의학 박사학위 논문

A Pseudoknot Improves Selection Efficiency in Ribosome Display

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A Pseudoknot Improves Selection Efficiency in Ribosome Display

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- ABSTRACT -

A Pseudoknot Improves Selection Efficiency in Ribosome Display

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, I tested a pseudoknot, originating from the genomic RNA of infectious bronchitis virus (IBV), a member of the positive-stranded coronavirus group. I used the previously-isolated anti-DNA scFv, 3D8, as a target protein. During in vitro translation in rabbit reticulocyte lysate, I 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.

Key words: Antibody, Iinfectious bronchitis virus, Library, Pseudoknot, Ribosome display, scFv, Selection efficiency

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ABBREVIATION

ABTS	2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
ARM complex	Antibody-ribosome-mRNA complex
BSA	Bovine serum albumin
DEPC	Diethyl pyrocarbonate
ELISA	Enzyme-linked immunosorbent assay
HRP	Horseradish Peroxidase
IBV	Infectious Bronchitis Virus
PCR	Polymerase chain reaction
RIA	Radioimmunoassay
RT	Reverse transcriptase
scFv	Single chain variable fragments

I. INTRODUCTION

Ribosome display is an efficient strategy for screening and evolution of peptides or proteins(Takahashi et al., 2002; He et al., 2004; Schimmele et al., 2005; Rothe et al., 2006). This system overcomes the limitations on library size that transformation efficiency places on cell-based display methods (Hanes et al., 1998; He et al., 1999; Schaffitzel et al., 1999; Irving et al., 2001). Improvement to library diversity permits the selection of higher affinity ligands and provides a mechanism for protein evolution (Hanes et al., 2000; Hanes et al., 2000).

Genotype and phenotype are linked in ribosome display, enabling efficient selection and identification of proteins (Gersuk et al., 1997; Hanes & Pluckthun, 1997; He & Taussig, 1997). 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 (He & Taussing., 2005). Much research has focused on improving complex stability, as this relates directly to the library diversity and efficiency of ribosome display (Zhou et al., 2002; Sawata & Taira, 2003). 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 (Kontos et al., 2001). For example, the pseudoknot is found in genomic RNA from infectious bronchitis virus (IBV), a member of the positive-

stranded coronavirus group (Paul et al., 1993). 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 (Paul et al., 1993). In fact, pausing at a pseudoknot has proved a more effective barrier to ribosomal elongation than the simple stem-loop structure (Kontos et al., 2001).

In this paper, I 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.

II. MATERIALS AND METHODS

A. Plasmid construction

In order to construct pRD3D8, which was based on anti-DNA scFv (3D8) (Lee et al., 2004), I 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'-GACCACCATGGACCATCATCATCATCATCATGAGGTCCAGCTGCAGCAG-3') and 3D8/for (5'-GTTGGTGCAGCATCAGCCCGTTTTATTTCCAGCTTGGTC-3'). Mouse Cκ was prepared as described previously (Lee et al., 2004). In brief, RNA was isolated from mouse spleen and Cκ DNA was amplified by RT-PCR using the primers Ck/back (5'-AAACGGGCTGATGCTGCA-3') and Ck/for_XmaI (5'-TCCCCCCGGGCTCTAGAACACTCATTCCTGTTGGAGCT-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. 1. The predicted secondary structure of pseudoknot was shown as Fig. 2. The gene III spacer region was amplified from pCANTAB5E (GE Healthcare BioSciences, Piscataway, NJ, U.S.A.). The pseudoknot-containing spacer region

was amplified using the primers PK/back (5’-TCCCCCCGGGCGGGGTATCAGTCAGGCTCGGCTGGTACCCCTTGCAAAGCGAGCCTTCCTCAACCTCCTGTCAATG-3’) and g3p/for (5’-ATACCCAAGCTTTATCACCAGTAGCACC-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 HindIII/XmaI site in pRD3D8 (Fig.3 and 4)

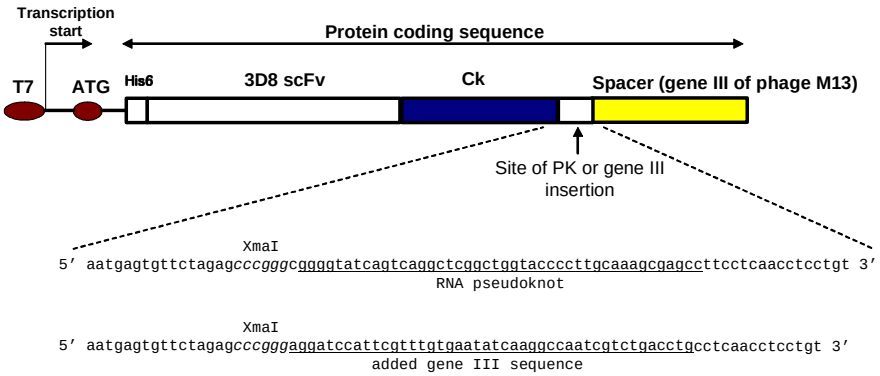

Fig. 1. DNA structures and sequences for investigating the effect of a pseudoknot on ribosome display. A T7 promoter (T7) and a protein initiation sequence (ATG) are introduced for *in vitro* transcription and translation. Arrows indicate the transcriptional start and protein coding sequence. I introduced the minimal IBV pseudoknot and part of gene III between the sequences encoding 3D8VH/K and the spacer generating plasmids p3D8pk and p3D8g3, respectively.

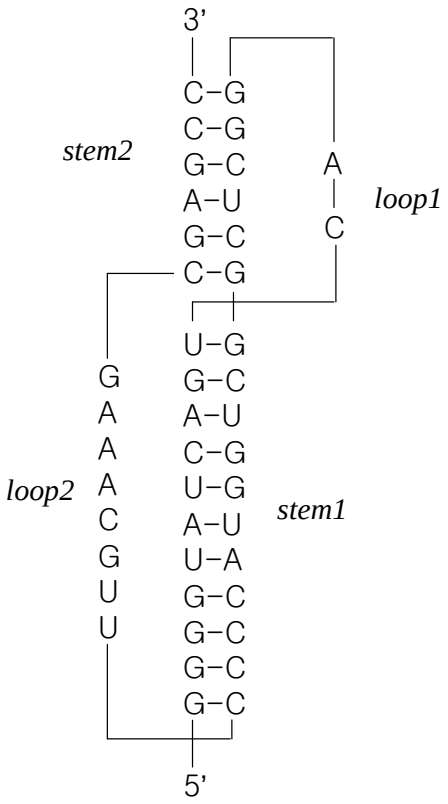

Fig. 2. Predicted secondary structure of the IBV pseudoknot (Kontos et al., 2001).

Fig. 3. Nucleotide sequences of the minimal IBV pseudoknot introduced into p3D8pk. Restriction enzyme sites are in italics, the similarly-sized sequences corresponding to the minimal IBV pseudoknot are in bold, and the predicted amino acid sequences are indicated below the nucleotide sequences.

Fig. 4. Nucleotide sequences of the portion of gene III introduced into p3D8g3. Restriction enzyme sites are in italics, the similarly-sized sequences corresponding to the section of gene III are in bold, and the predicted amino acid sequences are indicated below the nucleotide sequences.

B. *In vitro* transcription.

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

C. *In vitro* translation

The 25 µL reaction mixtures contained 70 mM KCl, 1.6 mM MgCl2, 1 µL [35S] 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, U.S.A.), 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 markers (GE Healthcare BioSciences, U.S.A.) were used as standards and proteins were detected by autoradiography.

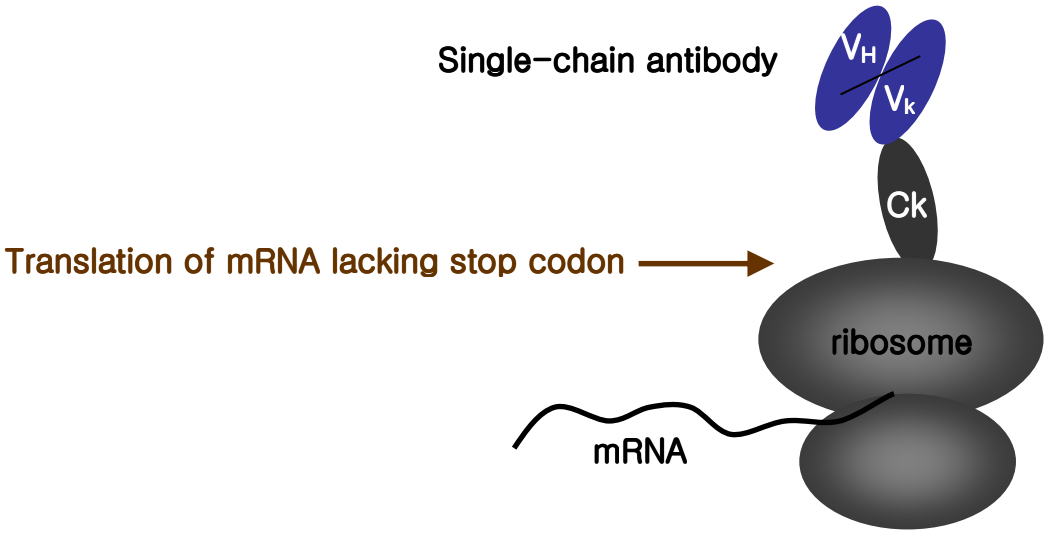

Fig. 5. The structure of antibody-ribosome-mRNA complex (ARM) complex. The absence of stop codon prevents release of mRNA and nascent antibody from the ribosome. The constant region of light chain as a spacer region makes the single-chain antibody fold correctly.

D. Affinity selection

Microtiter plates were coated overnight using 50 µL calf thymus DNA (10 µg mL-1 in PBS; Sigma-Aldrich, Milwaukee, MI, U.S.A.) at 4 °C. Plates were then washed with PBS, blocked with sterilized 3 % (w/v) BSA for 2 h at room temperature, washed 3 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 3 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, U.S.A.).

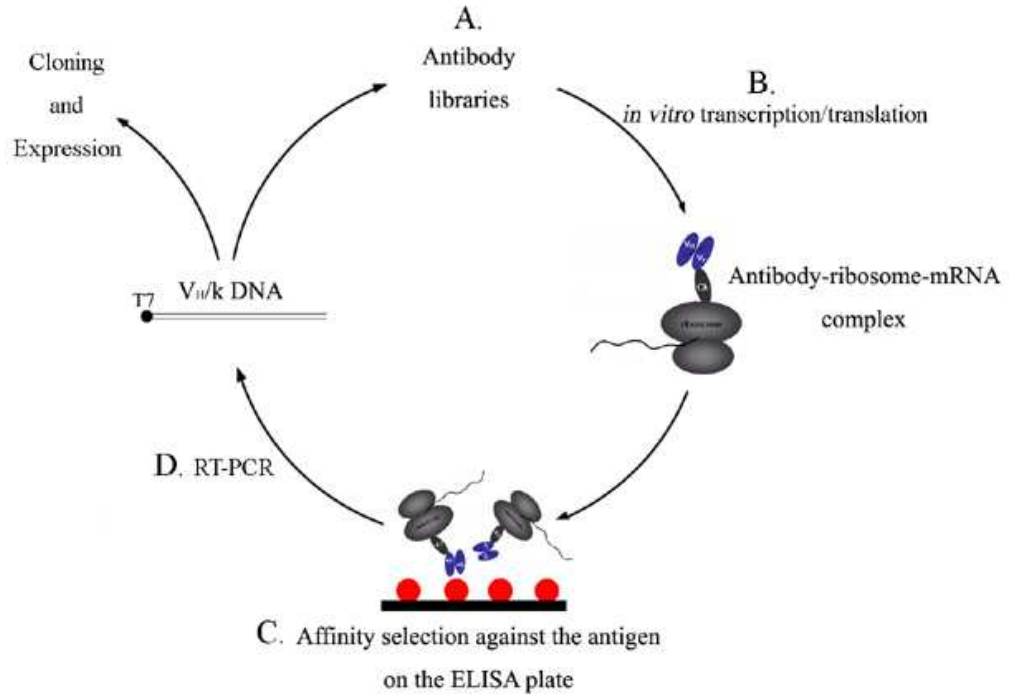

Fig. 6. Affinity selection of ribosome display.

E. RT-PCR

Selected mRNA was reverse transcribed using SuperScript™ III reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) 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' GACCACCATGGACCATCATCATCATCATCATGAGGTCCAGCTGCAGCAG 3') and 3D8re/for (5' CAGCCAGGGAGGATGGAGAC 3').

F. 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 3 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 3 times with PBST, after which anti-His antibody (1:1,000; Qiagen, Valencia, CA, U.S.A.) was added and the plates incubated for 1 h at room temperature. Plates were washed 3 times with PBST and incubated with HRP-conjugated anti-mouse IgG antibody (Zymed Laboratories, San Francisco, CA, U.S.A.) for 1h at room temperature. Following further washing as described above, 100 μL ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); GE Healthcare BioSciences, U.S.A.) substrate solution was added to each well and absorbance measured at A405.

III. RESULTS

A. Plasmid construction

I used a previously-isolated anti-DNA scFv (3D8) as the translated product for this experiment (Kim et al., 2006). I constructed a single-chain fragment (VH/K) comprising the 3D8 scFv heavy chain variable domain (VH) 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, I inserted two different similarly-sized spacers at the 3' terminus of VH/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. 1). The predicted pseudoknot secondary structure is depicted in Fig. 2 (Kontos et al., 2001).

B. Ribosomal pausing at the pseudoknot

In a previously study, translational pausing was demonstrated using an IBV-derived pseudoknot inserted into influenza virus PB1 reporter mRNA (Paul et al., 1993). I investigated whether or not ribosomal pausing was induced at the predicted site in our construct. I used plasmids that contained the IBV pseudoknot and part of the filamentous 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. 7). No translational intermediate was detected from p3D8g3 mRNA, which does not contain a pseudoknot (Fig. 8).

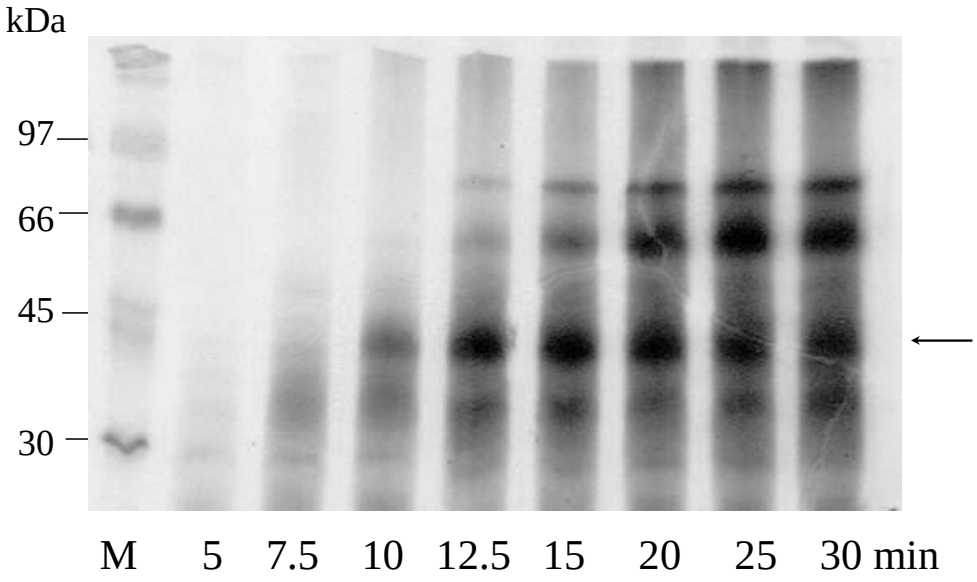

Fig. 7. Ribosomal pausing at the pseudoknot. Time courses of translation in RRL of mRNAs from (A) p3D8pk, representing the pseudoknot-containing constructs. Translation was performed at 26 °C in presence of [^{35}S] methionine and translational products were visualized by autoradiography. The intermediate product that resulted from pausing at the pseudoknot is indicated by an arrow. The full-length protein and intermediate product are predicted to be 58 kDa.

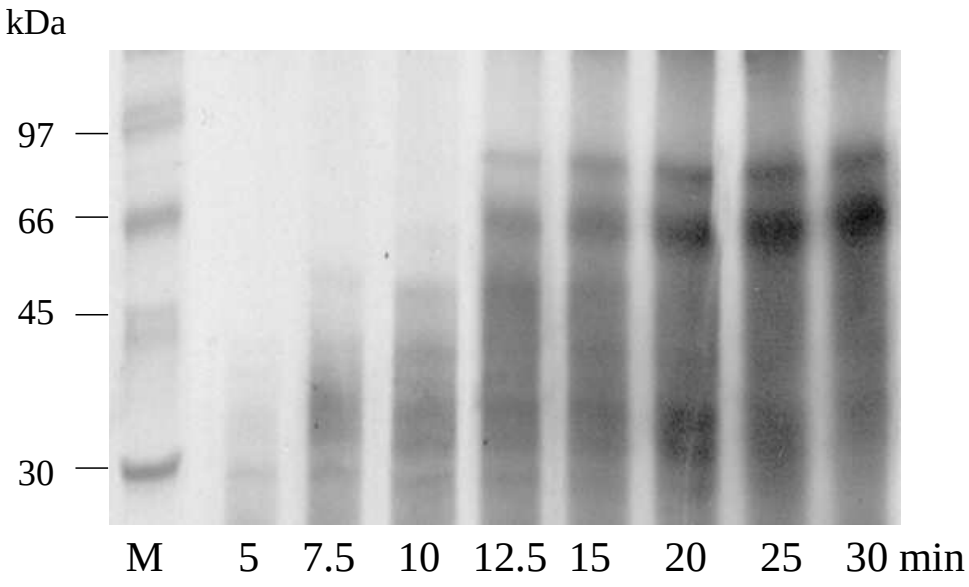

Fig. 8. No ribosomal pausing at the control construct. Time courses of translation in RRL of mRNAs from p3D8g3, representing the control constructs. Translation was performed at 26 °C in presence of [^{35}S] methionine and translational products were visualized by autoradiography. The full-length protein and intermediate product are predicted to be 43 kDa.

C. Binding activity of translated proteins

As p3D8pk and p3D8g3 contain 3D8 scFv, I 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, I 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. 9). In addition, the translated products exhibited similar levels of binding reactivity, indicating little difference in the quantity of product from the two different mRNAs.

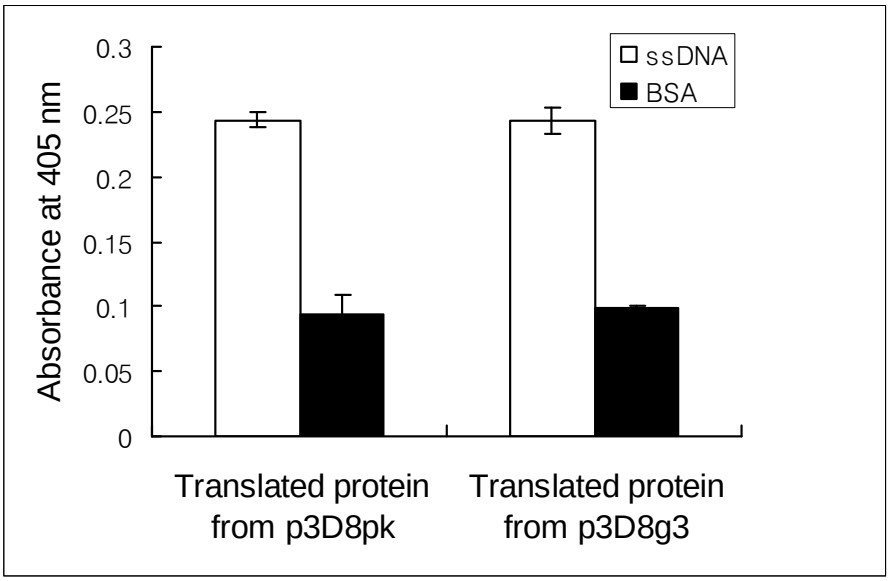

Fig. 9. Binding activity of proteins translated from p3D8pk and p3D8g3. After 15 min of in vitro translation, the mixtures were added to microtiter wells coated with calf thymus DNA or BSA. Bound proteins were detected by HRP-conjugated anti-His antibody. Binding assays were performed in triplicate and the mean values are shown.

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

To compare enrichment in ribosome display, I translated the mRNAs from p3D8pk and p3D8g3, which differed only in the presence of a pseudoknot or gene III sequence. Since I 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. 10). This suggests that pausing induced by the pseudoknot increases the efficiency of ribosome display.

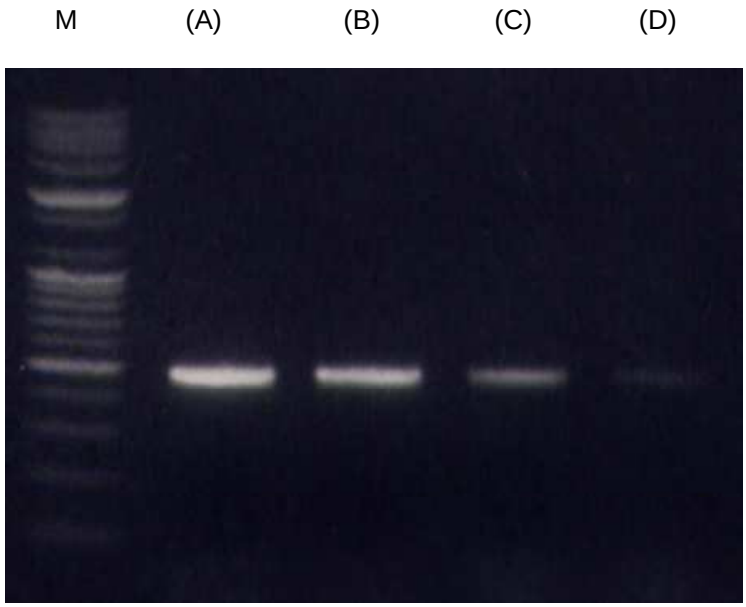

Fig. 10. RT-PCR analysis comparing selection of p3D8pk and p3D8g3. mRNA from p3D8pk and p3D8g3 was translated in RRL for 10 or 20 min, then selected by binding to DNA-coated microtiter plates. Selected mRNA was eluted with EDTA, amplified by RT-PCR, then analyzed by agarose gel electrophoresis. Lanes 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.

IV. 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. 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 (Roberts et al., 1997; Nemoto et al., 1997; Takahashi, 2003). 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. Improved fusion synthesis was achieved though continued efforts to simplify the preparation of the mRNA-protein fusion (Liu et al., 2000; Kurz et al. 2000). 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. 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 (Hanes & Pluckthun 1997; Hanes et al. 1998), 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 I 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 (Paul et al. 1993). 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 (*K*d) of purified 3D8 scFv is 17-74 nM (Kim et al. 2006), I 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.

V. CONCLUSION

In this paper, I 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, RNA secondary structure, a pseudoknot resulted in pausing of translation and an increase in selection efficiency in ribosome display. 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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- 국문요약 -

RNA pseudoknot의 Ribosome Display 선별 효율 향상

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Ribosome display의 라이브러리의 크기와 다양성은 리보솜, mRNA 그리고 번역된 단백질 간에 형성된 복합체의 안정성에 달려있다. mRNA의 이차 구조가 이 복합체의 안정성을 향상시킬 수 있을 것인가를 알아보기 위하여 코로나바이러스 중 하나인 infectious bronchitis virus (IBV)의 게놈에서 유래한 pseudoknot를 사용하였다. 또한 기존에 선별된 항-DNA scFv인 3D8을 기본구조물로 사용하였다. Rabbit reticulocyte lysate에서 in vitro 번역과정을 거치는 동안, mRNA로 pseudoknot가 삽입되어 번역과정 동안 일시적으로 리보솜의 번역이 중지되는 것을 관찰하였다. mRNA pseudoknot를 포함한 구조물은 항체 선별이 더 효율적으로 이루어지는 것을 확인하였고 이는 pseudoknot가 리보솜, mRNA 그리고 번역된 단백질 간에 안정성이 향상되었다는 것을 의미한다. 비교적 짧은 이 pseudoknot가 항체 선별 효율 향상을 위해 ribosome display에 적용될 수 있을 것이다.

핵심어: 항체, Iinfectious bronchitis virus, 라이브러리, Pseudoknot, Ribosome display, scFv, 선별효율