Additional RNA–Protein Interactions Facilitate in vitro Selection by Ribosome Display

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By introducing specific RNA–protein interaction into ribosome display method, further stabilization of RNA–ribosome–protein complex was achieved. The amount of mRNA of interest recovered after one round of selection correlates with the strength of RNA–protein interaction. This noncovalent but strong interaction should provide a simple and effective strategy for selections of functional proteins from large libraries.

Various methods exist for screening libraries for peptides or proteins with specific binding activity. For example, ribosome display,[1–3] which is based on the translation of proteins from mRNA from which the termination codon has been removed, exploits formation of a ternary complex, which consists of a protein, a ribosome and an encoding mRNA, for affinity enrichment in vitro. In vivo, phage display and yeast two-hybrid systems are widely used for isolation of proteins with binding affinity for target molecules,[4,5] but in vitro systems have advantages because larger libraries can be analyzed than in vivo systems and expression of proteins that are toxic to host cells is also possible.

We developed two methods that complement ribosome display, namely, RIDS (Ribosome-Inactivation Display System),[6] in which ribosomes are inactivated by ricin, and a method that includes direct RNA–protein interactions in addition to linkage via ribosomes (Figure 1a).[7–9] We postulated that introduction of strong RNA–protein interactions would stabilize RNA–ribosome–protein ternary complexes and, thus, enhance a selection. We also postulated that stronger RNA–protein interactions would increase the amounts of mRNA recovered. In the present study, to test our hypothesis, we compared the amounts of recovered mRNA from one round of selection after constructing several expression systems with RNA-protein interactions of different strengths by introducing different RNA motifs with different affinities for the nascent protein (Figure 1b).

Figure 1c shows the construct that we used to compare the effects of RNA–protein interactions. Tat protein of HIV-1 is a trans-activating protein that binds to a specific regulatory RNA element TAR (trans-activation response region) and regulates expression of viral protein.[10] The dissociation constant (K_d) for Tat and TAR is only 20 nM. Previously, we isolated an aptamer (Tat aptamer) that binds to Tat protein more strongly than the natural TAR RNA, with a K_d of only 120 pM.[11] To compare the effects of RNA–protein interactions exclusively, we tested a non-interacting RNA motif, C-variant (Cv),[12] as a control in addition to the two interacting RNA motifs, because a stem-loop structure at the end of mRNA has a protective effect against exonucleases.[2] We chose the FLAG peptide as the target protein of the ribosome display because an appropriate immobilized antibody was available. Since this antibody was immobilized on

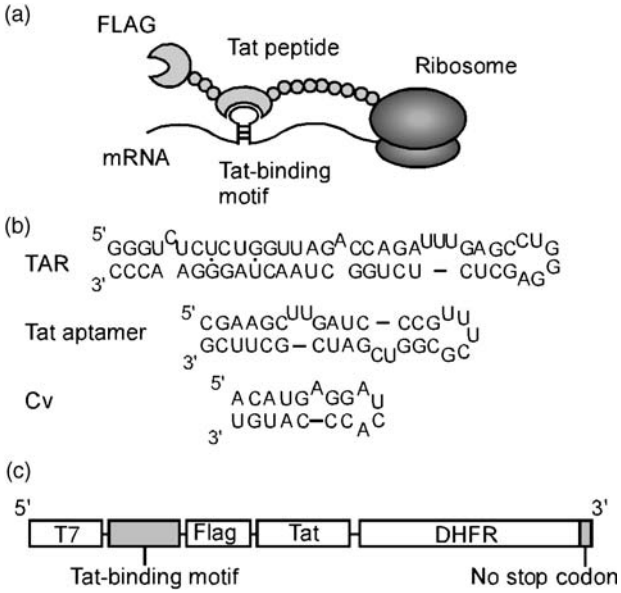

Figure 1. (A) Schematic representation of the ribosome display system with an additional RNA–peptide interaction. (B) Predicted secondary structures of RNA motifs with different affinities for Tat peptide. (C) Constructs prepared as templates for in vitro transcription. T7 indicates the T7 promoter. Translation starts with FLAG.

agarose beads, it was easy to purify FLAG peptide by centrifugation. As Tat peptide, we used a Tat-derived peptide that consisted of amino acid residues 49–86 of Tat protein. We used dihydrofolate reductase (DHFR) as a spacer protein to facilitate interactions between the RNA motif and nascent Tat peptide. The stop codon of DHFR mRNA was removed to stall the ribosome at the end of DHFR protein and to tether the peptide and mRNA. In addition to these three constructs, we constructed three constructs without a gene for FLAG as negative controls whose products do not bind to the resin with attached FLAG-specific antibodies. Each stop codon-deficient DNA fragment was amplified by PCR and transcribed in vitro using RiboMAX™ Large Scale RNA Production Systems (Promega) with Ribo m^7G Cap Analog (Promega) and [α-^{32}P]CTP (Amersham Biosciences) for detection and quantification. Transcribed mRNAs were purified with an RNeasy™ Mini Kit (Qiagen).

We used a Flexi® Rabbit Reticulocyte Lysate System (Promega) for in vitro translations, following the manufacturer's instructions. The concentrations of mRNA in samples were equalized by monitoring radioactivities. Translation was allowed to proceed at 30 °C for 20 min and then the reaction mix-

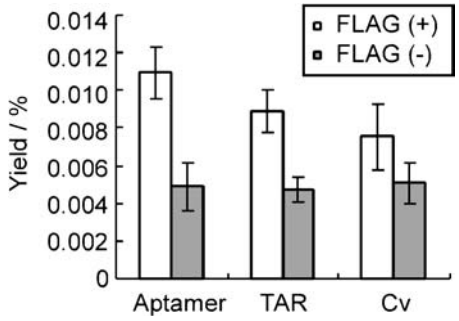

Figure 2. Effects of RNA motifs with different affinities on the amounts of mRNA bound to agarose beads. The vertical scale shows the relative yield, defined as the ratio of the mRNA with a FLAG gene to that without a FLAG gene.

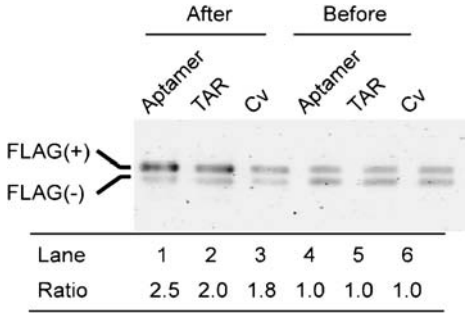

Figure 3. Enrichment of FLAG-encoding mRNA from a mixture with FLAG-non-coding mRNA by ribosome display. Equal amounts of mRNA were mixed in the first library (Lanes 4–6). After affinity selection by ribosome display, the mRNA was amplified by RT-PCR and analyzed by gel electrophoresis (Lanes 1–3).

tures were immediately chilled on ice to avoid degradation of mRNA.[3] The reaction products were mixed with Anti-FLAG® M2-Agarose (SIGMA) in binding buffer, as described elsewhere,[8] and incubated at 4 °C for 1 h with gentle mixing. Samples were then centrifuged at 700 × g for 30 s and supernatants were carefully removed. The Anti-FLAG® agarose beads were resuspended in 200-μL aliquots of binding buffer that contained 2 mM heparin sodium salt (SIGMA) with gentle mixing. This washing step was repeated three times. After the third removal of the supernatant, the amount of mRNA remaining attached to agarose beads was determined by scintillation counting. Figure 2 shows the yields, defined in each case as the ratio of radioactivity associated with FLAG-specific antibody to the total initial input of radiolabeled mRNA. As anticipated, the yield of Tat aptamer-containing mRNA was highest, followed by TAR, and then by Cv, whereas yields of mRNA without the gene for FLAG (controls) were similar for each RNA motif. Supporting our hypothesis, the affinity for the Tat peptide and the yield of mRNA showed similar trends (Figure 2). Thus the direct RNA–protein interaction in the RNA–ribosome–protein complex appeared to increase the stability of the ternary complex and to enhance the efficacy of the ribosome display system.

Our results demonstrated clearly that the translated peptide interacted with mRNA in the ribosome display complex, but it was possible that the translated peptide had interacted intermolecularly with an RNA motif within another mRNA that was not the exact template for this peptide.[7] Such intermolecular interactions would complicate the relationship between an mRNA (genotype) and its protein (phenotype) in the selection of functional proteins from a library. To examine this possibility, we mixed identical amounts of selection-positive (with FLAG) and selection-negative (without FLAG) mRNA for each RNA motif and repeated the selection. After three washes, we denatured the Anti-FLAG® antibody agarose beads in the denaturing buffer supplied with the RNeasy™ Mini kit and isolated mRNA. We reverse-transcribed the purified mRNA using M-MLV reverse transcriptase (Promega) and then amplified the cDNA by PCR with TaKaRa Ex Taq™ DNA polymerase (TaKaRa) and appropriate primers for 22 cycles. We analyzed the products of PCR by agarose gel electrophoresis and staining with SYBR Gold (Molecular Probes). Figure 3 shows that the mRNA encoding FLAG (upper band) was recovered at a consistently higher level during selection than the FLAG-non-encoding counterpart (lower band) for all RNA motifs. Among the three motifs, the Tat aptamer was the most effective, followed by TAR and then Cv. These results are consistent with the results in Figure 2. Moreover, the intensities of bands of selection-negative mRNA were almost identical for all three RNA motifs, whereas those of selection-positive mRNA were different. Thus, the RNA–peptide interactions were intramolecular and not intermolecular.

The results described here demonstrate that strong RNA–peptide interactions enhance the stability of RNA–ribosome–peptide ternary complexes and should facilitate selection. The noncovalent but strong interactions exploited here provide a simple strategy for improved selection of functional proteins from large libraries.

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