A pseudoknot improves selection efficiency in ribosome display

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Abstract The size and diversity of ribosome display libraries depends upon stability of the complex formed between the ribosome, mRNA and translated protein. To investigate if mRNA secondary structure improves stability of the complex, we tested a pseudoknot, originating from the genomic RNA of infectious bronchitis virus (IBV), a member of the positive-stranded coronavirus group. We used the previously-isolated anti-DNA scFv, 3D8, as a target protein. During in vitro translation in rabbit reticulocyte lysate, we observed that incorporation of the pseudoknot into the mRNA resulted in production of a translational intermediate that corresponded to the expected size for ribosomal arrest at the pseudoknot. Complexes containing the mRNA pseudoknot exhibited a higher efficiency of affinity selection than that those without, indicating that the pseudoknot improves stability of the mRNA-ribosome-antibody complex in a eukaryotic translation system. Thus, in order to improve the efficiency of selection, this relatively short pseudoknot sequence could be incorporated into ribosome display.

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Introduction

Ribosome display is an efficient strategy for screening and evolution of peptides or proteins [1–4]. This system overcomes the limitations on library size that transformation efficiency places on cell-based display methods [5–8]. Improvement to library diversity permits the selection of higher affinity ligands and provides a mechanism for protein evolution [9, 10].

Genotype and phenotype are linked in ribosome display, enabling efficient selection and identification of proteins [11–13]. This linkage is accomplished by removing the mRNA stop codon, causing the ribosome to stall at the 3′ end. The ribosome forms a relatively stable complex with the mRNA and translated protein, because in the absence of a stop codon, there is a reduction in the rate at which protein is released [14]. Much research has focused on improving complex stability, as this relates directly to the library diversity and efficiency of ribosome display [15, 16]. In our opinion, the introduction of additional mechanisms for ribosomal pausing during translation might improve complex stability and selection efficiency.

mRNA secondary structure represents one way to induce ribosomal pausing and thus influence ribosomal elongation [17]. For example, the pseudoknot is found in genomic RNA from infectious bronchitis virus (IBV), a member of the positive-stranded coronavirus group [18]. During in vitro translation using rabbit reticulocyte lysate (RRL), insertion of a pseudoknot-forming sequence within influenza virus PB1 mRNA resulted in production of a new translational intermediate corresponding in size to that expected for ribosomal arrest at the pseudoknot [18]. In fact, pausing at a pseudoknot has proved a more effective barrier to ribosomal elongation than the simple stem-loop structure [17].

In this paper, we investigate whether or not the addition of mRNA secondary structure, in the form of a pseudoknot, enhances the efficiency of ribosome display. In an RRL system, insertion of a pseudoknot comprising 44 nucleotides resulted in pausing of translation and an increase in selection efficiency in ribosome display. This simple modification has the potential to be applied more widely in ribosome display, such as in the selection of higher affinity ligands.

Materials and methods

Plasmid construction

In order to construct pRD3D8, which was based on anti-DNA scFv (3D8) [20], we inserted the mouse kappa chain constant region (Cκ) at the 3' terminus of 3D8 scFv, generating a spacer that enabled the scFv fragment to fold outside of the putative ribosome tunnel. For in vitro transcription and translation, a T7 promoter and translational start site were added using an upstream primer. The 3D8 scFv DNA was amplified from pIg20 3D8 using the primers HIS3D8/back (5′-GACCACCATGGACCATCAT CATCATCATCATGAGGTCCAGCTGCAGCAG-3′) and 3D8/for (5′-GTTGGTGCAGCATCAGCCCGTTTTATTT CCAGCTTGGTC-3′). Mouse Cκ was prepared as described previously [19]. In brief, RNA was isolated from mouse spleen and Cκ DNA was amplified by RT-PCR using the primers Ck/back (5′-AAACGGGCTGATGCTG CA-3′) and Ck/for_XmaI (5′-TCCCCCCGGGCTCTAGA ACACTCATTCCTGTTGGAGCT-3′). 3D8/for and Ck/ back were designed with identical sequences corresponding to scFv at the 3' end and spacer DNA at the 5' end, enabling the two fragments to be linked by PCR and ligated into pUC18. To investigate the effects of a pseudoknot on

ribosome display, p3D8PK and p3D8g3, containing the pseudoknot and gene *III*, respectively, were prepared from pRD3D8 as presented schematically in Fig. 1A. The gene *III* spacer region was amplified from pCANTAB5E (GE Healthcare BioSciences, Piscataway, NJ). The pseudoknot-containing spacer region was amplified using the primers PK/back (5′-TCCCCCCGGGCGGGGTATCAGTCAGG CTCGGCTGGTACCCCTTGCAAAGCGAGCCTTCCT-CAACCTCCTGTCAATG-3′) and g3p/for (5′-ATACCCA AGCTTTATCACCAGTAGCACC-3′). This similarly-sized control spacer was amplified using the primers g3/back (5′-TCCCCCCGGGAGGATCCATTCGTTTGTG-3′) and g3p/ for. The amplified products were then ligated into the *Hind*III/*Xma*I site in pRD3D8 (Fig. 2A and 2B)

In vitro transcription

The plasmids pRD3D8pk and pRD3D8g3 were linearized by *Sma*I digestion (3'-terminal region) and transcribed with the T7 Cap Scribe kit (Roche Applied Sciences, Indianapolis, IN), according to the manufacturer's instructions. Transcripts were precipitated with 5 M ammonium acetate and resuspended in diethyl pyrocarbonate (DEPC)-treated water.

In vitro translation

The 25 μl reaction mixtures contained 70 mM KCl, 1.6 mM MgCl$_2$, 1 μl [^{35}S] L-methionine (10 μCi ml^{-1}), 0.5 μl 1 mM of each amino acid except methionine, 16.5 μl Flexi RRL (Promega, Madison, WI), 10 U RNasin (Promega) and sterile DEPC-treated water. Prior to addition to the reaction mix, 1.0 μg mRNA was incubated for 5 min at 65°C, then 10 min at 25°C. Translation was performed at 26°C, after which the mixture was subjected to 12% SDS-PAGE. Rainbow [^{14}C] methylated protein molecular weight

Fig. 1 DNA structures and sequences for investigating the effect of a pseudoknot on ribosome display. (**A**) A T7 promoter (T7) and a protein initiation sequence (ATG) are introduced for in vitro transcription and translation. Arrows indicate the transcriptional start and protein coding sequence. We introduced the minimal IBV pseudoknot and part of gene *III* between the sequences encoding 3D8V$_H$/K and the spacer generating plasmids p3D8pk and p3D8g3, respectively. (**B**) Predicted secondary structure of the IBV pseudoknot [17]

A 3D8 V$_H$/K   *Xma*I
→*CCCGGG***CGGGGTATCAGTCAGGCTCGGCTGGTACCCCTTGCAAAGCGAGCC**
     R   A   G   Y   Q   S   G   S   A   G   T   P   C   K   A   S   L
TTCCTCAACCTCCTGTCAATGCTGGCGGCGGCTCTGGTGGTGGTTCTGGTGGCGGCTC
  P   Q   P   P   V   N   A   G   G   G   S   G   G   G   S   G   G   G   S
TGAGGGTGGCGGCTCTGAGGGTGGCGGTTCTGAGGGTGGCGGCTCTGAGGGTGGCGGT
  E   G   G   G   S   E   G   G   G   S   E   G   G   G   S   E   G   G   G
TCCGGTGGCGGCTCCGGTTCCGGTGATTTTGATTATGAAAAAATGGCAAACGCTAATA
  S   G   G   G   S   G   S   G   D   F   D   Y   E   K   M   A   N   A   N   K
AGGGGGCTATGACCGAAAATGCCGATGAAAACGCGCTACAGTCTGACGCTAAAGGCAA
  G   A   M   T   E   N   A   D   E   N   A   L   Q   S   D   A   K   G   K
ACTTGATTCTGTCGCTACTGATTACGGTGCTGCTATCGATGGTTTCATTGGTGACGTT
  L   D   S   V   A   T   D   Y   G   A   A   I   D   G   F   I   G   D   V
TCCGGCCTTGCTAATGGTAATGGTGCTACTGGTGATA*AAGCTT*GGCAC
  S   G   L   A   N   G   N   G   A   T   G   D   K   A   W   H

B 3D8 V$_H$/K   *Xma*I
→*CCCGG***GAGGATCCATTCGTTTGTGAATATCAAGGCCAATCGTCTGACC**
     R   E   D   P   F   V   C   E   Y   Q   G   Q   S   S   D   L
TGCCTCAACCTCCTGTCAATGCTGGCGGCGGCTCTGGTGGTGGTTCTGGTGGCGGCTC
  P   Q   P   P   V   N   A   G   G   G   S   G   G   G   S   G   G   G   S
TGAGGGTGGCGGCTCTGAGGGTGGCGGTTCTGAGGGTGGCGGCTCTGAGGGTGGCGGT
  E   G   G   G   S   E   G   G   G   S   E   G   G   G   S   E   G   G   G
TCCGGTGGCGGCTCCGGTTCCGGTGATTTTGATTATGAAAAAATGGCAAACGCTAATA
  S   G   G   G   S   G   S   G   D   F   D   Y   E   K   M   A   N   A   N   K
AGGGGGCTATGACCGAAAATGCCGATGAAAACGCGCTACAGTCTGACGCTAAAGGCAA
  G   A   M   T   E   N   A   D   E   N   A   L   Q   S   D   A   K   G   K
ACTTGATTCTGTCGCTACTGATTACGGTGCTGCTATCGATGGTTTCATTGGTGACGTT
  L   D   S   V   A   T   D   Y   G   A   A   I   D   G   F   I   G   D   V
TCCGGCCTTGCTAATGGTAATGGTGCTACTGGTGATA*AAGCTT*GGCAC
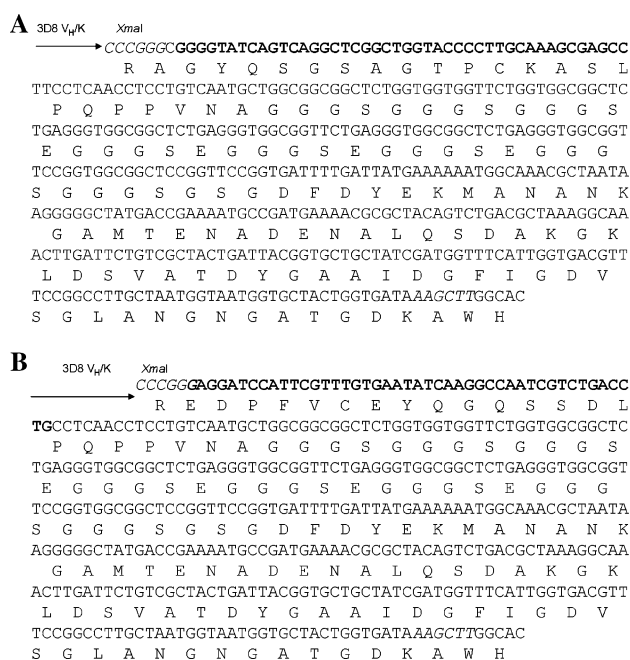 S   G   L   A   N   G   N   G   A   T   G   D   K   A   W   H

Fig. 2 Nucleotide sequences of the minimal IBV pseudoknot and portion of gene *III* introduced into p3D8pk and p3D8g3. Restriction enzyme sites are in italics, the similarly-sized sequences corresponding to the minimal IBV pseudoknot (**A**) and section of gene *III* (**B**) are in bold, and the predicted amino acid sequences are indicated below the nucleotide sequences

markers (GE Healthcare BioSciences) were used as standards and proteins were detected by autoradiography.

Affinity selection

Microtiter plates were coated overnight using 50 μl calf thymus DNA (10 μg ml^{-1} in PBS; Sigma-Aldrich, Milwaukee, MI) at 4°C. Plates were then washed with PBS, blocked with sterilized 3% (w/v) BSA for 2 h at room temperature, washed three times with PBSM (PBS containing 5 mM MgCl$_2$), then incubated on ice for at least 10 min. The translated mixture was mixed with ice-cold buffer (PBS containing 5 mM MgCl$_2$, 1 μg cycloheximide and 1.5% [w/v] BSA) and added to the antigen-coated microtiter wells. Plates were incubated for 1 h in a cold room on ice, washed three times with ice-cold PBSTM (PBSM and 0.05 % [v/v] Tween 20®) and twice with ice-cold PBSM. The ribosomal complexes adhering to the wells were dissociated in 200 μl EB20 buffer (PBS and 20 mM EDTA) at 65°C for 10 min. mRNA was isolated using an RNA isolation kit (Roche Applied Sciences).

RT-PCR

Selected mRNA was reverse transcribed using SuperScript™ III reverse transcriptase (Invitrogen, Carlsbad, CA)

and cDNA was amplified with *Taq* DNA polymerase (Genesis, Daejun, Korea) for 25 cycles (20 s at 95°C, 40 s at 55°C, 30 s at 68°C) in a 50 μl PCR mixture using the primers HIS6S2/back (5′-GACCACCATGGACCATCAT CATCATCATCATGAGGTCCAGCTGCAGCAG-3′) and 3D8re/for (5′-CAGCCAGGGAGGATGGAGAC-3′).

ELISA of translated proteins

ELISA was used to determine the binding activity of translated proteins to DNA. Microtiter plates (Costar, High Wycombe, UK) were coated with 50 μl calf thymus DNA solution (10 μg ml^{-1} in PBS) or PBS (negative control) at 4°C overnight, then washed three times with PBS and blocked with 3% (w/v) BSA in PBS for 1 h at 37°C. After a further washing with PBS, translated protein in ice-cold PBSM was added to the wells and incubated for 1 h at 4°C. The wells were washed three times with PBST, after which anti-His antibody (1:1,000; Qiagen, Valencia, CA) was added and the plates incubated for 1 h at room temperature. Plates were washed three times with PBST and incubated with HRP-conjugated anti-mouse IgG antibody (Zymed Laboratories, San Francisco, CA) for 1 h at room temperature. Following further washing as described above, 100 μl ABTS [2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); GE Healthcare BioSciences] substrate solution was added to each well and absorbance measured at A$_{405}$.

Results

Plasmid construction

We used a previously-isolated anti-DNA scFv (3D8) as the translated product for this experiment [20]. We constructed a single-chain fragment (V$_H$/K) comprising the 3D8 scFv heavy chain variable domain (V$_H$) and the complete constant region of the mouse κ light chain (K), which enabled the target protein to fold outside the putative ribosomal tunnel. In order to investigate the effect of a pseudoknot on selection in ribosome display, we inserted two different similarly-sized spacers at the 3' terminus of V$_H$/K. The control spacer region was amplified from gene III of the filamentous phage M13 and the pseudoknot from the genomic sequence of IBV (Fig. 1A). The predicted pseudoknot secondary structure is depicted in Fig. 1B [17].

Ribosomal pausing at the pseudoknot

In a previous study, translational pausing was demonstrated using an IBV-derived pseudoknot inserted into influenza

virus PB1 reporter mRNA [18]. We investigated whether or not ribosomal pausing was induced at the predicted site in our construct. We used plasmids that contained the IBV pseudoknot and part of the filamentous phage M13 gene *III* (p3D8pk and p3D8g3, respectively) between the sequences encoding 3D8 V_H/K and a spacer. The mRNA was prepared by in vitro transcription, and then translated in an RRL system. To detect pausing, translation reactions were harvested every 2.5 min and the reaction mixtures subjected to SDS-PAGE. Translated proteins were observed using autoradiography. In comparison to p3D8g3 (control), we observed that p3D8pk mRNA produced a translational intermediate of ca. 45 kDa (Fig. 3). As with a previous study [18], this was a paused intermediate and not a dead-end product. The intermediate was detected initially at 10 min and could be observed clearly at 15 min. The fully translated protein (ca. 60 kDa) was detected at 12.5 min and increased in abundance over time (Fig. 3A). No translational intermediate was detected from p3D8g3 mRNA, which does not contain a pseudoknot (Fig. 3B).
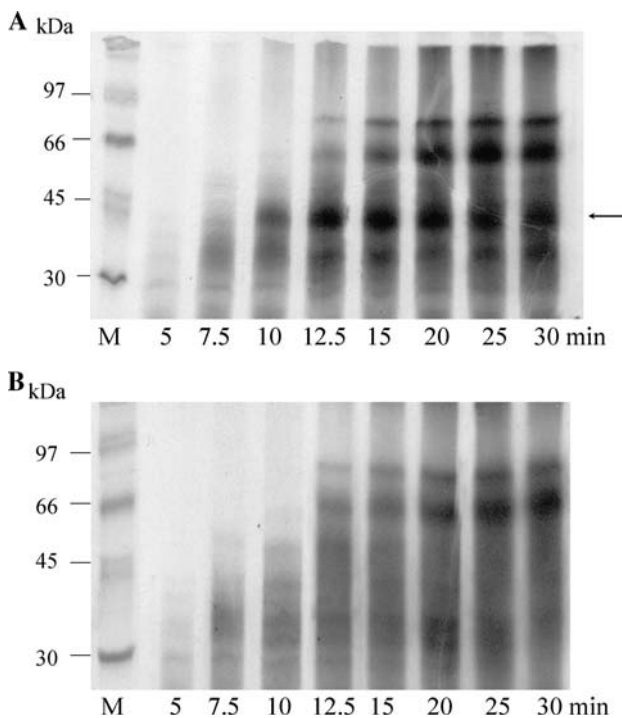

Fig. 3 Ribosomal pausing at the pseudoknot. Time courses of translation in RRL of mRNAs from (**A**) p3D8pk and (**B**) p3D8g3, representing the pseudoknot-containing and control constructs, respectively. Translation was performed at 26°C in presence of [^{35}S] methionine and translational products were visualized by autoradiography. The intermediate product that resulted from pausing at the pseudoknot is indicated by an arrow (**A**). The full-length protein and intermediate product are predicted to be 58 and 43 kDa, respectively

Binding activity of translated proteins

As p3D8pk and p3D8g3 contain 3D8 scFv, we investigated whether or not the 3D8 translated in vitro could bind antigen (DNA). To determine binding activity of proteins translated from p3D8pk and p3D8g3 mRNAs, we performed an ELISA assay with calf thymus DNA and BSA as a negative control. After 15 min of translation, both translated products (3D8pk and 3D8g3) exhibited specific binding to calf thymus DNA, relative to non-specific binding to BSA (Fig. 4). In addition, the translated products exhibited similar levels of binding reactivity, indicating little difference in the quantity of product from the two different mRNAs.

Comparison of ribosome display selection efficiency between the pseudoknot- and gene III-containing mRNAs

To compare enrichment in ribosome display, we translated the mRNAs from p3D8pk and p3D8g3, which differed only in the presence of a pseudoknot or gene *III* sequence. Since we had observed translational pausing at between 10 and 20 min in p3D8pk, the mRNA was translated for the same time periods. After affinity selection, mRNA was isolated from bound ribosomal complexes and reverse transcribed. Comparison of the resulting PCR bands indicated that irrespective of time, the band derived from the pseudoknot-containing construct was more abundant than that from the gene *III*-containing construct (Fig. 5). This suggests that pausing induced by the pseudoknot increases the efficiency of ribosome display.
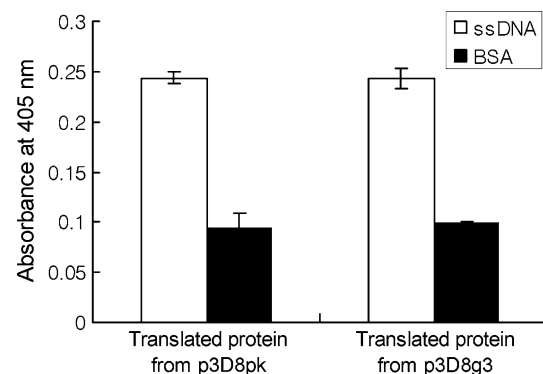

Fig. 4 Binding activity of proteins translated from p3D8pk and p3D8g3. After 15 min of in vitro translation, the mixtures were added to microtiter wells coated with calf thymus DNA or BSA. Bound proteins were detected by HRP-conjugated anti-His antibody. Binding assays were performed in triplicate and the mean values are shown
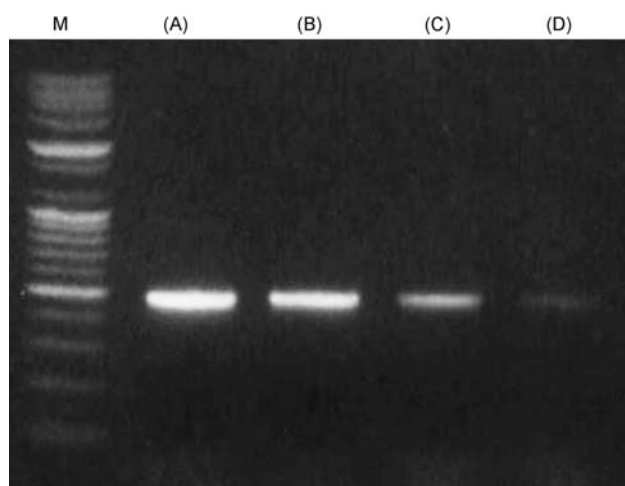
Fig. 5 RT-PCR analysis comparing selection of p3D8pk and p3D8g3. mRNA from p3D8pk and p3D8g3 was translated in RRL for 10 or 20 min, and then selected by binding to DNA-coated microtiter plates. Selected mRNA was eluted with EDTA, amplified by RT-PCR, then analyzed by agarose gel electrophoresis. Lanes **A–D** show the RT-PCR products of selected mRNAs from products translated from p3D8pk for 10 and 20 min, and p3D8g3p for 10 and 20 min, respectively. M, molecular weight markers

Discussion

In vitro display techniques are potentially more advantageous than in vivo display. Very large libraries can be conveniently handled and proteins can be made to evolve through the iteration of random mutagenesis and selection with considerable ease [21]. Two methods of in vitro display, mRNA-protein fusion and ribosome display, are generally used and both allow the complete in vitro synthesis and selection of proteins. The most significant difference between these methods is the way in which the protein is linked to mRNA. mRNA-protein fusions link mRNA and protein by a covalent bond through the use of a small adaptor molecule, typically puromycin [22–25]. In the initial description of this strategy, a complex enzymatic step requiring ligation of puromycin to the mRNA led to low yields of the mRNA-protein fusion [24, 25]. Improved fusion synthesis was achieved though continued efforts to simplify the preparation of the mRNA-protein fusion [26, 27]. In ribosome display, the absence of a stop codon causes ribosomes to stall on the mRNA. As it is difficult to represent the complete diversity of all translated mRNAs using this method, research has focused on improving the stability of the ribosome-mRNA-protein complex. In order to induce ribosome stalling, Zhou et al. [15] introduced the ricin A subunit (RTA) and observed that the stalled complex remained relatively stable even at room temperature. In the prokaryotic ribosome display system [5, 12], the stem-loop segments from T7 gene 10 and *E. coli* lipoprotein

genes have been introduced at the 5′- and 3′-ends of mRNA. However, secondary structure has not been applied to eukaryotic ribosome display systems, and so we investigated the applicability of incorporating a pseudoknot for increasing stability of the complex.

The pseudoknot originated from the genomic RNA of infectious bronchitis virus (IBV), a member of the positive-stranded coronavirus group. This mRNA secondary structure is known to induce ribosomal arrest during in vitro translation in RRL [18]. In our construct (V_H/K-pseudoknot-spacer), ribosomal arrest was observed at the pseudoknot sequence and although there was no significant difference between the binding of the two constructs, the pseudoknot-containing construct showed an increased efficiency of affinity selection. Thus, ribosomal pausing induced by the pseudoknot affects stability of the antibody-ribosome-mRNA complex. Although the equilibrium dissociation constant (Kd) of purified 3D8 scFv is 17–74 nM [20], we observed a lower than predicted level of translated protein binding. This suggests a lower abundance of translated protein, since the translational time was short and ribosomal recycling was limited by the pseudoknot and absence of a stop codon.

This research suggests that the relatively short (44 nucleotide) pseudoknot sequence could be applied easily to ribosome display and that its incorporation would improve the stability of the ribosome complex, as well as the efficiency of affinity selection.

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