. With regard to the structural changes that occur upon substrate binding, the aptamers that were successfully used for the receptor module in this study are not the rare examples of a large number of aptamers obtained to date by the in vitro selection method. The Rev peptides modified with a variety of fluorophores can be used to provide the fluorescent reporter modules. Thus, the excitation and the detection wavelengths of the fluorescent RNP are tunable. Although an aptamer with a pre-organized binding site is not suitable for use as a receptor module in the construction of an effective modular RNP assembly for fluorescence sensing, the use of the modular RNP strategy reported here would be ideal for many RNA aptamers that undergo structural changes upon substrate binding. A combination of these two modules affords a facile strategy for the construction of fluorescent sensors with desired excitation and/or detection wavelengths.

Acknowledgments

Supplementary Material

Experimental details, titration of the relative fluorescence intensity of GTP-RRE RNP complexed with 7mC-Rev under the condition used for in-line probing experiments (Fig. S1), nucleotide sequence of the ATP aptamer-conjugated ATP-RRE (Fig. S2a), saturation curves for relative fluorescence intensity changes in ATP-RRE complexed with 7mC-Rev (Fig. S2b), nucleotide sequence of the RNA component of An33/Rev ATP-binding RNP receptor (Fig. S3a), and a saturation curve for the relative changes in the fluorescence intensity of An33/7mC-Rev (Fig. S3b). This material is available free of charge via the Internet at http://www.elsevier.com. Supplementary data associated with this article can be found, in the online version, at
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Facile conversion of RNA aptamers to modular fluorescent sensors with tunable detection wavelengths

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E-mail address: t-morii@iae. kyoto-u.ac.jp
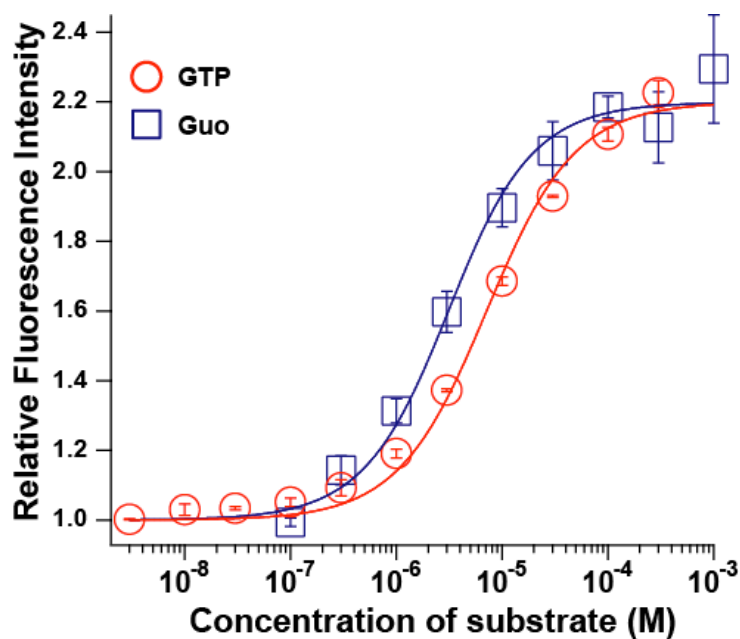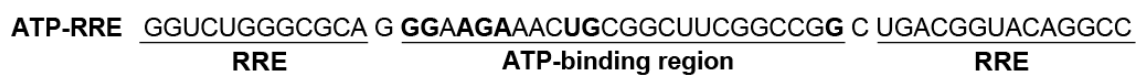
Supplementary Material

Figure S1. Titration of the relative fluorescence intensity of GTP-RRE RNP complexed with 7mC-Rev. Fluorescence intensity was measured in the presence of indicated concentration of GTP (red) or Guo (blue) in a buffer containing 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$, and 0.005% Tween20 at 25 °C, which was used for the in-line probing experiments to afford equilibrium dissociation constants (K_D) for GTP and Guo of 6.6 and 2.6 µM, respectively, and maximum changes in the relative fluorescence intensity change (I/I_0) of 2.2.

a.

ATP-RRE <u>GGUCUGGGCGCA</u> G **GGAAGAAACUGCGGCUUCGGCCGG** C <u>UGACGGUACAGGCC</u>
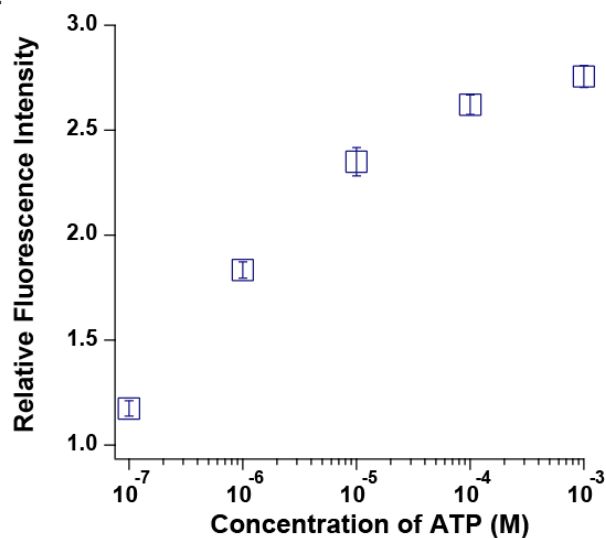   **RRE**   **ATP-binding region**   **RRE**

b.

Figure S2. (a) Nucleotide sequence of the ATP aptamer-conjugated RRE RNA (ATP-RRE). The A5:U32 base pair lying adjacent to the ATP-binding pocket in the aptamer[13] was made to overlap with A52:U66 of wild-type RRE RNA. (b) A saturation curve for the change in the relative fluorescence intensity (I/I_0) of ATP-RRE complexed with 7mC-Rev (ATP-RRE/7mC-Rev) with an increasing ATP concentration in a buffer containing 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$, and 0.005% Tween20 at 25 °C. A maximum change in I/I_0 of ATP-RRE/7mC-Rev upon addition of ATP is 2.6.

a.

An33 GGUCUGGGCGCA C GU**AGUGG** AA U**GUGUGUG** G UGACGGUACAGGCC

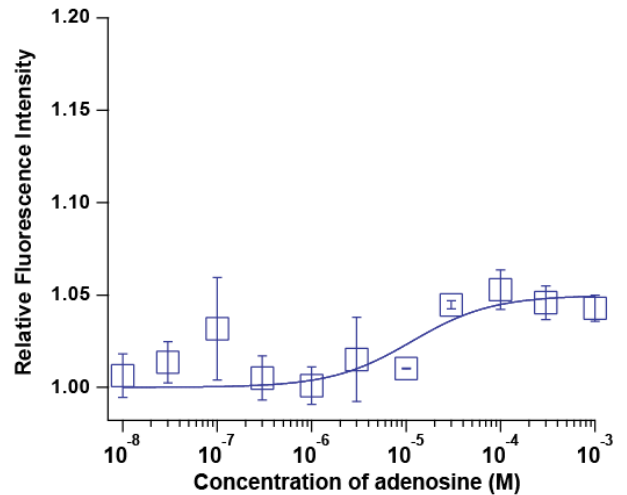RRE ATP-binding region RRE

b.
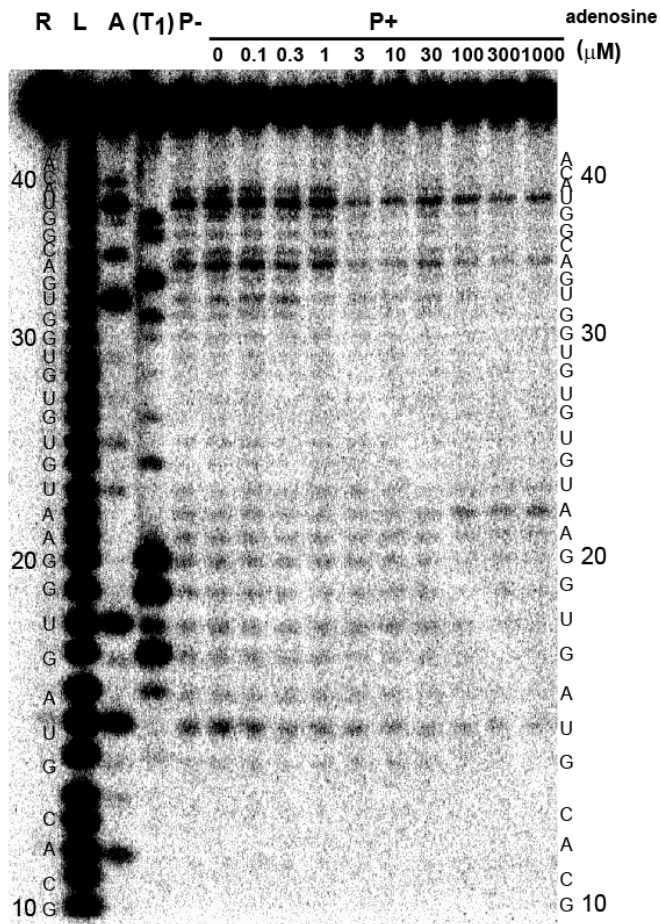
c.

Figure S3. (a) Nucleotide sequence of the RNA component of An33/Rev ATP-binding RNP that is isolated from the RNP library.[6] (b) An autoradiogram shows the cleavage pattern of in-line probing[11] for the RNP complex (An33/Rev) with an increasing Guo concentration (25 °C, 70 hours) in a buffer containing 50 mM sodium cacodylate (pH 7.0), 100 mM NaCl and 10 mM $MgCl_2$. R: intact RNA, L: RNA hydrolyzed on heating in the presence of Mg^{2+}, A: pyrimidine nucleotide markers cleaved by RNase A, T1: G nucleotide markers cleaved by RNase T1. The concentration of Guo used for the incubation is indicated on the top of each lane (0, 0.1, 0.3, 1, 3, 10, 30, 100, 300 and 1000 µM). (c) A saturation curve for the change in the relative fluorescence intensity of An33/7mC-Rev in the presence of an increasing Ado concentration in a buffer containing 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$, and 0.005% Tween20 at 4 °C shows little change in the relative fluorescence intensity (I/I_0=1.1). The equilibrium dissociation constant (K_D) is 11 µM.

Materials and Methods

Materials.

The DNA templates and primers of GTP-RRE RNA and ATP-RRE RNA were purchased from Gene Design. Nucleotides (ATP, Ado, GTP) were purchased from Sigma-Aldrich. N-Fmoc-protected amino acids, HBTU (2-(1Hbenzotriazole-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate), 1-hydroxybenzotriazole (HOBt), DIEA (N, N-diisopropylethylamine), TFA (trifluoroacetic acid), and distilled DMF (N, N-dimethylformamide) were obtained from Watanabe Chemical Industry. Fmoc-PAL-PEG resin (0.38 mmol/g) was purchased from Applied Biosystems. Gel electrophoresis grade acrylamide and bisacrylamide were obtained from Nakalai tesque. All other chemicals were reagent grade and used without further purification.

Synthesis of Oligopeptides and Fluorophore Coupling Reactions.

The Rev peptide modified with fluorophore (7mC-Rev, NBD-Rev, 5FAM-Rev) was synthesized as described previously.[2d] Peptides were synthesized on a Shimadzu PSSM-8 peptide synthesizer according to the Fmoc chemistry protocols by using Fmoc-PAL-PEG resin, protected Fmoc-amino acids, and HBTU. Fluorophore with activated group was directly coupled to N-terminal deprotected Rev peptide on the resin in DMF containing 5% (v/v) DIEA. The labeled peptides were then deprotected and cleaved from the resin, gel filtrated with Sephadex G-10 resin (GE Healthcare), purified by a reversed phase HPLC, and characterized by MALDI-TOF MS spectrometry (AXIMA-LNR, Shimadzu Biotech). Peptide concentrations were determined using absorbance with the modified fluorophore.

Preparation of the RNA Subunit of the Ribonucleopeptide Receptor.

The original double-stranded DNA were constructed by PCR amplification to add the promoter for T7 RNA polymerase using PrimeSTAR DNA polymerase (TaKaRa) with 5'-DNA primer (5'-TC<u>TAATACGACTCACTATA</u>GGTCTGGGCGCA-3': T7 RNA promoter is underlined) and 3'-DNA (5'-GGCCTGTACCGTC-3'). RNA transcription was performed using an AmpliScribeT7 kit (Epicenter) for 3 h at 37 °C, according to the supplier's recommended protocols. The resulting RNA was phenol/chloroform extracted, precipitated with ethanol, and pelleted by centrifugation. The RNA was purified by denaturing polyacrylamide gel electrophoresis and eluted. Concentrations of RNAs were determined by UV spectroscopy.

Fluorescence Measurements on the Microplate.

The 96-well fluorescence measurements were performed on a Wallac ARVOsx 1420 multilabel counter. A binding solution (100 µL) containing 1 µM of fluorescent RNP in 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$, 0.005% Tween 20 or 10 mM potassium-phosphate (pH 6.2), 200 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.005% Tween 20 with indicated concentration of ligand was gently swirled for a few minutes and allowed to sit for 30 min at 25 °C. Emission spectra were measured with an appropriate filter set for each fluorophore. Excitation and emission wavelengths for 7mC-Rev were 355 and 390 nm, for 5FAM-Rev were 485 nm and 535 nm and for NBD-Rev were 475 nm and 535 nm respectively. Images of the fluorescence intensity of wells were obtained by using the Wallac 1420 software version 2.00. Color-coded images of fluorescent intensity of each well were obtained by using the Wallac 1420 software version 2.00.

Determination of Binding Affinity by Fluorescence Titrations.

The binding affinities of fluorescent RNP to ATP, GTP, adenosine (Ado) and guanosine (Guo) were obtained by fitting the substrate titration data using the equation:

$$F_{obs} = A(([\text{FRNP}]_T + [\text{substrate}]_T + K_D) - (([\text{FRNP}]_T + [\text{substrate}]_T + K_D)^2 - 4[\text{FRNP}]_T[\text{substrate}]_T)^{1/2}) \diagup 2[\text{FRNP}]_T$$

where A is the increase in fluorescence at saturating substrate concentrations (F_{max} — F_{min}), K_D is the equilibrium dissociation constant, and [FRNP]$_T$ and [substrate]$_T$ are the total concentrations of fluorescent RNP and the substrate, respectively.

In-line Probing Procedures.

The in-line probing reactions were carried out as described previously.[11] A solution containing 5'-^{32}P-labeled RNA (120000 cpm) and non-labeled RNA (1.0 μM) was heated at 80 °C for 3 min then chilled on ice in 50 mM sodium cacodylate (pH 7.0), 100 mM NaCl, 10 mM MgCl$_2$, with Rev (1.2 μM) and the indicated concentration of substrate. The solution was incubated at 25 °C for 3 or 5 days. An aliquot (5 μL) of the reaction solution was diluted with 5 μL of a loading solution containing 8 M urea and 10 mM EDTA, and the 4 μL of the loading solution was run on a 12% denaturing polyacrylamide/8 M urea gel, then analyzed by a STORM phosphor imager (GE).