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A Novel Screening System for Self-mRNA Targeting Proteins

Bei-Wen Ying[1], Tsutomu Suzuki[1], Yoshihiro Shimizu[2] and Takuya Ueda[*,1]

[1]Department of Integrated Biosciences, Graduate School of Frontier Sciences, and [2]Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, FSB-401, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562

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Here we describe the application of an *in vitro* translation system for genetic screening, to identify RNA-binding proteins that bind to their own mRNAs. It is a relatively novel system designed using an advanced cell-free translation system reconstructed with purified translational components. Due to the absence of nucleases and proteases, the complex of mRNA and nascent polypeptide synthesized in this system is expected to exhibit high stability ensuring the following efficient selection toward the protein. *Escherichia coli* ribosomal protein S15, which is known to bind to its own mRNA, was employed as a model molecule to evaluate the system. Wild-type S15 mRNA specifically isolated from a mutant mRNA lacking the secondary structure responsible for binding the S15 protein accumulated markedly after several rounds of selection–amplification. The success of this selection demonstrates the potentiality of the systematic screening of self-mRNA targeting proteins through direct and functional selection. This strategy as a method to identify peptides or proteins that bind to their own mRNAs, is of general interest and has different potential applications, such as, the identification of new regulatory proteins or peptide motifs for RNA recognition, the study of self-mRNA–protein interactions, *etc*.

Key words: cell-free translation, *in vitro* selection, ribosomal protein S15, screen, self-mRNA binding.

RNA–protein interactions play significant roles in many fundamental biological processes. Most RNAs exist and function by interacting with RNA-binding proteins in cells. The characterization of these proteins has led to the identification of several RNA-binding motifs, among which are the RNP motif and the arginine-rich motif (ARM). In contrast, while a number of proteins that interact with their cognate mRNAs have been studied, most probably remain unrevealed. A perusal of the literature indicates that the self-mRNA binding proteins characterized so far are largely confined to ribosomal proteins, for instance, the *Escherichia coli* ribosomal protein S8, which specifically interacts with its own mRNA at a putative site responsible for translational feedback regulation (*1*). Among non-ribosomal proteins, *E. coli* threonyl-tRNA synthetase is negatively autoregulated at the translational level, and appears to be the sole known case in which the binding of a protein to its own mRNA inhibits its entry onto the ribosome (*2*). It is likely, however, that a number of self-mRNA targeting proteins, which might be regulated in a similar manner, remain undiscovered on genomes. A systematic approach, therefore, is required to select the genes for proteins capable of interacting with their own RNAs from genomes.

In vitro translation systems have proved to be highly useful for studying regulatory mechanisms (*3*) due to their relatively straightforward approaches. Cell-free translation systems are based on crude cell extracts, for example, *E. coli* S30 extract (*4*, *5*), wheat germ extract (*6*, *7*), or reticulocyte lysate (*6*, *8*), along with all necessary components. Using such cell-free systems, some screening techniques of great potential have been developed (*9*, *10*). Two different approaches to interlinking a phenotype and its genotype have been reported: ribosome display and mRNA display (*11*). The ribosome display method utilizes a non-covalent ternary complex of mRNA, ribosome and nascent polypeptide (*12*, *13*), while the mRNA display method is based upon a covalent RNA–puromycin linkage (*14*, *15*). These two methods have been shown to be useful for isolating mRNAs encoding proteins that bind to a target molecule specifically.

If a complex of nascent peptide and transcribed mRNA formed *via* a specific interaction is stably isolated using these methods, the genes of proteins with RNA binding properties could be consequently selected from a DNA library. A multiple repeat of this selection will result in the elimination of proteins that bind to other mRNAs, because the target RNA is not transcribed due to the loss of template.

In these selection methods, the cell-free translation system should be of high performance. However, the degradation of proteins and nucleic acids that inevitably occurs when crude cell extracts are used sometimes impedes the attainment of reproducible results. To address this problem, we recently developed PURE (*Protein synthesis Using Recombinant Elements*), an advanced cell-free translation system, reconstructed from purified histidine-tagged translation factors and enzymes (*16*, *17*), which we believe offers a suitable approach for *in vitro* selection. Here, we describe a model system that

[*]To whom correspondence should be addressed. Tel: +81-471-36-3641, Fax: +81-471-36-3642, E-mail: ueda@k.u-tokyo.ac.jp

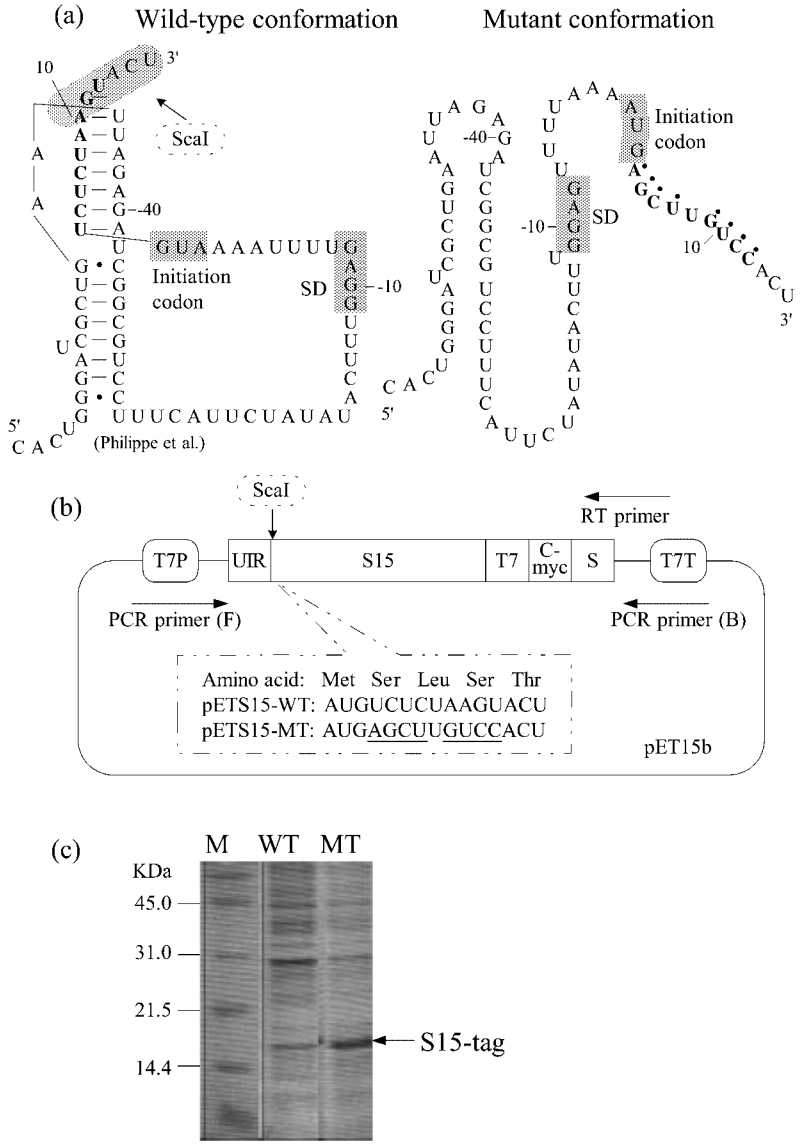

Fig. 1. **Structures of wild-type and mutant S15 genes and *in vivo* expression.** (a) Secondary structures of mRNA 5′-regions: pseudoknot structure in the wild type [from Philippe *et al.* (1990)] and open stem–loop structure in the mutant. The Shine–Dalgarno sequence, AUG initiation codon, and ScaI restrictive site are shaded. The numbering starts from the initiation codon of the reading frame. The mutations between positions +4 and +12 are shown by bold and dot-marked capitals. (b) Construction of plasmids used for *in vitro* selection of self-mRNA targeting proteins: T7P, T7 promoter; T7T, T7 terminator; S15, translated region of S15; T7, C-myc, S, polypeptide tag; UTR, 5-untranslated region of S15; SD, Shine–Dalgarno sequence; pETS15-WT, wild-type construct; pETS15-MT, mutant construct. The mutated sequences are underlined. The position of the ScaI restriction digestion site is indicated by a vertical arrow. The locations of the primers for RT-PCR are shown by horizontal arrows: RT primer, PCR primer (F), and PCR primer (B) respectively denote the S15-RT-3′-in, S15-RT-5′ and S15-RT-3′-out primers. (c) *In vivo* expression of the S15-tag protein. The wild-type and mutant S15 protein genes were expressed *in vivo* and analyzed by SDS–PAGE. WT and MT, respectively, indicate the expression of the wild-type and the mutant constructs after IPTG induction. The arrow denotes the position of the S15-tag protein. M indicates the protein molecular mass.

employs the PURE methodology for screening self-mRNA targeting proteins, some of which might be under translational regulation.

One case of autoregulation at the translational level is the *E. coli* ribosomal protein S15, which has been demonstrated to be controlled by binding to its own mRNA (*18*, *19*). A peculiar secondary structure in the 5′ upstream region of this protein has been shown to be responsible for feedback regulation and to show a structural resemblance to the S15 binding region of 16S rRNA (*20*). Hence, we decided to employ S15 as a model molecule for selecting a self-mRNA targeting protein in order to evaluate the feasibility of using the PURE system to study the autoregulation of translation through a screening approach.

RESULTS

Design of an S15 Gene for the Selection System—The *E. coli* ribosomal protein S15 binds to its own mRNA surrounding the Shine–Dalgarno region, and this binding stabilizes a distinctive pseudoknot structure through an interaction between the regions –45 to –39 and +4 to +10 (Fig. 1a) (*21*). The sequence following the initiation codon, 5′-TCTCTAAGT-3′ (located from +4 to +12), was replaced by 5′-AGCTTGTCC-3′. The resultant mutant S15 gene has the amino acid sequence Ser-Leu-Ser, which is identical to that of the wild type, but was expected to be deprived of pseudoknot structure formation (see Fig. 1a). In addition, this substitution, which causes the disappearance of the ScaI site in the wild-type S15 mRNA, enables us to discriminate the wild type from the mutant DNA. Prior to the introduction of the mutant, a T7-C-myc-S tag sequence was inserted in the C terminal region of the S15 gene for recovery of the S15–mRNA complex, which simultaneously provided a spacer in order to ensure the folding of the polypeptide responsible for RNA binding capability (Fig. 1b).

To examine the suppression of autoregulation of the mutant S15 gene, its expression was evaluated by an *in vivo* experiment. The mutant was highly expressed in cells containing the plasmid encoding the S15 gene after

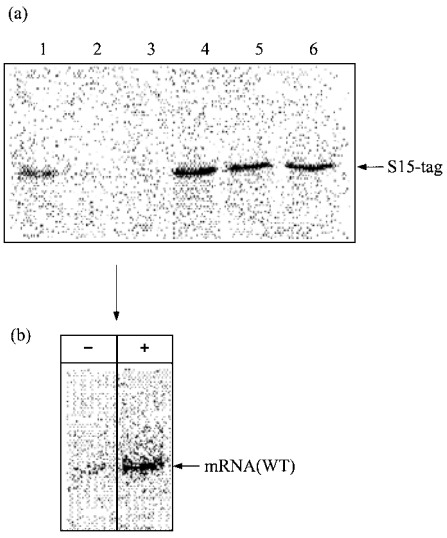

Fig. 2. **Translational repression and self-mRNA binding activity of S15 protein *in vitro*.** (a) Translation of wild-type and mutant S15 genes. The wild-type (lanes 1–3) or mutant (lanes 4–6) S15 gene was translated in the PURE system in the absence of additional protein (lanes 1 and 4), in the presence of purified S15-tag protein (1 μg/50 μl) (lanes 2 and 5), or in the presence of 5 μg purified native S15 protein (5 μg/50 μl) (lanes 3 and 6). The arrow indicates the ^{35}S-labeled translation products. (b) Binding of S15 protein to wild-type mRNA. ^{32}P-labeled mRNA was purified by T7-antibody agarose beads in the presence (+) or absence (–) of S15-tag protein, which was purified from the over-expressed *E. coli* cells, and analyzed by 4% denaturing PAGE. The arrow indicates the position of the ^{35}P-labeled wild-type mRNA.

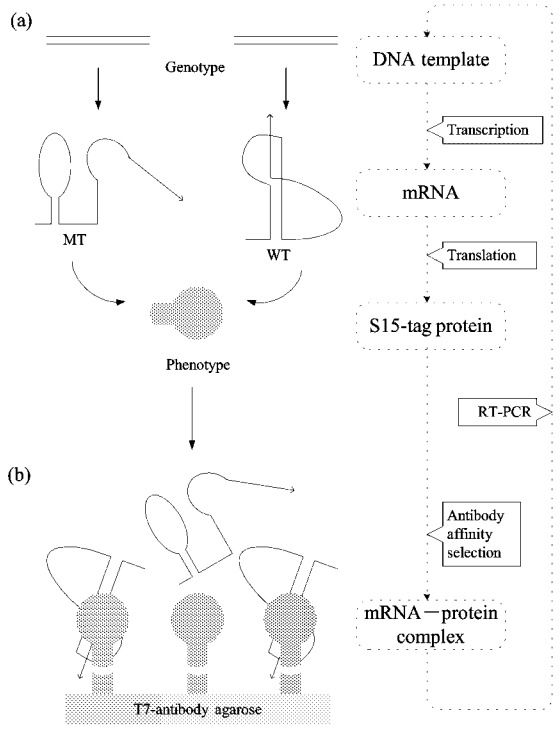

Fig. 3. **Schematic depiction of the wild-type S15 selection process through the T7-antibody affinity motif.** (a) mRNAs transcribed from the wild-type (WT) and mutant (MT) DNAs are translated to polypeptides. (b) Ternary complex of the wild-type mRNA, nascent tagged protein, and T7-antibody agarose formed during the affinity reaction. The flow chart outlines the screening procedure.

IPTG induction, whereas the wild type was definitely inhibited (Fig. 1c). This indicated that the recombinant S15 protein with an additional polypeptide tag is capable of repressing its translation *in vivo*, and suggested that the replacement of the sequence downstream of the AUG initiation codon would cause a loss of the distinctive secondary structure, resulting in constitutive expression. Because of its high level of expression, the mutant S15-tag gene was expressed in *E. coli* BL21 (DE3) and the S15-tag protein was purified (data not show) for further *in vitro* experiments.

Repression of S15 Translation and Formation of the mRNA–S15 Complex in Vitro—To clarify the correlation between translational repression and protein binding of the mRNA, the protein was synthesized *in vitro* in the presence or absence of the S15 protein. While both constructs were translated in the PURE system, much more of the S15-tag protein was produced with the mutant gene than with the wild type (Fig. 2a, lanes 1 and 4). Moreover, the expression of the S15-tag wild-type gene was drastically repressed in the presence of either the native S15 or S15-tag protein (Fig. 2a, lanes 2 and 3), whereas the mutant S15 gene was efficiently translated despite the presence of the S15 protein (Fig. 2a, lanes 5 and 6). The results of this *in vitro* experiment using the PURE system thus clearly indicate that S15 gene expression proceeds under negative regulation and is dependent upon the presence of the pseudoknot structure in the mRNA. Since this finding was consistent with the results of the *in vivo* expression experiment, we considered the mutant mRNA to be an appropriate molecule for wild-type mRNA screening through the *in vitro* selection procedure described below.

Binding of the S15 protein to the wild-type mRNA was subsequently examined in isolation using T7-antibody agarose beads. Labeled mRNA was recovered only in the presence of the S15-tag protein specifically bound to the T7-antibody agarose beads; without the S15-tag protein, no mRNA was detected (Fig. 2b). This result indicates that the resultant complex is sufficiently stable during the isolation procedure using the T7-antibody. Furthermore, the results of these *in vitro* experiments indicate that both the transcribed mRNAs and the translated proteins have the correct folding for complex formation in the PURE system, suggesting that the following specific selection without any covalent linkage would be achievable.

In Vitro Selection System for Self-mRNA Targeting Proteins—To verify the validity of selection using the PURE system, a mixture of wild-type and mutant mRNAs was employed as a starting sample for the selection procedure depicted in Fig. 3. Because the wild-type DNA has the SacI site, which was replaced by an uncut sequence in the mutant DNA, the wild type and mutant were easily discriminated after the RT-PCR reaction (Fig. 1). As expected, a few cycles of selection and amplification led to marked condensation of the wild-type mRNA (Fig. 4). T7-antibody affinity beads selection was thus shown to be effective for the isolation of an mRNA–pro-

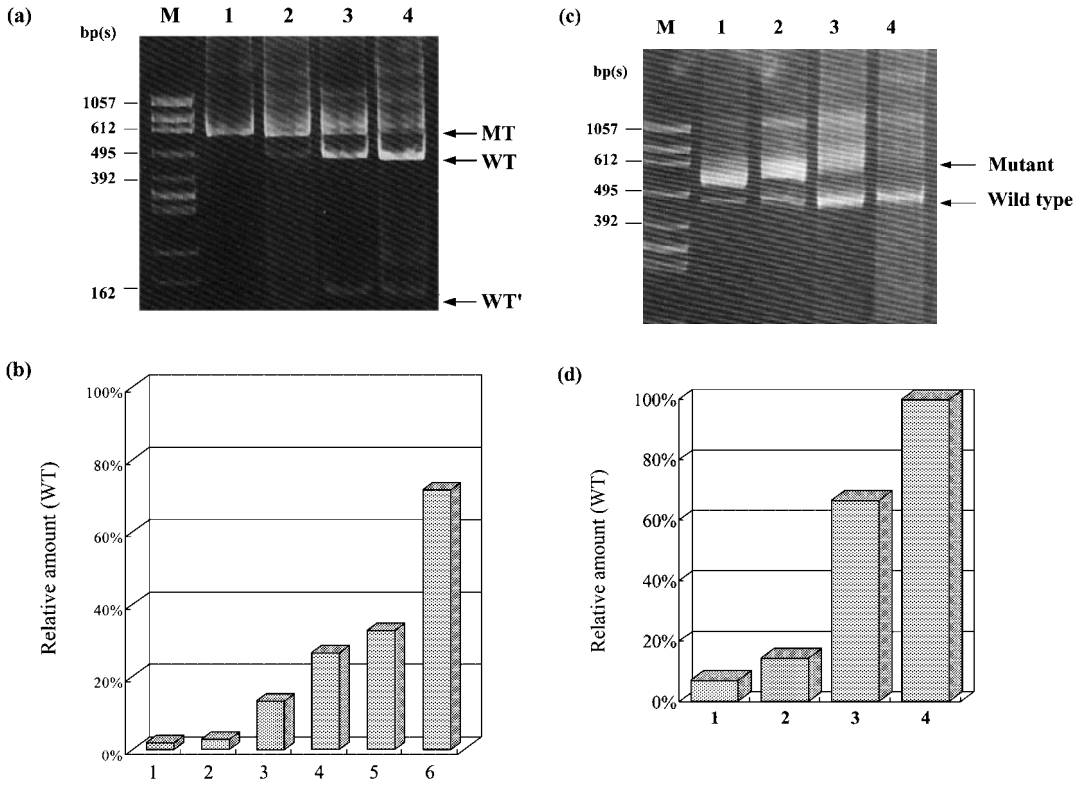

Fig. 4. **Accumulation of wild-type mRNA in the *in vitro* selection process.** (a) and (c) Analysis of amplified DNA by native PAGE: M, molecular marker of DNA; lane 1, mixture of wild-type and mutant DNAs; lanes 2, 3, and 4, RT-PCR products obtained from the 2nd, 4th, and 5th rounds of selection in (a) and the 1st, 2nd, 3rd rounds in (c), respectively. Arrows correspond to the wild-type and mutant DNAs after ScaI digestion. MT, mutant with a full size of 600 bps; WT and WT′, wild type with digested fragments of 470 bps and 130 bps, respectively [shown only in (a)]. (b) and (d) Histograms of the accumulation of wild-type DNA in the selection procedures shown in (a) and (c). Each bar indicates the wild-type DNA content in the total RT-PCR product resulting from each cycle of selection. Bar 1 shows the starting sample before selection and bars 2–6 or 2–4 represent the RT-PCR products after 1–5 or 1–3 rounds of screening, respectively.

tein complex from an unbound mutant mRNA. In the isolation procedure, we blocked the T7-antibody agarose with BSA in advance. Without such blocking, non-specific binding of mRNA to the resin occurred, resulting in inefficient mRNA recovery. As shown in Figs. 4, a and b, after 5 rounds of the selection process, the wild-type recombinant was successfully enriched more than 70% from a starting mixture containing less than 2% of the wild-type DNA. In the case of a starting mixture containing about 7% of the wild-type DNA, three rounds of selection were sufficient to reach 100% homogeneity (Fig. 4, c and d). These results clearly indicate that the selection system for self-mRNA targeting proteins is possible using the PURE system.

DISCUSSION

We have described a newly designed system for screening proteins that bind to their own mRNAs using our recently developed PURE cell-free system, and have demonstrated the efficient recovery of an RNA–protein complex with RNA unattached to the product through multiple rounds of the selection process. Based on our results using S15 mRNA, we propose the screening system depicted schematically in Fig. 5. DNA pools, derived either from a living organism or randomized DNA synthesis, are first created in fusion form with a specific affinity tag for the selection step. All of the DNAs are transcribed by RNA polymerase and translated into peptides by the PURE system. Functional selection from the translation mixture is achieved using an immobilized selection motif. In the selection step, the mRNA binding protein can be fished out contingent upon the formation of an mRNA–peptide complex. The RNA obtained is then converted to double-stranded DNA *via* RT-PCR for the next cycle of screening. The RNAs, which are bound by general RNA binding proteins or other proteins, are, of course, amplified in the first cycle of selection. However these RNAs are diminished in the next cycle of the selection process, because the loss of genetic information for the partner proteins results in the failure of complex formation. Thus, we believe that multiple rounds of the screening process will bring about the efficient recovery of self-targeting RNA from a random pool or DNA library.

The PURE system comprises highly purified components, ensuring high mRNA and protein stability compared to conventional cell-free translation systems utilizing crude extracts (*16*, *17*). The efficient recovery of mRNA is of particular significance in this screening process. Another advantage of the PURE system is that unidentified proteins binding to the target mRNA in cell extracts need not be taken into account, because all the

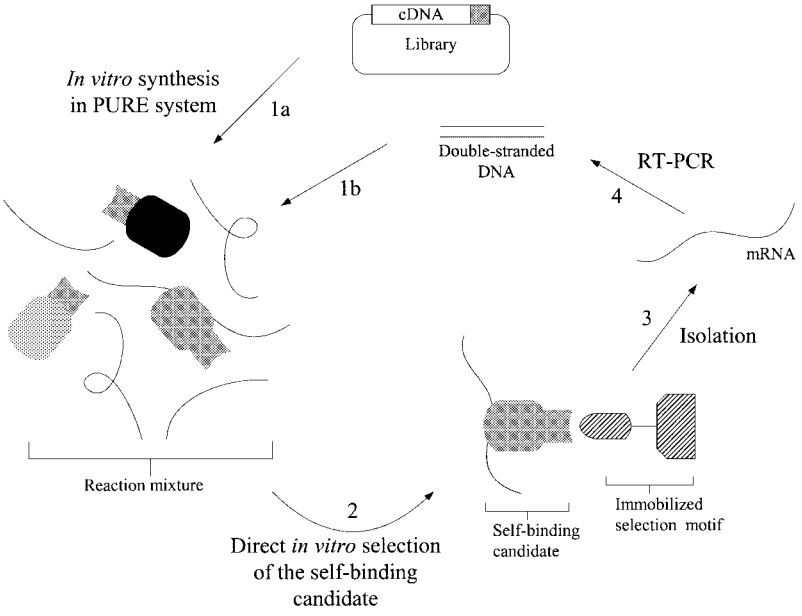

Fig. 5. **Schematic representation of the system designed to screen self-mRNA targeting proteins.** The principle of *in vitro* selection is shown as four steps. Step 1, cell-free transcription and translation of tagged candidates using the PURE system, starting from a cDNA library or double-stranded DNAs; step 2, direct screening of self-mRNA targeting proteins through a selection motif; step 3, recovery and purification of bound mRNAs; step 4, RT-PCR amplification. The DNA products obtained are employed in the next round of selection. Curved lines and shaded objects indicate random mRNAs and the corresponding polypeptides, respectively.

components are well comprehended, allowing a straightforward interpretation of the results. Hence, screening using the PURE system will be an extremely useful technique for research into RNA–protein interactions, especially translation control, which is limited to a large extent *in vivo*. We also believe that this novel and unique means of direct and functional selection of self-mRNA targeting proteins will provide a platform for the study of protein–nucleic acid interactions.

One shortcoming might be the inefficient productivity of a target protein that is translated under negative control. In the experiment described here, we fished out the wild-type S15 mRNA from the mutant mRNA, both of which produce the same protein. In this case, the mutant mRNA, which was not under negative regulation, supplied sufficient protein product for the formation of an mRNA–protein complex. Therefore, the accumulation increased from 2% to 71.6% of the total fraction after only five rounds of selection. To select a target protein that is negatively regulated via binding of the protein to the mRNA, many more cycles will be required. Of course, this mode of selection depends on either the affinity or the specificity; therefore, if the capability of a protein to bind to its own mRNA is strong enough, much more efficient accumulation can be achieved.

Because proteins have structural and catalytic roles in biology, technology for selecting proteins with a particular function *in vitro* has received considerable attention as a fundamental issue to be focused on in biochemistry and biotechnology (*9*). At present, complex procedures are needed to analyze even a single interaction between an RNA and a protein; in the post-genomic era, a means of dealing promptly with extensive data on RNA–protein networks is strongly required (*22*, *23*). In establishing an *in vitro* protein selection process, the genotype should be linked to the phenotype. Successful examples described previously include an mRNA–protein–ribosome complex and an mRNA–protein–puromycin fusion. Our system is simpler than these *in vitro* selection approaches, especially for self-mRNA targeting proteins in which an mRNA is directly attached to a peptide or protein without any covalent binder, and offers the prospect of relatively straightforward research into the protein translation process. We believe that this method will lead to the discovery of various new motifs for RNA–binding, which in turn will provide a more definitive identification of the general determinants for self-mRNA recognition by proteins.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—The gene encoding ribosomal protein S15 was amplified by PCR from the genome of *E. coli* strain A19 with the primers XbaI-S15–5′ (5′-gggcctctagaacactgggatcgctgaattagag-3′) and S15-T7-3′ (5′-gtccaccagtcatgctagccatgcgacgcagacccaggcgc-3′). A DNA fragment with the 7-C-myc-S tag sequence was synthesized by PCR elongation with two synthesized primers, 5′-atggctagcatgactggtggacagcaaatgggtatggaacagaagttgatttccgaagaagacctcgag-3′ (forward) and 5′-gctgtccatgtgctggcgttcgaatttagcagcagcggtttctttctcgaggtcttcttcggaaatcaa-3′ (backward), and then inserted into the C terminus of the S15 sequence by PCR ligation with the primers XbaI-S15–5′ and S-BamHI-3′ (5′-gcgcgggatccttagctgtccatgtgctggcgttc-3′). The polypeptide tagged fragment S15-tag was subsequently cloned into the vector pET15b (Novagen) with XbaI and BamHI restriction sites, resulting in the wild-type plasmid. The mutant plasmid was constructed using a QuikChange® site-directed mutagenesis kit (Stratagene) with the primers 5′-AGCTCATTTTAAAACTCCAAAGTATATAG-3′ (S15-MT-up) and 5′-TGTCCACTGAAGCAACAGCTAAAATCGTTTC-3′ (S15-MT-down). The DNA sequence was ascertained using an ABI PRISM 310 genetic analyzer (Applied Biosystems).

Cell-Free Translation in the PURE System—The standard translation mixture was prepared in reaction buffer to a total volume of 50 μl as described previously by Shimizu *et al.* (*16*). However, *E. coli* 70S ribosome was at a higher concentration—up to about 80 pmol per reaction. The reaction mixture containing essential elements

and energy sources was pre-incubated at 37°C for 5 min. After adding the DNA templates, the *in vitro* transcription–translation reaction was carried out at 37°C for 2 h.

In Vivo Expression and Protein Purification—The recombinant plasmids were transformed into *E. coli* strain BL21 (DE3) by electroporation (BioRad). Expression of the S15-tag protein was induced in the presence of 1 mM isopropyl-1-thio-β-D-galactoside (IPTG) at 37°C for 4 h. *In vivo* expression of the two constructs was analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The *in vivo*-expressed S15-tag protein was then purified by ion-exchange chromatography using FPLC mono S® (Amersham Biosciences) by elution with a linear gradient from Buffer A (20 mM Hepes–KOH, 10 mM KCl, 5 mM MgCl2, 0.2% β-mercaptoethanol, pH 7.6) to Buffer B (20 mM Hepes–KOH, 500 mM KCl, 5 mM MgCl2, 0.2% β-mercaptoethanol, pH 7.6).

Autoradiographic Analysis—The cell-free transcription–translation coupled reaction was performed with [^{35}S]-methionine in the presence or absence of the purified native S15 or S15-tag protein. Following SDS–PAGE, autoradiography was carried out using a FujiFilm BAS imager. To examine the binding of the S15-tag protein to mRNA after labeling the mRNA with α-^{32}P cytidine 5′-triphosphate (CTP) by means of an *in vitro* transcription reaction, the mRNA–S15-tag protein complex was recovered on T7-antibody agarose beads. After extensive washing, the agarose beads were subjected directly to denaturing PAGE (7 M urea, 4%). The mRNA bound to the beads was visualized by autoradiography as described above.

Antibody Affinity Selection—T7-antibody agarose (Novagen) was utilized for affinity selection by the batch method. In advance, 50 μl of T7-antibody agarose (50% slurry) was blocked with 5% BSA at room temperature for 1 h. Subsequently, 60 μl of the *in vitro* translation mixture treated with DNase I (RNase free, Amersham Biosciences) at 37°C for 10 minutes, was incubated with pre-blocked T7-antibody–agarose at room temperature for 20 min. The batch mixture was then washed with 500 μl of a wash buffer [20 mM Tris (PH7.5), 100 mM KCl, 10 mM $MgCl_2$] followed by centrifugation at 1,500 rpm for 5 min, repeated three times. The agarose was subjected directly to the next step of mRNA recovery.

Purification of Bound mRNAs and RT-PCR—Bound mRNAs were isolated directly from the agarose beads and purified by ISOGEN (NipponGene) according to the manufacturer's protocol. Purified mRNAs were subsequently used for reverse transcription and PCR amplification (PCR201 Kit, Toyobo), leading to double-stranded DNAs adapted for the next round of selection. The following RT-PCR primers containing the T7 promoter or T7 terminator sequences were designed. S15-RT-3′-in: 5′cgttttagaggccccaaggggttatgctagttattgctcagcg3′ for RT; S15-RT-3′-out: 5′-gcgtacaaaaaaccccctcaagacccgtttagaggccccaaggg-3′ and S15-RT-5′: 5′-ccattaatacgactcactataggg-3′ for PCR amplification (Fig. 1).

Evaluation of Screening Efficiency—The DNAs (RT-PCR products) obtained were digested by the restriction enzyme ScaI followed by native PAGE (4% gel) of the digestion products. The band intensities of the respective DNAs were estimated using NIH imaging software.

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