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One-Pot Preparation of mRNA/cDNA Display by a Novel and Versatile Puromycin-Linker DNA

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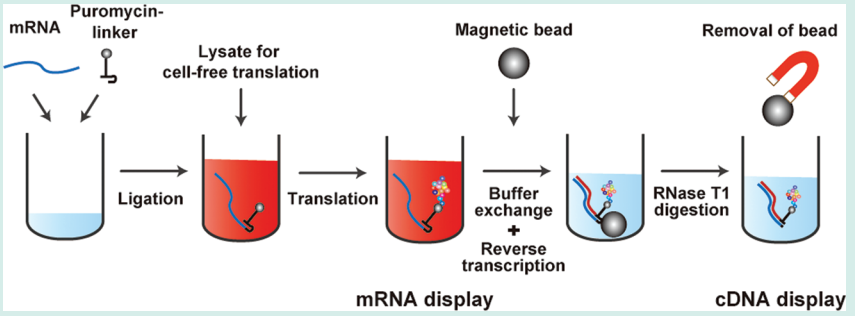
ABSTRACT:



A rapid, easy, and robust preparation method for mRNA/cDNA display using a newly designed puromycin-linker DNA is presented. The new linker is structurally simple, easy to synthesize, and cost-effective for use in "in vitro peptide and protein selection". An introduction of RNase T1 nuclease site to the new linker facilitates the easy recovery of mRNA/cDNA displayed protein by an improvement of the efficiency of ligating the linker to mRNAs and efficient release of mRNA/cDNA displayed protein from the solid-phase (magnetic bead). For application demonstration, affinity selections were successfully performed. Furthermore, we introduced a "one-pot" preparation protocol to perform mRNA display easy. Unlike conventional approaches that require tedious and downstream multistep process including purification, this protocol will make the mRNA/cDNA display methods more practical and convenient and also facilitate the development of next-generation, high-throughput mRNA/cDNA display systems amenable to automation.

KEYWORDS: mRNA display, cDNA display, in vitro selection, protein engineering, cell-free translation, peptide aptamer

INTRODUCTION

In vitro display technologies (e.g., ribosome display) using cell-free translation systems are powerful tools for designing functional proteins, such as single chain variable fragments (scFv), in an evolutionary fashion.[1,2] Among the in vitro display technologies available, the "in vitro virus"[3] (or mRNA display[4]) approach is one of the most promising genotype-phenotype linking technologies and involves an mRNA covalently bound to its coding protein through a puromycin molecule in a cell-free translation system. The mRNA-puromycin-protein fusion is the simplest genotype-phenotype linking molecule, and its library size can be theoretically expanded to 10^{14} molecules. Although the in vitro virus (mRNA display) technology has particular key advantages in comparison with other in vitro display technologies, it remains challenging to perform the selection cycle.[5] In particular, the low efficiency of the ligation of an mRNA to a puromycin-linker and the degradation of mRNA due to its instability before the selection process are major issues for researchers unfamiliar with using this technology. These difficulties can primarily be overcome through the introduction of more sophisticated puromycin-linkers.[6−9] Moreover, to stabilize the genotype-phenotype linkage, we have developed the "cDNA display", which involves binding the cDNA and its coded protein via a puromycin of a linker that includes a biotin site and a restriction enzyme site.[10,11] The puromycin-linker enables the rapid purification of mRNA-protein fusion constructs from the cell-free translation lysate and the successive removal of the ribosomes from the mRNA-protein fusion using streptavidin-coated magnetic beads. Thus, the cDNA can be synthesized efficiently on magnetic beads using this method. Although

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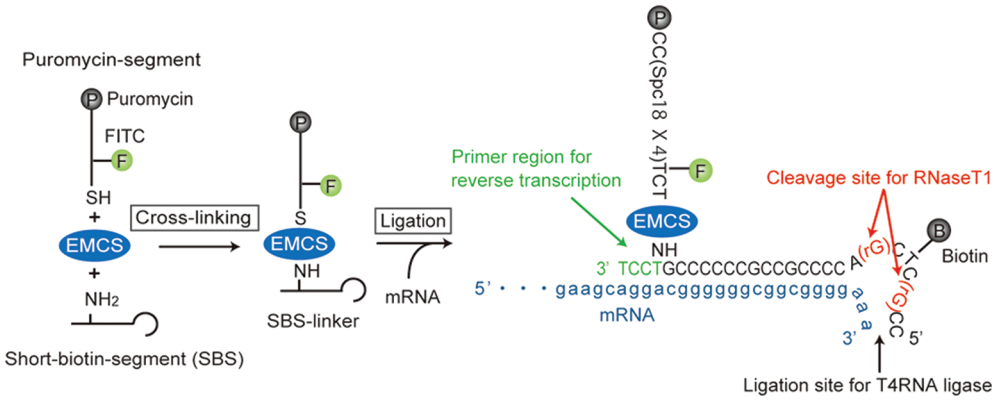

Figure 1. Schematic diagram of the puromycin-linker DNA construct. The puromycin-linker is synthesized with two fragments (puromycin segment (PS) and the short biotin segment (SBS)) by chemical cross-linking using EMCS (*N*-(6-maleimidocaproyloxy) succinimide). The final linker construct comprises four parts: a ligation site for mRNA, a primer region for reverse transcription, a biotin moiety for immobilization of the mRNA-puromycin-linker conjugate to a solid surface using biotin-streptavidin chemistry, and two cleavage sites for RNase T1 to release the mRNA/cDNA−protein fusion moiety from the solid surface. In addition, the linker includes puromycin (for covalent linking of the expressed protein to mRNA) and FITC (for detection and quantification). The 3′-region of mRNA is shown in lower case letters.

"cDNA display" has been proposed previously, real "cDNA display" had not been realized prior to the development of our cDNA display method. In Tabuchi's method,[8] ribosomes on an mRNA-protein fusion inhibit the reverse transcription reaction because the reverse transcription reaction is performed in the cell-free translation lysate without the purification of the mRNA-protein fusions. Besides, in the psoralen ligation strategy,[6] UV irradiation inhibits the reverse transcription reaction.[12] For these reasons, only our method should be suitable for performing the efficient cDNA display in in vitro directed evolution. However, to achieve both user-friendly handling and application in a high-throughput system, further improvements are required in these mRNA/cDNA display technologies.

In this study, we significantly simplified the preparation scheme of the mRNA/cDNA display by skipping the purification process of the ligation product (i.e., mRNA-puromycin-linker conjugate) using a novel versatile puromycin-linker. This novel "one-pot" preparation method omits the time-consuming purification process [i.e., (i) silica-based column purification of the mRNA-puromycin-linker conjugate; (ii) ethanol precipitation of the eluate; (iii) drying of the pellet; (iv) dissolution of the pellet in an appropriate volume of water; and (v) absorbance measurements of the templates by UV spectroscopy] as required previously. Thus, this new method enables researchers performing an in vitro peptide/protein selection to easily handle mRNA/cDNA display, as well as ribosome display.

Some automated systems have been developed for the in vitro selection of RNA or DNA aptamers;[13,14] however, there have been no reports of fully automated systems for in vitro protein selection (including phage display) because automation requires easy handling of the stable genotype-phenotype linking molecule. In this regard, our method may also facilitate the development of mRNA/cDNA display methods applicable to various high-throughput systems.

RESULTS AND DISCUSSION

Characteristics of the Novel Puromycin-Linker DNA. We previously succeeded in improving the robustness and handiness of the mRNA display method by converting "mRNA display" to "cDNA display" (e.g., through achieving efficient ligation of an mRNA to a puromycin-linker and the ability to rapidly purify products from lysate contaminated with nucleases).[10,11] Even with these advances, the mRNA/cDNA display method required a great deal of care, especially in regard to the purification processes, which were costly both in terms of time and lost product. Moreover, the laboriousness of the purification processes (e.g., ethanol precipitation) represented a significant barrier that prohibited the development of high-throughput systems necessary for exploiting the huge sequence space in evolutionary molecular engineering (directed evolution). Consequently to further improve each preparation process (i.e., in particular the ligation process including the annealing reaction, the purification of the ligated product, and the recovery of mRNA/cDNA−protein fusion from a magnetic-bead), we designed a novel puromycin-linker DNA (Figure 1).

The puromycin-linker DNA has two distinct differences from the linker described by Yamaguchi et al.[10] and Naimuddin et al.[11] First, the RNase T1 cleavage site is contained within the puromycin-linker DNA instead of the *Pvu*II restriction endonuclease cleavage site. Such a cleavage site facilitates the release of the mRNA/cDNA−protein fusion construct from the immobilization site on the magnetic beads. Second, as a DNA double-stranded region in the previously reported linker is not required for RNase T1 recognition, we were able to design a considerably shorter linker to expedite synthesis and handling.

In our linker, G-ribonucleotides (rGs) are incorporated into the short biotin segment (SBS) as RNase T1 cleavage sites. We therefore investigated where rGs should be located around the biotin-T base in the single-stranded loop. We synthesized three SBS DNAs in which the location of the rGs varied (Figure 2a). After these SBS DNA fragments were ligated with an mRNA and purified, the SBS-mRNA ligation products were immobilized on streptavidin-coated magnetic beads by biotin−avidin chemistry. Following reverse transcription of the mRNA on the magnetic beads, the mRNA/cDNA-SBS fusion products were digested with RNase T1 (Figure 2b). Any undigested fusion products remained intact on the beads. The digested products in the supernatant were analyzed by denaturing PAGE (Figure 2c). We found that RNase T1 was incapable of digesting the 3′-phosphodiester bond of rGs next to the biotin-dT but could digest rGs next to the cytosine. Consequently, this observation indicates

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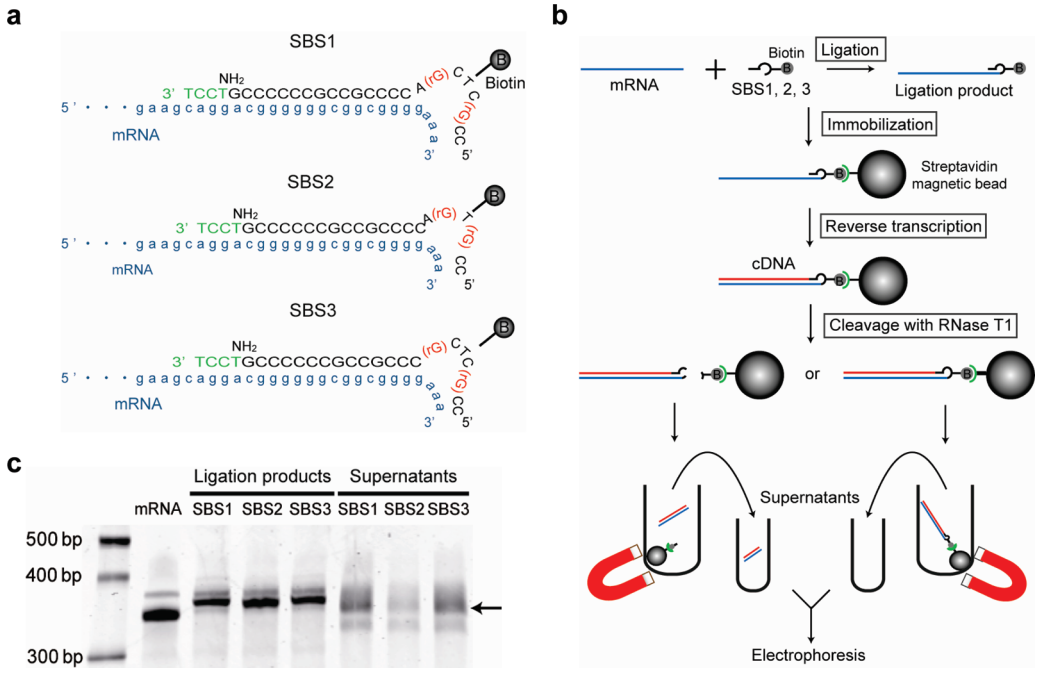

Figure 2. Optimum ribonucleotide G (rG) location for RNase T1 cleavage. (a) Three patterns of rG location around the biotin site of the puromycin-linker. (b) Experimental scheme for examining RNase T1 cleavage activity against rG around the biotin site. After an mRNA was ligated with each SBS DNA fragment, the ligated product was immobilized onto a streptavidin-coated magnetic bead. A cDNA was then synthesized on the bead using reverse transcriptase. The cDNA/mRNA hybridized double-stranded region was released from the bead by RNase T1 cleavage (left), while the double-stranded region remained immobilized on the bead when RNase T1 could not cleave the rG around the biotin site (right). After RNase T1 treatment, the supernatants include the cDNA/mRNA double-stranded regions were analyzed by PAGE. (c) The results of RNase T1 treatment against rGs in the linker DNA. After the Pou-coding mRNA was ligated with the SBS linker fragments 1, 2, or 3, each ligated product was then treated according to the experimental scheme b. Supernatants including released cDNA and mRNA were resolved by 8 M urea denaturing 6% PAGE, and the gels were stained with SYBR-Gold. The arrow indicates the digested cDNA.

that the single-stranded loop region of the linker including the two rGs and biotin-dT should be longer than six nucleotides. Despite this, the total length of our linker (28 nucleotides) can be half the length of the linker used in previous methods (58 nucleotides). Thus, we synthesized a novel puromycin-linker DNA with SBS DNAs 1 and 3 for use in subsequent experiments. To examine the performance of the novel puromycin-linker, the efficiency of each step of the protocol generating the final product (cDNA−protein fusion) was tested (Figure 3a). In the first step (Figure 3a (i)) of the protocol, more than 90% of the mRNA was ligated with the puromycin-linker within 5 min and nearly 100% of the mRNA was ligated within 20 min (Figure 3b). In the case of the previous long biotin segment (LBS)-linker,[10,11] the ligation reaction time (about 60 min) is too long for use in a high-throughput system. In general, the long length of the LBS-linker may hinder the smooth hybridization of the mRNA without nonspecific interaction because it is harder to optimize the annealing conditions for the LBS-linker and an mRNA in comparison with an SBS-linker (data not shown). Moreover, the LBS-linker must hybridize in close proximity to the 3′-terminal region of mRNA following self-hybridization and formation of the loop structure. Thus, the LBS-linker may take longer than the SBS-linker to hybridize precisely with the given region of the mRNA, resulting in the requirement of longer reaction times and a reduction in the final ligation product yield of the LBS-linker (Table 1). We found that in the second step (Figure 3a (ii)) more than 95% of the cDNA was synthesized within 5 min of the start of the RT reaction, without the initial heating step (Figure 3c). The cleavage efficiency of RNase T1 was also evaluated (as shown in Figure 3a (iii)). After the RT reaction, the mRNA/cDNA-linker product was digested with RNase T1, resulting in the production of a cDNA-linker fragment and a short fragment including biotin. We found that nearly 100% of the mRNA/cDNA-linker product was cleaved by RNase T1 within 5 min (Figure 3d). On the other hand, *Pvu*II cleaves double-stranded DNA in the LBS-linker completely, in approximately 1 h. We considered that the inefficiency of *Pvu*II in cleaving the LBS-linker may be related to torsion stress in the double-stranded DNA induced by the biotin-containing loop structure. Because RNase T1 can cleave only at rG ribonucleotides in single-stranded DNA,[15] we used RNase T1 to remove the biotin region of the linker instead of *Pvu*II. RNase T1 degrades an mRNA-protein fusion but not an mRNA/cDNA−protein fusion after reverse transcription, which results in the final product only being the mRNA/cDNA−protein fusion (cDNA display). To confirm this, we evaluated the yield of the purified cDNA−protein fusion by SDS-PAGE containing 8 M urea (Figure 3e), which was about 1% of the input mRNA-linker. The final yield may appear to be considerably smaller than the yield that was expected from the yield of the mRNA-protein fusion product (around 25% of the input mRNA-linker; see Figure 4b). On the other hand, in the case of mRNA display, the overall yield (mRNA-protein fusion after reverse transcription) through all steps from translation to right before the affinity selection step was estimated to be around 3% of the input mRNA-linker, based on the efficiency of each step (e.g., fusion formation, dT purification, ethanol precipitation, reverse transcription) and the loss of material to tubes, tips, etc.[12] In addition, the mRNA-protein

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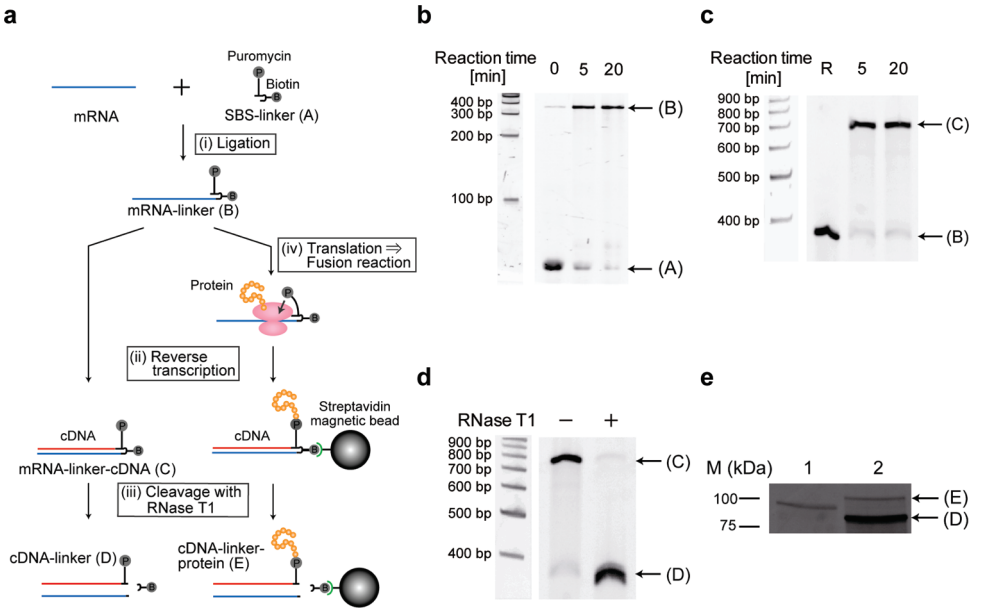

Figure 3. Characteristics of the novel puromycin-linker DNA (SBS-linker). (a) Experimental scheme for the synthesis of the cDNA display using the novel linker. In the left branch of the scheme, (ii) reverse transcription and (iii) cleavage with RNase T1 without translation process are performed to evaluate the efficiency of each step. The right branch of the scheme shows a usual process with (iv) translation and fusion reaction. In panels b−e, each arrow indicates the band correcponding to A−E. (b) After an mRNA was ligated with a SBS-linker using T4 RNA ligase, the ligated product was analyzed by 8 M urea denaturing 5% PAGE. (c) After the ligated product (R lane) was reverse transcribed, the mRNA-linker-cDNA product was analyzed by 8 M urea denaturing 5% PAGE. (d) The mRNA-linker-cDNA product was treated with RNase T1 for 5 min and analyzed by 8 M urea denaturing 5% PAGE. (e) According to the right scheme of (a), the mRNA/cDNA−protein fusion was synthesized and treated with RNase T1. The final product was analyzed by 8 M urea denaturing 6% SDS-PAGE and detected using FITC fluorescence. Lane 1 represents 0.15 pmol of the mRNA-linker as a reference. Lane 2 represents the whole final product after the fusion reaction and reverse transcription reaction as 15 pmol of the mRNA-linker was initially used in the cell-free translation reaction. The efficiency of the cDNA-linker-protein fusion formation was evaluated by comparing band intensities between the band arising from the cDNA-linker-protein fusion and the reference band (lane 1).

Table 1. Comparison of the Efficiency of Puromucin-Linker Types

puromycin-linker type	this paper (2011)	cDNA display (2009)[a,b]	mRNA display (2009)[c] (in vitro virus) (2003)[d]	mRNA display (2009)[e]
total ligation reaction (annealing reaction + ligation reaction) time	10 (min)	90 (min)	900−2400 (min)[f] (15−40 h)	30−60 (min)
enzyme for ligation reaction	T4 RNA Ligase	T4 RNA Ligase	T4 RNA Ligase	T4 DNA Ligase
mRNA/puromycin-linker ration for ligation reaction	1:1−1.5	1:4	1:200	1:1
final yield of ligated product	>95%	>90%	80−90%	20%
purification after ligation reaction for translation reaction (method)	unnecessary	necessary (silica-based column)	necessary (silica-based column)	necessary (PAGE[g])

[a] Yamaguchi et al.[10] [b] Naimuddin et al.[11] [c] Tabata et al.[21] [d] Miyamoto-Sato et al.[22] [e] Takahashi et al.[12] [f] Ligation reaction only. [g] PAGE: Polyacrylamid gel electrophoresis.

fusion was isolated at 1.3% yield based on the amount of initial input mRNA-linker after purification by mRNA display using the psoralen linker.[6] Considering the above previous study, the productivity of mRNA/cDNA−protein fusion using the novel linker may be almost equal to that of the mRNA-protein fusion. In addition, the short SBS-liker will also aid users because this approach involves a reduction in the use of the puromycin linker by around 75% (see Table 1) and the cost-effective synthesis of the puromycin-linker DNA; the cost was reduced by ∼50% compared with the costs associated with synthesis of the LBS-linker. The cost-effectiveness of puromycin-linker is especially important in high-throughput systems because in general a high-throughput system demands a large amount of puromycin-linker.

One-Pot Preparation of mRNA-Protein Fusion Products (mRNA Display). Until now, mRNA/cDNA display methods required purification of the mRNA-linker ligation product to remove excess linker (which was present at almost a 2-fold molar excess over the input mRNA) because of the inefficiency of the ligation reaction. However, by improving the ligation efficiency with the novel puromycin-linker, our method eliminates the need for purification of the ligation product because very little excess puromycin-linker would be expected to remain in the ligation reaction buffer when the mRNA and linker are added at a 1.5:1 molar ratio.

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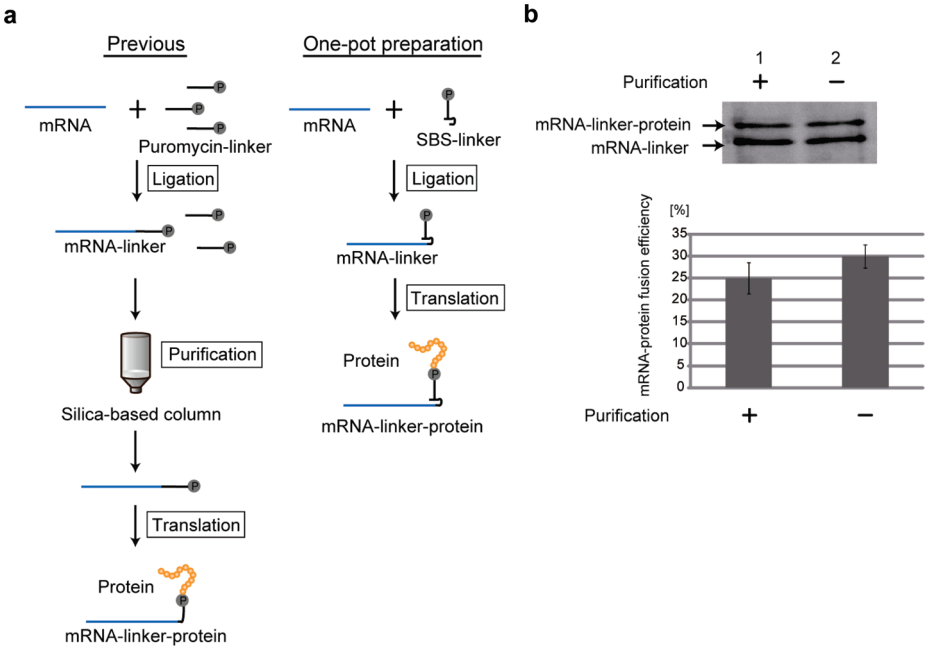

Figure 4. Synthesis of mRNA-protein fusion with and without purification of the ligation product. (a) Experimental scheme of the previous and the one-pot preparation method for the synthesis of the mRNA-protein fusion. After an mRNA is ligated to a puromycin-linker using T4 RNA ligase, the ligated product (mRNA-linker) is purified using a silica-based column to remove the ligation buffer, the enzyme and the excess linker (previous method). The purified ligation product is translated using rabbit reticulocyte lysate. In the one-pot preparation method presented here, an aliquot of the ligation reaction buffer including the ligated product is directly added to a rabbit reticulocyte lysate without purification and translated. (b) The synthesized mRNA-protein fusion with the purification step (lane 1) and without the purification step (lane 2) were resolved by 8 M urea containing 6% SDS-PAGE and detected using FITC fluorescence (upper). The same experiment was repeated three times and the mRNA-protein fusion efficiency with and without the purification step were calculated (bottom). The efficiency was calculated by using the following equation: % efficiency =100 × $a/(a + b)$, where a is the intensity of the band representing the mRNA-linker-protein (upper band) and b represents the mRNA-linker (lower band). Columns: Mean of three independent experiments performed in duplicate. Bars: Standard deviation.

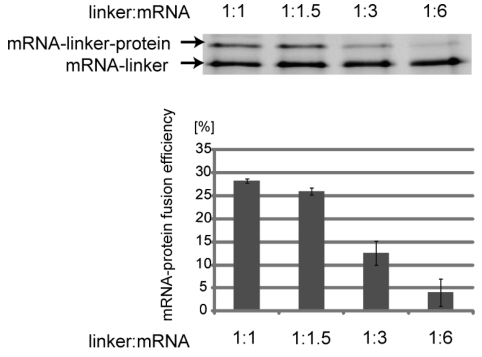

Figure 5. Effect of excess mRNA in the mRNA-protein fusion reaction. After the linker was ligated with the B domain of protein (BDA) coding mRNA at different ratios (linker/mRNA = 1:1, 1:1.5, 1:3, 1:6), each mRNA-linker was translated using a rabbit reticulocyte lysate. The products (mRNA-linker-protein) were resolved by 8 M urea containing 6% SDS-PAGE and detected using FITC fluorescence (upper). The histogram indicates the efficiency of mRNA-protein fusion formation in each ratio (linker/mRNA= 1:1, 1:1.5, 1:3, 1:6) (bottom). The efficiencies were calculated by using the following equation: % efficiency = 100 × $a/(a + b)$, where a is the intensity of the band representing the mRNA-linker-protein (upper band) and b represents the mRNA-linker (lower band). Columns: Mean of three independent experiments performed in duplicate. Bars: Standard deviation.

In general, mRNA should be of high purity in a cell-free translation system to improve translation efficiency.[16] Previous methods required purification of the ligation product (i.e., the mRNA-linker) to remove the excess puromycin-linker. Consequently, we examined whether utilizing the ligation reaction buffer containing unpurified mRNA-linker had an effect on mRNA-protein fusion synthesis as compared with conventional methods involving purification of the mRNA-linker (Figure 4a). The method employing unpurified mRNA-linker exhibited the same mRNA-protein fusion efficiency as the conventional method (Figure 4b). Moreover, we found that excess mRNA (under a 1.5-fold molar excess over the linker) did not reduce the efficiency of mRNA-protein fusion (Figure 5), allowing us to use the costly puromycin-linker without concern for losses. Consequently, we found that the unpurified ligation product is suitable for direct use in cell-free translation systems for the synthesis of mRNA-protein fusions. That also indicates that the concentration range for mRNA in our new method (i.e., from 1-fold to 1.5-fold molar excess over the linker) can be broad and makes the method well suited to robust, high-throughput applications.

From these results, we propose a "one-pot" mRNA display preparation method that enables easier and more rapid preparation of mRNA-protein fusions than existing methods. The total reaction time required to ligate an mRNA to the linker and translate this product without the post-translational fusion reaction (i.e., the high-salt incubation) is ∼30 min, which is significantly shorter than the 120 min required in other methods.[10,11,17] Furthermore, after the mRNA-protein fusion is converted to an mRNA/cDNA−protein fusion using the primer region of the novel puromycin-linker, the product can be easily purified in the

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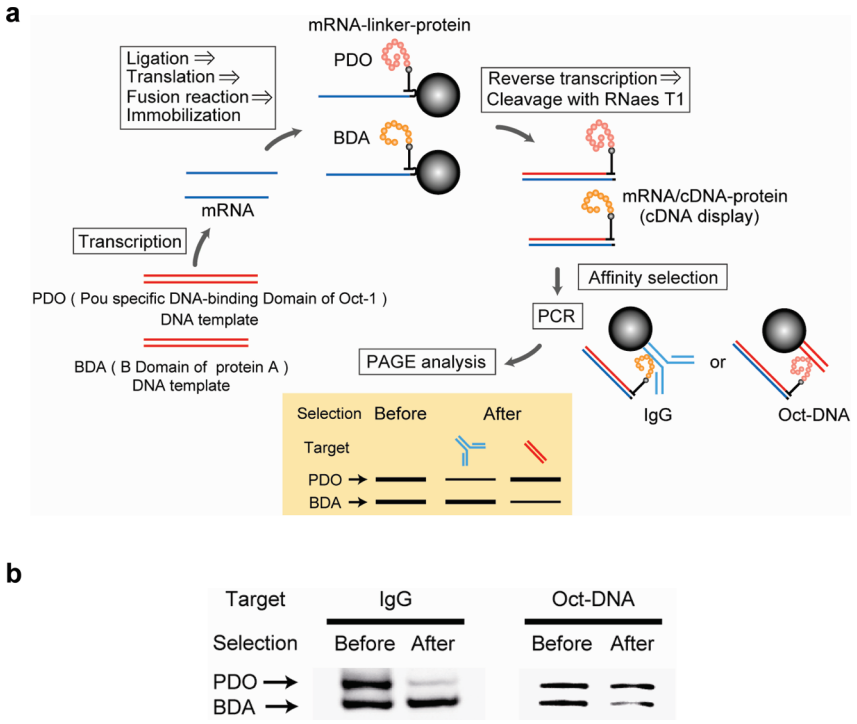

Figure 6. Validation of the cDNA display method with the novel puromycin-linker DNA using affinity selection. (a) Selection scheme using cDNA display. DNA templates encoding the Pou-specific DNA-binding domain of Oct-1 (PDO) or the B domain of protein A (BDA) were used as templates to generate mRNA/cDNA−protein fusion products (cDNA display molecules) using the following the steps: transcription, ligation of the mRNA to the puromycin-linker, translation, fusion with mRNA, immobilization on streptavidin-coated beads, reverse transcription and RNase T1 cleavage. The cDNA display mixture consisted of equal molar amounts of PDO and BDA and was subjected to affinity selection against IgG or Oct-DNA. The cDNA moieties of the selected molecules were then amplified by PCR from the eluted fraction after several washes and then the PCR products analyzed by PAGE. Selection against IgG enriches BDA (lower band) or against Oct-DNA enriches PDO (upper band). (b) Results of the affinity selection with the IgG (left) or Oct DNA (right). The initial molar ratio of PDO to BDA in the cDNA display binary mixture was 1:1. After a single affinity selection against IgG or Oct-DNA, the PCR products of the cDNA display molecules before and after selection, were analyzed by 8 M urea denaturing 5% PAGE. PDO and BDA are shown by arrows.

same tube using magnetic separation and released from the beads by using RNase T1. The total time required for preparation of an mRNA/cDNA−protein fusion from an mRNA-protein fusion is about 20 min. Therefore, we believe anyone with experience in performing phage display or ribosome display can perform a mRNA/cDNA display with ease using the presented one-pot preparation method.

Affinity Selection Using cDNA Display with the Novel Puromycin-Linker. To examine the performance of the cDNA display method with the new puromycin-linker in the selection of a target molecule, we constructed two kinds of DNA templates (Figure 6a).[10] The B domain of protein A (BDA)[18] and the Pou-specific DNA-binding domain of Oct-1 (PDO)[19] interact specifically with IgG and Oct-DNA, respectively, and can thus be specifically selected from a mixed pool. The validity of our method was evaluated by semiquantitatively estimating the ratio between PDO and BDA selected from a mixed pool against a target molecule.

In preliminary experiments, we investigated the exponential amplification phase of the PCR process. The same primers were used for PCR of each PDO and BDA template so that the ratio of the amount of amplified template to the initial amount of DNA template would be proportional by consuming the primers in the exponential amplification phase. We attempted to select for both IgG and Oct-DNA from a pool of cDNAs displaying PDO and BDA in a 1:1 ratio.

Each cDNA displaying molecule that interacted with its target molecule was amplified in a single round of selection (Figure 6b). The reproducibility of this in vitro screening approach was confirmed by three identical selection experiments (data not shown). Finally, the affinity selection model was successfully used when employing the cDNA display method with the novel puromycin-linker, as shown in previous work.[10]

The critical need to exploit an extremely large sequence space in evolutionary molecular engineering has stimulated the demand for high-throughput screening systems for functional proteins in recent years. In the development of high-throughput phage display methods, all steps should ideally be designed as "bacteria-free".[20] Phage particles synthesized under bacteria-free conditions would thus be very stable "in vitro viruses" covered with coat proteins. However, the laborious and time-consuming preparation of the genotype-phenotype linking molecule characteristic of the present in vitro virus (mRNA display) technologies creates a bottleneck that prohibits high-throughput screening. Ribosome display is an alternative method for generating mRNA-protein complexes using a cell-free translation system.[2] In fact, the ribosome display molecule can be more easily prepared using a simple protocol compared with mRNA display. However, the selection conditions are significantly restricted by the instability of mRNA and the weak connection between mRNA and its coding protein (noncovalent bond).

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The new linker we report here overcomes these problems and should enable the development of an automated high-throughput "evolution reactor" screening system for evolutionary protein engineering (directed evolution).

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. The modified oligonucleotides "puromycin segment (PS)" and "short biotin segment (SBS)" were obtained from Geneworld (Tokyo, Japan). The PS had the following composition: 5′-(S)-TC(F)-{(Spc18) × 4}-CC-(Puro)-3′, where (S) = 5′-thiol-modifier C6, (F) = fluorescein-dT, (Puro) = puromycin CPG, and (Spacer18) = spacer phosphoramidite 18. The SBS had the following composition: 5′-CC-(rG)C(T-B)C(rG)ACCCCGCCGCCCCCCG(T)CCT-3′, where (T) = amino-modifier C6 dT, (T-B) = biotin-dT, and (rG) = riboG. EMCS [N-(6-Maleimidocaproyloxy) succinimide] was purchased from Dojindo (Kumamoto, Japan).

Vectors, Oligonucleotides, and Library Construction. The B domain of protein A was obtained from the pEZZ 18 protein A gene fusion vector (GE Healthcare). The forward primer incorporated a T7 promoter, the tobacco mosaic virus "omega" 5′-untranslated region, a Kozak sequence, and an ATG start codon. The reverse primer harbored a hexa-histidine tag, a spacer sequence (GGGGGAGGCAGC), and a complementary sequence (AGGACGGGGGGCGGGGAAA) for the puromycin-linker DNA at the 3′-terminus to enable ligation between the mRNA and the puromycin-linker DNA. In the case of the Pou-specific DNA-binding domain of Oct-1 (PDO), the template was generated by replacing the B domain region with PDO.

Synthesis of Puromycin-Linker DNA. Immediately prior to use, 20 nmol of the PS 5′-thiol group was reduced with 0.1 M DTT in 50 μL of 1 M phosphate buffer (pH 7.0) for 1 h at room temperature and then desalted on a NAP-5 column (GE Healthcare). A total of 10 nmol of SBS and 2 μmol EMCS were added to 100 μL of 0.2 M sodium phosphate buffer (pH 7.0), and the mixture was incubated for 30 min at 37 °C, precipitated with ethanol and coprecipitant (Quick-precip Plus, Edge BioSystems), and dissolved in diethylpyrocarbonate (DEPC)-treated water. The reduced PS was immediately added and the mixture was stirred at 4 °C overnight. The reaction was terminated by adding DTT to a final concentration of 50 mM and incubating for 30 min at room temperature.

Ethanol precipitation at room temperature was performed to remove the excess PS. To remove the SBS and the un-cross-linked SBS-EMCS complexes, the ethanol precipitate was dissolved with DEPC-treated water and purified with a C18 HPLC column using the following conditions: column, AR-300, 4.6 × 250 mm (Nacalai Tesque, Japan); solvent A, 0.1 M triethylammonium acetate (TEAA); solvent B, acetonitrile/water (80:20, v/v); gradient, B/A (15−35% over 33 min); flow, 0.5 mL/min; detection, absorbance at 254 and 490 nm. The fraction corresponding to the last peak at an absorbance of 254 nm (corresponding to a single peak at an absorbance of 490 nm) was collected. The fraction was dried and the puromycin-linker DNA was resuspended in DEPC-treated water and stored.

Ligation of mRNA to Puromycin-Linker DNA. The 3′-ends of mRNA molecules were hybridized to the complementary strands of the puromycin-linker DNAs under annealing conditions (heating to 90 °C for 2 min followed by incubation at 70 °C for 1 min, then cooling to 4 °C) in ligation buffer (50 mM Tris-HCl pH 7.5, containing 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP). Following the addition of T4 RNA ligase (6 U/pmol mRNA, Takara) and T4 polynucleotide kinase (2 U/pmol mRNA, Takara), the ligation reactions were performed at 25 °C for 5 min. The ligation products were analyzed using 8 M urea denaturing 7% polyacrylamide gel electrophoresis (PAGE). The ligated products were visualized by FITC fluorescence using a fluoroimager (Pharos Fx, BioRad).

Reverse Transcription and Biotin Site Cleavage. Ligated products were purified using the RNeasy MinElute Cleanup Kit (Qiagen) and then added to the reverse transcription (RT) reaction mixture (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 50 mM DTT, 0.5 mM dNTP mix, and SuperScriptIII reverse transcriptase (Invitrogen) [27 U/pmol ligated product]) and incubated at 40 °C. After RT, products were incubated at 70 °C for 15 min to inactivate the reverse transcriptase. To cleave the biotin site on the linker, RNase T1 (2 U/pmol RT product, Ambion) was added, and the reaction mixture was incubated at 37 °C. The reverse transcribed single-stranded cDNA fused linker DNA-mRNA and cDNA-puromycin conjugates were analyzed by 8 M urea denaturing 5% PAGE and detected using FITC fluorescence.

Cell-Free Translation. The mRNA-puromycin conjugates (15 pmol) were added to 125 μL of the cell-free translation extract (Ambion) and the mixture was incubated at 30 °C for 20 min. To increase the yield of mRNA-protein fusions, the post-translation fusion reaction was incubated for an additional 60 min at 37 °C in the presence of a high concentration of salts (KCl and $MgCl_2$ at final concentrations of 800 and 80 mM, respectively).

Immobilization of mRNA-Protein Fusions on Magnetic Beads. Streptavidin (SA)-coated 2.8-μm magnetic beads (Dynabeads M270 streptavidin, Invitrogen) were washed twice with solution A (DEPC-treated water, 0.1 M NaOH, 0.05 M NaCl) and once with solution B (DEPC-treated water, 0.1 M NaCl) according to the manufacturer's instructions. The washed SA beads (1.0 mg) and 2× binding buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 2 M NaCl, 0.2% Triton X-100) were added to the mixture containing mRNA-protein fusions and incubated for 15 min at room temperature. The beads were subsequently washed three times with 1× binding buffer. RT was performed at 40 °C for 10 min by adding 40 μL of the RT reaction mixture (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 50 mM DTT, 0.5 mM dNTP mix), and 200 U of SuperScriptIII reverse transcriptase (Invitrogen) to the beads. To release the mRNA/cDNA−protein fusion molecules from the beads and remove mRNA, 20 U of RNase T1 (Ambion) and 60 U of RNase H (Takara) was added, then incubated at 37 °C for 10 min followed by separation on a 6% SDS-PAGE gel containing 8 M urea. Gel bands were viewed and quantified using a fluoroimager (Pharos Fx, BioRad) based on the detection of the FITC label attached to the puromycin-linker.

Target Preparation. Biotin labeling of IgG was performed using NHS-SS-biotin (Pierce) according to the manufacturer's instructions. The resultant mixture was dialyzed extensively against the buffer to remove free NHS-SS-biotin molecules, and the protein concentration was estimated by SDS-PAGE using known concentrations of IgG as standards.

The double-stranded (ds) DNA harboring the Pou binding site (Oct-DNA) was prepared by hybridization of the oligonucleotides 5′-CCAGAATATGCAAATTATTAAGGGCAAAAA-biotin-3′ and 5′-TTTTTGCCCTTAATAATTTGCATATTC-TGG-3′. The underlined sequences indicate the PDO binding site.

In Vitro Affinity Selection. Two different templates (BDA and PDO) were prepared for test screening by cDNA display

with the one-pot preparation. The cDNA display library was prepared by translating the linker-conjugated mRNAs in a 1:1 mixture of BDA and PDO, incubating with high salt, reverse transcription and RNase T1 cleavage. The BDA/PDO libraries were added to IgG-coated magnetic beads in 50 μL of selection buffer (50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 500 mM NaCl, 0.1% Tween 20). The mixture was incubated at room temperature for 30 min with rotation and the beads were then recovered and washed several times with selection buffer. The bound cDNA display molecules were removed from the beads with 50 μL of elution buffer (50 mM DTT containing selection buffer). Also, the BDA/PDO library was incubated with Oct-DNA at room temperature for 30 min. Then, the Oct-DNAs were captured by magnetic beads and the beads were washed several times with selection buffer. The bound cDNA display molecules were removed from the beads with 8 M urea containing selection buffer.

Each supernatant was precipitated with ethanol and coprecipitant (Quick-precip Plus, Edge BioSystems) and dissolved in 12 μL of DEPC-treated water. An aliquot of 4 μL was amplified by PCR using the following primers at 0.2 μM: 5′-(FITC)-CAACAACATTACATTTTACATTCTACAACTACAAGC-CACC-3′ and 5′-TTTCCCCGCCGCCCCCCGTCCTGCTT-CCGCCGTGATGATGATGATGGCTGCCTCCCCC-3′. The PCR conditions were as follows: 25 cycles of denaturation (at 95 °C) for 25 s, annealing (at 68 °C) for 20 s, and elongation (at 72 °C) for 30 s. PCR products were quantitatively analyzed on a fluorimager following 8 M urea denaturing 5% PAGE.

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Notes

I have no conflict of interest related to this paper.

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