Stabilized Self-Sustained Sequence Replication against Evolutionary Instability

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When self-sustained sequence replication, which is an isothermal amplification method of DNA/RNA, was applied to in vitro evolution experiments, it often showed evolutionary instability. A method to prevent this instability was devised using a DNA oligomer of promoter sequence with modified 3'-end.

Self–sustained sequence replication (3SR,[1] or NASBA[2]) is an isothermal amplification method of DNA/RNA in one pot. It is known widely as an effective method of detecting HIV-1 and other etiological agents.[3] Reaction schemes of 3SR and related RNA-Z hairpin amplification[4] are shown in Figure 1. The specific growth rate of a sample DNA in the polymerase chain reaction (PCR) is dependent mainly on the period of the temperature cycle, whereas that of 3SR is dependent mainly on the length and the base sequence itself of the sample DNA. Thus the "fitness" of a DNA molecule in 3SR can be set to be its specific growth rate. Based on this "fitness," the "natural selection"-type evolution reactors[5] can be made by the use of the Darwinian principle, that is, "survival of the fittest molecule" in the reactor. A serial transfer process has been used to emulate this type of reactor and applied to some evolution experiments of DNA/RNA molecules.[4,6–8]

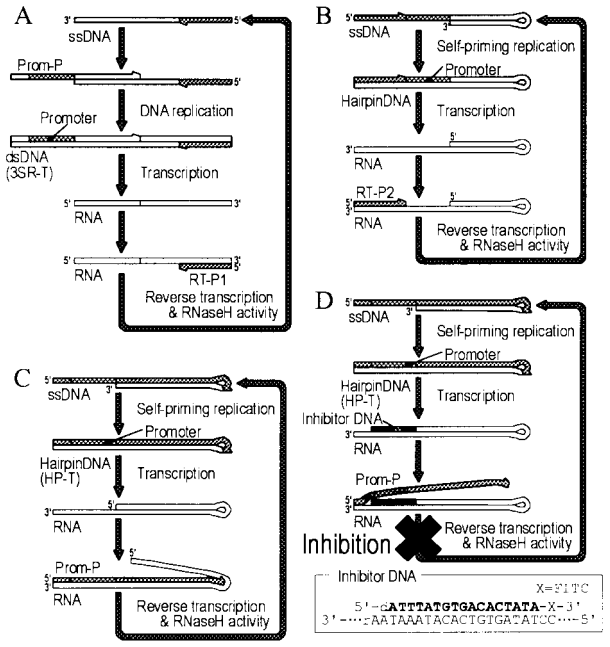

Figure 1. Reaction schemes of 3SR (A) and RNA-Z hairpin amplification (B). In our experiments, the transcription was performed with RNA polymerase from bacteriophage SP6 (SP6-RNAP, Takara Shuzo). The reverse transcription, RNaseH catalysis and DNA replication were performed with a single enzyme, human immunodeficiency virus reverse transcriptase[11] (HIV-1 RT, Worthington Biochemical). C: Reaction scheme of a hairpin amplification which emerged as an intermediate step in our 3SR evolution reactor. D: Inhibition of the reaction shown in C by the excess inhibitor DNA. It has SP6-RNAP promoter sequence (bold) with FITC at 3'-end. It hybridizes to the complementary sequence of the promoter on the hairpin RNA, and suppresses the reverse transcription because the Prom-P hybridizes to the RNA with only 4 bases at 5'-end. Abbreviations are defined in the text.

In the results of the serial transfer experiments, we found the indication of evolution from 3SR amplification mechanism to RNA-Z-like hairpin amplification mechanism and also to CATCH amplification mechanism,[6,7] when we used a template with a random sequence region.[8] Elliger et al. found similar phenomena.[9] This evolutionary phenomenon occurred more frequently in the case of the template with a longer random region. Although RNA-Z hairpin amplification has been used to optimize the promoter sequence,[4] its applicability is limited because of its single-stranded nature.

Therefore, we have to prevent the evolution from the 3SR template to the RNA-Z-like hairpin template in order to use 3SR stably in in vitro selection/evolution experiments of the natural selection type. Analysis of the evolution pathway showed that there was an intermediate hairpin molecule which was amplified according to a scheme as shown in Figure 1-C.[10] The hairpin template in this figure was amplified only with the promoter-primer. It emerged through hybridization of 3'-end of the promoter-primer to the random region of the 3SR-RNA transcript followed by reverse transcription and self-priming hairpin formation. Thus we have to suppress this intermediate hairpin amplification.

The RNA transcript of 3SR contains neither a promoter sequence nor its complementary sequence. On the other hand, that of the RNA-Z hairpin and that of the intermediate hairpin surely contain a complementary promoter sequence for its amplification (Figures 1-B and C). The promoter sequence is recovered in 3SR by hybridization of a primer, which contains the promoter sequence, in order to regenerate the double-stranded DNA (Figure 1-A). Based on this difference, we designed a reagent to inhibit the hairpin amplification specifically, that is, a DNA oligomer containing the promoter sequence and the modified 3'-end as shown in Figure 1-D.

As an experimental demonstration of the effectiveness of the inhibitor DNA, we performed two couples of batch experiment. The 3SR DNA template used (3SR-T, 120 nts) consisted of the sequence of a primer Prom-P (41 nts), a random sequence (59 nts) and the complementary sequence of a primer RT-P1 (20 nts): 5'-GATTATTTATGTGACACTATAG-GAATATTAGAAAACAACTC-N_{59}-TTCCGCTCATTCTA-GAAACG-3'. The hairpin template used (HP-T, 81 nts) consisted of the sequence of the primer Prom-P and the complementary sequence (minus one base at 5'-end) of it: 5'-GATTATTTATGTGACACTATAGGAATATTAGAAAACA ACTCAGTTGTTTTCTAATATTCCTATAGTGTCACATAA ATAATC-3'. Primers Prom-P (41 nts) and RT-P1 (Reverse

transcription primer, 20 nts) were 5'-GATTATTTATGTGA-CACTATAGGAATATTAGAAAACAACTC-3' and 5'-CGTTTCTAGAATGAGCGGAA-3', respectively.

Experimental results were as follows. The hairpin template (HP-T) was able to be amplified starting from the 500 pM (where, 1 M = 1 mol dm^{-3}) template using only the Prom-P (200 nM). This amplification reaction was suppressed by the presence of 2 μM inhibitor DNA as shown in Figure 2-A. Namely, the inhibitor, which had a molar ratio of 10:1 to the primer and a molar ratio of 4000:1 to the template, suppressed the amplification of HP-T below the observable level for more than 60 min. We changed the concentration of the inhibitor DNA and analyzed 60 min products of the hairpin amplification reaction under the fixed concentration of HP-T (2.5 nM) and Prom-P (200 nM). At 25 nM inhibitor, there was no visible effect on the amplification reaction. At 250 nM inhibitor, the amplification was significantly suppressed. At 2.5 μM (and also at 25 μM) inhibitor, the amplification was completely suppressed. This concentration dependence is reasonable judged from the inhibition mechanism.

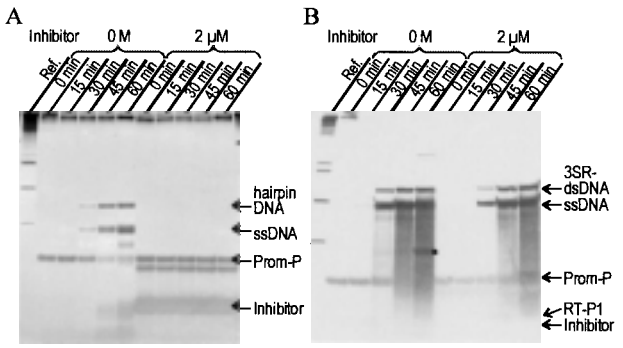

Figure 2. The effect of the inhibitor DNA on the hairpin amplification and on the 3SR amplification. A: A time course of the hairpin amplification starting from the 500 pM hairpin template (HP-T) with and without the inhibitor DNA (2 μM). The primer (Prom-P) concentration was 200 nM. B: A time course of the 3SR amplification starting from the 2 nM 3SR template (3SR-T) with and without the inhibitor DNA (2 μM). The concentration of Prom-P and RT-P1 was 200 nM each. 3SR-T and HP-T templates were amplified separately in 50 μL at 37 °C. The reaction mixtures contained 60mM Tris-HCl (pH 8.1), 10 mM KCl, 10 mM $(CH_3COO)_2Mg$, 2.5 mM DTT, 2 mM Spermidine, 1 mM each dNTP, 2 mM each NTP, 10% (v/v) DMSO, 0.9 U/μL SP6-RNAP, 0.3 U/μL HIV-1 RT, 200 nM Prom-P, 200 nM RT-P1 (for the case of 3SR-T, 0 M for the case of HP-T), each template and the inhibitor DNA. The buffer condition is a slight modification of the method described by Gebinoga et al.[11] An aliquot of 5 μL of the reaction mixture withdrawn every 15 min was added to 5 μL loading buffer (0.2% Bromophenol Blue, 99.8% Formamide). RNA was digested by addition of 1 μL 1 M NaOH at 90 °C for 10 min and DNAs was analyzed by 8% polyacrylamide gels (containing 8 M urea) electrophoresis followed by silver staining. Lane Ref. is for the reference of the DNA length. A band noted by a dot in B may be the emerging hairpin template.

The 3SR template (3SR-T) with a random sequence region was able to be amplified starting from the 2 nM template using two primers (Prom-P and RT-P1). As shown in Figure 2-B, the presence of the 2 μM inhibitor DNA did not affect the amplification reaction rate, but the absence of the inhibitor DNA led to emergence of the hairpin template. As for amplification reaction rate, six independent experiments using 3SR template from random pool or 3SR template of a specific sequence gave the nearly same result.

We observed also that, in a tube containing both the hairpin template and the 3SR template with a specific sequence, the inhibitor DNA of 10 times excess over the primer suppressed the amplification of the hairpin template, but did not affect the amplification of the 3SR template.

We applied this stabilized 3SR to a serial transfer experiment using random pool templates. We successfully performed the stable 3SR reaction over seven-fold transfer with 15 min incubation in each tube, that is, total 105 min reaction. The inhibitor DNA may hybridize to some RNA transcripts carrying a sequence homologous to the complementary promoter sequence, because we used the random pool 3SR templates. Then it may suppress the amplification of the sequence. Therefore our stabilized system may not explore the entire sequence space of DNA, but the fraction of the omitted region is estimated to be extremely small. The stabilized 3SR will make it feasible to perform a stable in vitro selection and evolution under the isothermal condition, instead of PCR.

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