An Efficient Ligation Method in the Making of an *in vitro* Virus for *in vitro* Protein Evolution

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ABSTRACT

The *"in vitro* virus" is a molecular construct to perform evolutionary protein engineering. The "virion(=viral particle)"(mRNA-peptide fusion), is made by bonding a nascent protein with its coding mRNA via puromycin in a test tube for *in vitro* translation. In this work, the puromycin-linker was attached to mRNA using the Y-ligation, which was a method of two single-strands ligation at the end of a double-stranded stem to make a stem-loop structure. This reaction gave a yield of about 95%. We compared the Y-ligation with two other ligation reactions and showed that the Y-ligation gave the best productivity. An efficient amplification of the *in vitro* virus with this "viral genome" was demonstrated.

INTRODUCTION

The Darwinian selection of proteins requires a linkage of phenotype with genotype. The *in vitro* virus is a molecular construct to realize such a linkage. A linkage of a nascent protein with its coding mRNA was made on a ribosome during *in vitro* translation via a puromycin attached to the 3' end of the mRNA (1,2). The *in vitro* virus dramatically increased the library diversity and the system flexibility of the *in vivo* version such as a phage display method. In fact, various aptamers have

been evolved starting from a random library (3,4) or a cDNA library (5) in this approach.

In the original *in vitro* virus the puromycin-linker was attached to an mRNA by simple enzymatic ligation (1). Recently, more efficient methods were developed such as an improved splint ligation method using T4 DNA ligase (6) and a photo-cross-linked ligation method using psoralen (7).

We developed a novel puromycin-linker attachment technology using the Y-ligation, which was a method of two single-strands ligation at the end of a double-stranded stem to make a stem-loop structure. The Y-ligation method (8) gave a high yield by increasing the local effective concentration of the two oligodeoxyribonucleotides and by using T4 RNA ligase. We applied this method in preparation of the "genome" of an *in vitro* virus and demonstrated a high yield in the "virion (=viral particle)" (mRNA-peptide fusion) formation of the *in vitro* virus and its amplification.

MATERIALS AND METHODS

Synthesis of DNA-oligomer with Puromycin-linker

Puromycin-linkers (Fig. 1) were synthesized by using a standard DNA synthetic method and purified in the reverse

phase HPLC. Puromycin-CPG and phosphoramidites were purchased from Glen Research and used according to the protocols recommended by the manufacturer. The sequences of linker-S, linker-Y and splint DNA were $(A)_{12}$-$(Spacer18)_2$-d(CC)-Puromycin, d(CCC)-$(GCCCCCGTCCC)_{OMe}$-$(Spacer18)_4$-$(CC)_{OMe}$-Puromycin and d$((T)_9$GCCCCCC-GTCCC), respectively. Here, Spacer18 is -$(OCH_2CH_2)_6PO_2O$-. The 5' end of puromycin-linkers was phosphorylated using T4 polynucleotide kinase (NEB). The base sequences of 3' end region (a GC clump) of mRNA were 5'….GGGACGGGGGGGC3' and 5'….GGGAC-GGGGGGGCAAAA3' for the splint ligation and the Y-ligation, respectively.
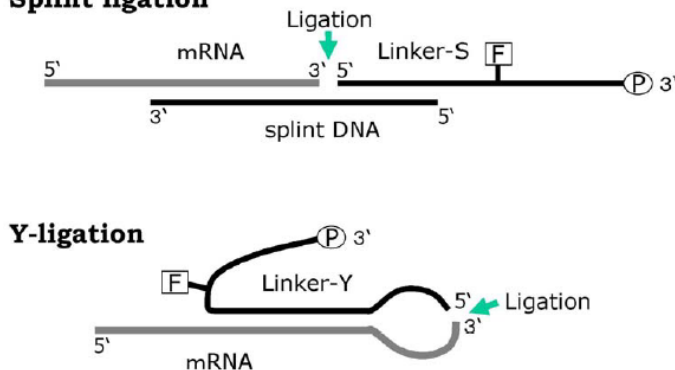

Fig. 1: Scheme of ligation methods of puromycin-linker to mRNA. P and F denote Puromycin and FITC, respectively. The sequence of "Y-ligation loop" is d(CCC) 5' and r(AAAA) 3'.

Ligation reaction

DNA "genomes" were prepared by PCR with different primer sets in two steps using the plasmid pETHisKGFP as an initial template. The plasmid pETHisKGFP was constructed by inserting the GFP gene of phGFPS65T (Clontech) into the pETHisK vector (9). The construct of genome has a T7 promoter, a translational enhancer of TMV 5' UTR (10), an initiation codon with good context (11), a coding sequence of His-tag (12), an N-terminus sequence in the GFP gene and a GC-clump sequence for hybridization to a GC-rich DNA-oligomer. The PCR primers and the mRNA sequence are the same as described in (13). Capped mRNAs were prepared using the DNA genomes and T7 RNA polymerase transcription kit (RiboMAX-T7; Promega).

Messenger RNAs (0.5 μM) were hybridized with each linker with or without a splint DNA in an annealing condition of 15 min / from-94-to-25 ºC in each ligation buffers. The ligation reaction with T4 DNA ligase (10 U/μl, NEB) was performed according to the supplier's recommendation at 25 ºC. The ligation reaction with T4 RNA ligase (1 U/μl, TAKARA) was performed in the supplier's buffer with 10% DMSO at 25 ºC. The ligation products were analyzed by 8 M urea 10% PAGE using TBE running buffer at 65 ºC and were visualized with fluorescence of FITC and then visualized again after staining

by SYBR Green II (FMC) using an fluorescence imager (Molecular Imager FX; BIO-RAD). Splint ligation products were then treated with T7 exonuclease, at 37 ºC 15 min, in order to digest the DNA splint and were purified by a spin column (S-200HR; Amersham Pharmacia).

Analysis of formation of *in vitro* virion

The mRNAs were translated in wheat-germ extract (TOYOBO) for 15 min at 25 ºC in standard condition and for 30 min at 10 ºC in a high salt condition (final 50 mM $MgCl_2$, 500 mM KCl). The products were analyzed using 6 M urea 10% SDS-Tricin PAGE (14) and visualized using the fluorescence imager.

Affinity columns of Ni-NTA agarose (QIAGEN; 200μl gel volume (Vc)) were used. The virion (mRNA-peptide fusion) was diluted in a TBS buffer (50 mM Tris-HCl, 150mM NaCl, 0.1% Tween20, pH 8.0) and incubated in each affinity column for 5 min at 25 ºC. The Ni-NTA agarose column was washed with 25 Vc of a buffer (50 mM Tris-HCl, 1 M NaCl, 0.1% Tween20, 10 mM imidazole, pH 8.0) and eluted with 2.5 Vc of a buffer (50 mM Tris-HCl, 0.1% Tween20, 500mM imidazole, pH 8.0). Eluted solution was desalted by P6 spin column (Bio-rad). Reverse transcription was performed using *C.therm*.Polymerase (Roche) for 30 min at 65 ºC. A PCR was performed (25 cycles) using KOD Dash polymerase (TOYOBO) and analyzed by electrophoresis on 3% agarose gel (MetaPhor agarose; FMC) using triethanolamine / Tricin running buffer (15) and were visualized using the imager.

RESULTS AND DISCUSSION

Ligation reaction between mRNA and puromycin-linker

We compared three puromycin-linker attachment methods, which are the splint ligation with T4 DNA ligase (6), the splint ligation with T4 RNA ligase and the Y-ligation with T4 RNA ligase (Fig. 1).

The splint ligation method with T4 DNA ligase gave a low product yield because of undesirable mRNA terminal heterogeneity originated from the T7 run-off transcription (6). This drawback was overcome by an improved splint ligation with a T4 DNA ligase (6). Two kinds of splint "N" and "N+1" were used in the improved method.

We tested another method against mRNA terminal heterogeneity, which was splint ligation with T4 RNA ligase. T4 RNA ligase can ligate nucleotides even if the 3' end of mRNA is not a complementary double strand. Thus, it is expected that mRNA with an N+1 terminus can ligate while total ligation yield increases.

Both splint ligations gave a relatively high yield of the ligation products. But the removal of the splint by PAGE (6) was troublesome. We removed the splint by treatment with T7 exonuclease. This removal method was rapid and efficient. However, some leftover splint DNAs inhibited the virion formation (7) and cleaved mRNA by RNaseH during translation (16).

We developed a puromycin-linker ligation method, using the Y-ligation. This method was simpler and did not require removing the splint DNA. We optimized reaction conditions of each method, that is, reaction buffers, temperature, molar ratios of [mRNA]:[splint]:[Linker-S], etc. Comparisons of the results of the three puromycin-linker attachment methods are shown in Fig. 2. The DNA ligase method gave a low yield. The RNA ligase method gave a yield of about 60%. The Y-ligation method had the best result and gave a yield of about 95%. It was reported that the improved splint ligation method using T4 DNA ligase and the photo-cross-linked ligation method using psoralen gave a yield of more than 70% (6) and exceeding 80% (7), respectively. The photo-cross-linked ligation method includes a process of a long time (15min) UV irradiation (7). This might make mRNA damaged. And some mismatched linkers might be also ligated. Y-ligation method does not have such problems.
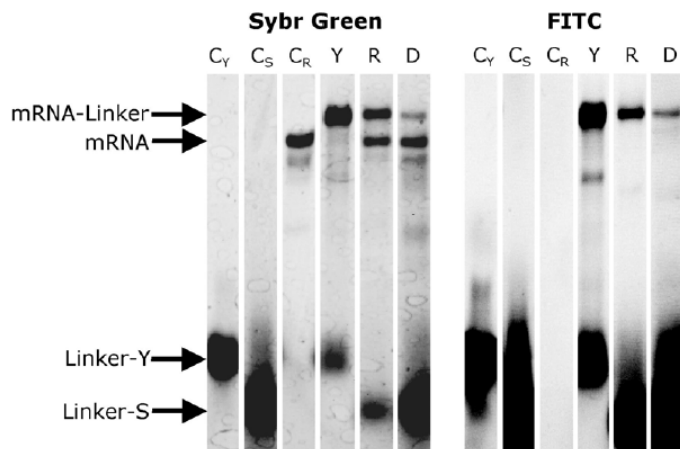

Fig. 2: Gel-electrophoresis of ligation products. The ligation products were electrophoresed on 8M Urea 10% PAGE at 65 ºC and were visualized with fluorescence of FITC and then visualized after staining by SYBR Green II using an imager. Lane C_Y: control, Linker-Y. Lane C_S: control, mRNA without ligation. Lane C_R: control, Linker-S. Lane Y: Y-ligation with T4 RNA ligase. Lane R: splint ligation with T4 RNA ligase. Lane D: splint ligation with T4 DNA ligase.

We also found that the dependence of the ligation efficiency on mRNA length was negligible up to 800 bases (data not shown). Time-courses of puromycin-linker attachment reactions are shown in Fig. 3. The Y-ligation reaction was very fast. Most of the templates were already ligated in five minutes incubation. The splint ligation reaction was slower, because the rate of the mRNA-splint-linker formation may be relatively low.

The dependence of the ligation efficiency on the molar ratio [mRNA]:[Linker-Y] is shown in Fig. 4. The Y-ligation reaction proceeded with a high efficiency, even in a 1:1 ratio. Moreover, the removal of excess linkers was not necessary.
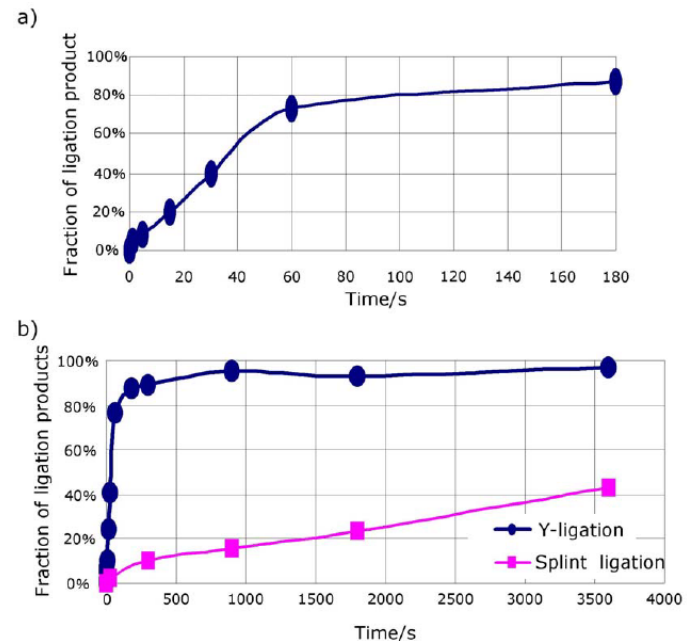

Fig. 3: Time course of ligation reactions. Concentrations were [mRNA] = 0.5 µM and [T4 RNA ligase] = 1 U/µl in the enzyme-supplier's buffer with 10% DMSO. At 25 ºC.
(a) Y-ligation, [mRNA]:[Linker-Y] = 1:1.5
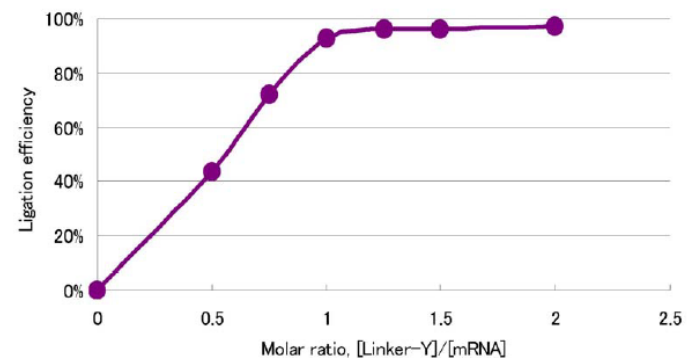(b) Splint ligation with T4 RNA ligase as compared with Y-ligation, [mRNA]:[Splint]:[Linker-S] = 1:1:1.5

Fig. 4: The dependence of the Y-ligation efficiency on the molar ratio, [Linker-Y]/ [mRNA]. Concentration of mRNA was [mRNA] = 0.5 µM. The ligation efficiency is defined as the final fraction (at incubation time 3,600s) of the ligation product.

Analysis of the formation of *in vitro* virion

We analyzed the virion formation of the genome that was prepared with the Y-ligation puromycin-linker attachment

method. The translation reaction was performed in a wheat-germ extract. The virion formation was detected by the fluorescence of the FITC labeled linker. The electrophoretic patterns showed the virion formation of about 40% (Fig. 5). This value was as high as those using a rabbit reticulocyte lysate for genomes prepared by other methods (6,16).
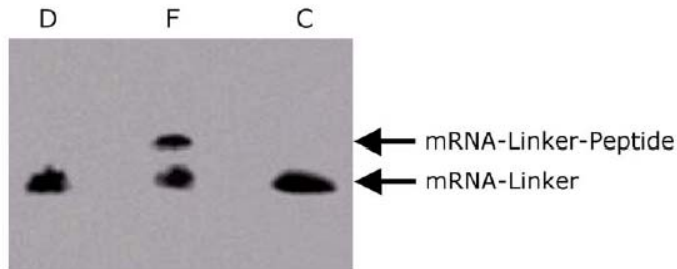

Fig. 5: Virion formation of mRNA·Linker-Y using the Y-ligation.Lane C: control, mRNA·Linker-Y without translation. Lane F: translation product. Lane D: digested translation product by proteinase K.

As a model system for demonstrating a selection process, a virion having a His-tag coding region was used. *In vitro* virus virion (mRNA-peptide fusion) was made and screened through a Ni-NTA-immobilized affinity column. Bound virion (mRNA-peptide fusion) molecules were eluted and their RNA moiety was amplified and analyzed. Two constructs with each type of the puromycin-linkers and a control without translation were examined. During a single turnover of the life cycle of the *in vitro* virus, both puromycin-linkers amplified the viral genomes (and also the virions (mRNA-peptide fusions)) well, and the genome prepared by the Y-ligation showed higher amplification than the genome prepared by the splint ligation (Fig. 6). The linker-S had a less flexible oligonucleotide moiety for splint ligation. As the Y-ligation genome has a more flexible linker, puromycin was probably able to approach the ribosome A-site efficiently. The amplification for the genome by the splint ligation was probably lowered also by residual splint DNA.
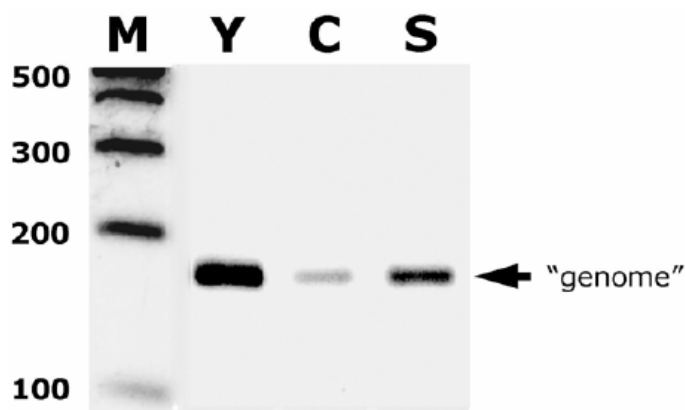

Fig. 6: PCR products from the virions after single turn-over of viral life cycle that had a step of the affinity trapping. In the reverse transcription on the virion, we used a primer (21mer)

hybridizing at 12 bases downstream from the hybridization site of the Linker-Y or -S. The PCR products of the cDNAs (168-mer both) were electrophoresed on 3% agarose gel, stained with ethidium bromide and visualized using the fluorescence imager. Lane M: 100 bp DNA ladder. Lane Y: using a genome with the Linker-Y. Lane S: using a genome with the Linker-S. Lane C: control, mRNA-Linker-Y without translation.

Is there any potential biohazard in the *in vitro* virus? In the present, the *in vitro* virus can amplify only under the very limited conditions and has not any potential of infection. The term "virus" in *in vitro* virus was coined based on the biophysical aspect of its evolution mechanism; in the biotechnological aspect, this is an mRNA-peptide fusion.

CONCLUSIONS

We compared three puromycin-linker attachment methods and concluded that the Y-ligation method gave the best productivity. The Y-ligation method is an easy process because any after-treatments such as removal of the splint DNA are not necessary. We demonstrated efficient amplification of the *in vitro* virus with this "genome." This method is also applied to the *in vitro* DNA virus [13], when higher stability of the linkage between mRNA and the primer-linker is required.

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PROTOCOLS

Y-ligation to make in vitro virus

1. Hybridize the mRNA (0.5 µM) with Linker-Y (0.6µM) in an annealing condition of 15 min / from-94-to-25 ºC in ligation buffers.
2. Ligate the mRNA with the hybridized Linker-Y using T4 RNA ligase (1 U/µl, TAKARA) in the supplier's buffer with 10% DMSO for 15 min at 25 ºC.
3. Analysis of the ligation products by 8 M urea 10% PAGE using TBE running buffer at 65 ºC.
4. Check the FITC fluorescent gel bands using a fluorescence imager (Molecular Imager FX; BIO-RAD), and then check again after staining by SYBR Green II (FMC).
5. Translate the mRNA in wheat-germ extract (TOYOBO) for 15 min at 25 ºC. And, for 30 min at 10 ºC in a high salt condition (final 50 mM MgCl2, 500 mM KCl).
6. Analysis the products using 6 M urea 10% SDS-Tricin PAGE and using the fluorescence imager.
7. After dilution of the virion (mRNA-peptide fusion) in a TBS buffer (50 mM Tris-HCl, 150mM NaCl, 0.1% Tween20, pH 8.0), incubate it in affinity column (Ni-NTA agarose; QIAGEN; 200 µl gel volume (Vc)) for 5 min at 25 ºC.
8. Wash the column with 25 Vc of buffer (50 mM Tris-HCl, 1 M NaCl, 0.1% Tween20, 10 mM imidazole, pH 8.0).
9. Elution with 2.5 Vc of buffer (50 mM Tris-HCl, 0.1% Tween20, 500mM imidazole, pH 8.0).
10. Eluted solution was desalted by P6 spin column (Bio-rad).
11. Reverse transcription using *C.therm*.Polymerase (Roche) for 30 min at 65 ºC.
12. PCR (25 cycles) the RT product using KOD Dash polymerase (TOYOBO). Electrophoresis on 3% agarose gel (MetaPhor agarose; FMC) using tri-ethanolamine/ Tricin running buffer and visualization using an imager.