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DNA Helicase Reduces Production of Aberrant Run-off Transcripts during *in vitro* RNA Synthesis with T7 RNA Polymerase

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In vitro transcription using the bacteriophage RNA polymerase is an efficient way to synthesize RNA molecules in a large scale. There are several RNA polymerases and each enzyme needs a different DNA promoter sequence for initiation of transcription.[1-3] Among the RNA polymerases used to synthesize RNAs *in vitro*, bacteriophage T7 RNA polymerase (T7 RNAP) is the most widely used due to its convenience and ample synthesis of RNA using DNA template harboring conserved sequences of upstream promoter.

T7 RNAP is a single subunit enzyme with a molecular weight of 98 kDa, which can catalyze RNA synthesis without any accessory proteins.[4-6] T7 RNAP binds to a variety of promoter sequences present in bacteriophage genome, all of which contain a 17-base-pair of consensus sequence as promoter.[6,7] In this study, forked DNA construct was designed, which contains the 5' and 3' overhangs comprised of thymine nucleotides and the 35-base-pair duplex harboring the T7 promoter sequence (Fig. 1(a)).

The fidelity of the *in vitro* transcription process is usually sufficient for low scale synthesis of RNAs. However, non-templated 3'-extension incorporating extra nucleotides at the 3'-terminal of nascent RNA strands has been often recognized as a propensity of T7 RNAP, producing aberrant run-off transcripts in some cases.[8,9] Especially, these incorrect run-off transcripts are often generated in a relatively short RNA synthesis less than 20 bases.[9] Thus, oligoribonucleotide synthesis with defined sequences entails verification of exact length as well as 3'-terminal sequences. Since translocation of the T7 RNAP along the DNA template is elemental for the nascent RNA synthesis, we hypothesized that T7 RNAP could be stalled at the end of DNA template without being released from the DNA template in the case of run-off products generation. This circumstance would make the T7 RNAP add a few more extra nucleotides to the transcript and generate aberrant run-off RNA products.

T7 bacteriophage gene gp4A encodes another class of DNA translocating proteins, T7 DNA helicase, which is a 63-kDa replicative primase-helicase protein[10] that assembles into a ring-shaped hexamer in the presence of dTTP.[11-13] T7 helicase can form both hexamer and heptamer, but hexamer

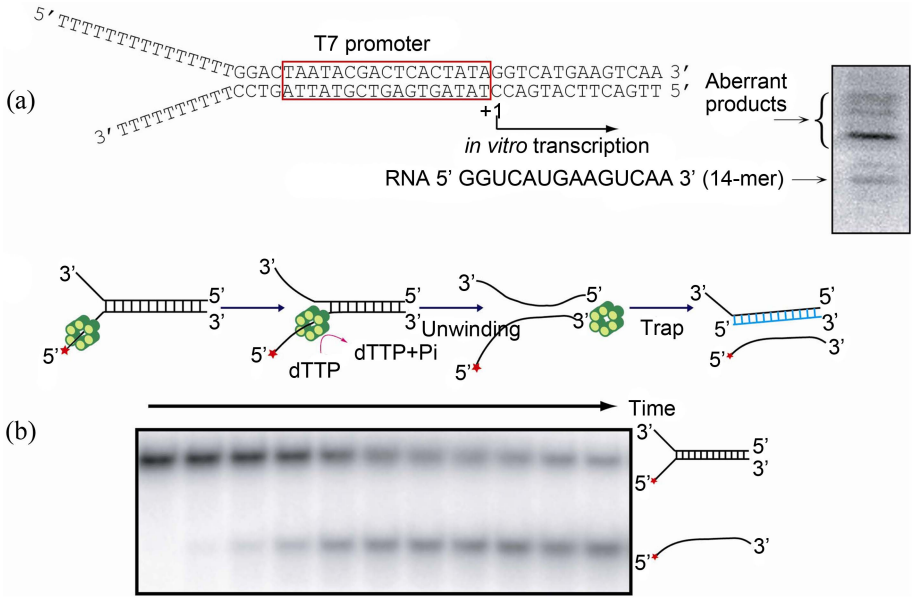

Figure 1. (a) Forked DNA template for both T7 RNAP and T7 helicase is shown, which contains T7 promoter sequence (indicated in a box) and two tails at the end of the template. *In vitro* transcription with the template generates aberrant RNA products as well as 14-mer RNA. +1 indicates initiation point of RNA synthesis. (b) Unwinding of the forked DNA substrate by T7 DNA helicase. ^{32}P-labeled forked DNA was used as a substrate, and an excess amount of unlabeled DNA was added as a trap DNA. Reaction aliquots of the unwinding reaction at increasing time points were analyzed on the native-PAGE (10%).

structure is a predominant form in the presence of nucleotide and single-stranded DNA (ssDNA).[14] T7 helicase binds preferentially to ssDNA and translocates unidirectionally.[15] This unidirectional movement along ssDNA at a rate of about 130 bases/s is fueled by dTTP hydrolysis (2 or 3-nt movement per one dTTP hydrolysis).[15] To unwind a duplex DNA substrate, T7 helicase requires two non-complementary tails at one end of the duplex DNA.[16] This enzyme interacts asymmetrically with the two tails of the forked DNA before initiation of DNA unwinding; the enzyme binds to only one strand of the duplex DNA at the 5'-end and excludes the complementary strand during DNA strand separation.[17]

Here, we combined T7 RNAP with T7 DNA helicase to lessen the generation of aberrant run-off transcripts. We hypothesized that addition of T7 helicase to the *in vitro* transcription reaction with T7 RNAP and the forked DNA templates would dissociate the stalled T7 RNAP from the end of DNA template. As a result, the correctly sized transcript would be produced with a less amount of aberrant run-off transcripts than the reaction performed by T7 RNAP alone.

We first carried out the *in vitro* transcription reaction with T7 RNAP using the designed forked DNA template. As shown in Figure 1(a), short RNA synthesis generates 3'-extended RNA transcripts with a few more bases longer than RNA with the expected length (i.e. 14-mer). The forked DNA template we designed contains T7 promoter sequences to be recognized by T7 RNAP and two non-complementary tails required for unwinding by T7 helicase (Fig. 1(a)). We next tested for duplex DNA unwinding by T7 helicase using the forked DNA substrate. To minimize reannealing of the displaced isotope-labeled ssDNA to its complementary ssDNA, an excess amount of the same unlabeled ssDNA was used in the reaction as a trap.[17,18] T7 DNA helicase efficiently unwound the forked DNA substrate as a function of time (Fig. 1(b)).

To verify our hypothesis, *in vitro* transcription reactions with T7 RNAP were performed using various auxiliary enzymes (Fig. 2(a)). When the T7 RNAP synthesizes RNA with the DNA template we designed (Fig. 1(a)), RNA products mainly appeared as aberrant RNAs in size, which are longer than 14-mer. However, combining the T7 helicase into the *in vitro* transcription reaction reduced incorrectly-sized run-off RNA products (Fig. 2(a)), whereas the other auxiliary enzymes including another class of RNA helicase (HCV NS3 helicase[19]) did not seem to change any patterns of the RNA products. Thus, only T7 helicase is effective in reducing the longer transcripts during *in vitro* transcription reaction with the forked DNA template. We suggest that the unwinding activity of T7 helicase reduces chances of stalling of T7 RNAP at the end of the duplex DNA template by disrupting the protein-nucleic acid interaction. Interestingly,

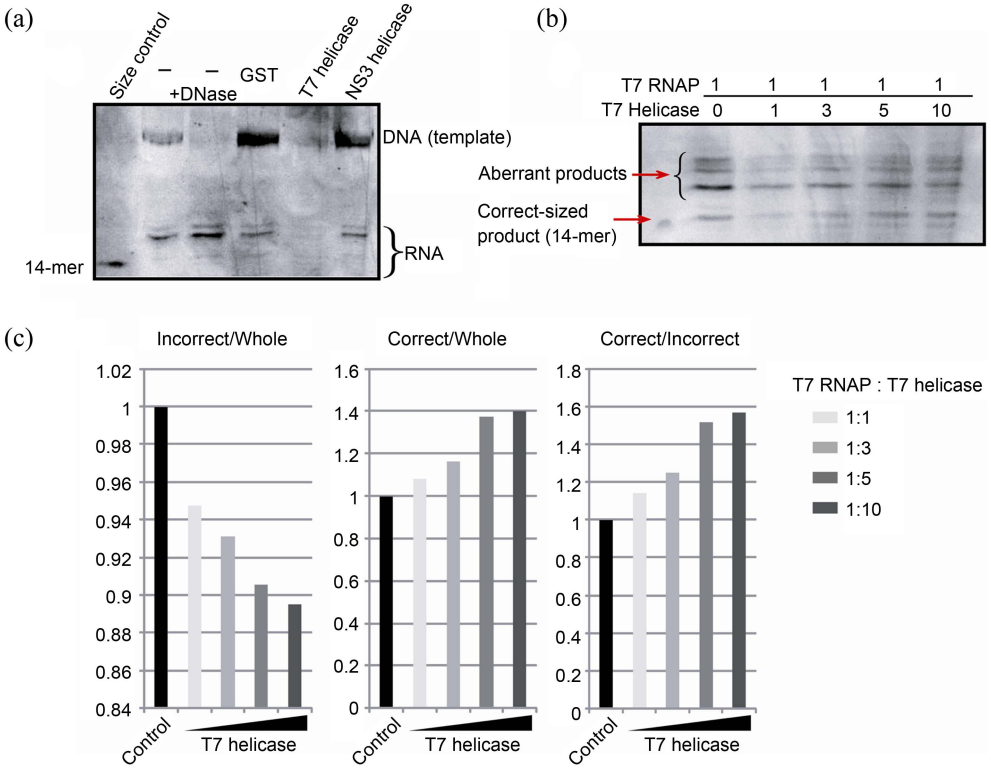

Figure 2. (a) Denaturing urea-PAGE (15%) analysis of RNAs synthesized by T7 RNAP. The forked DNA was used as a template and the auxiliary enzyme was added as indicated in each lane. –, only T7 RNAP; GST, Glutathione S-transferase; NS3 helicase, hepatitis C virus RNA helicase; +DNase, DNase was treated to the reaction containing only T7 RNAP. (b) Denaturing urea-PAGE (15%) analysis of RNAs synthesized by T7 RNAP in the presence of increasing amount of T7 DNA helicase. Ratio of T7RNAp and T7 DNA helicase is shown on top of the gel image. (c) Quantification of RNA products appeared in the gel shown in (B). Amounts of RNAs in "Control", which was performed in the absence of T7 helicase, were normalized. See the "Experimental section" for details.

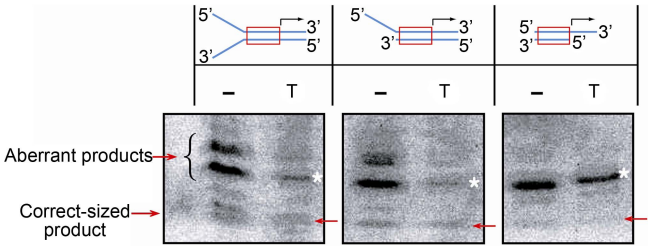

Figure 3. Denaturing urea-PAGE (15%) analysis of RNAs synthesized by T7 RNAP using various dsDNA templates. –, only T7 RNAP without any auxiliary enzyme; T, T7 helicase was added as an auxiliary enzyme. T7 promoter is indicated with a box. Broken arrow represents a direction of RNA synthesis. Asterisk shows aberrant RNA product that is longer than the correctly-sized RNA (14-mer, shown with arrow). T7 promoter annealing oligonucleotide was used to make the partial duplex DNA template.

the duplex DNA template seems to be also affected by the T7 DNA helicase, as shown by the decreased amount of DNA template remaining in the gel. We speculate that the reduced amount of remaining duplex DNA template was due to complete unwinding of duplex DNA template by the helicase during the *in vitro* transcription reaction.

We next performed a series of *in vitro* transcriptions using T7 helicase as an auxiliary enzyme at an increasing dosage. Without T7 helicase, the amount of correctly-sized 14-mer RNA product was less than that of the longer RNA products. However, rations of the 14-mer RNA product to the longer RNA products increased as the amount of T7 helicase was increased (Fig. 2(b)). Since the helicase is capable of unwinding the forked DNA template, T7 RNAP might have less chance to bind to intact duplex T7 promoter sequence during the *in vitro* transcription reaction. This will attribute to the decreased amounts of synthesized RNA products in the presence of T7 helicase. We quantified accumulation of produced RNAs in 3 different ways; the whole RNA products (whole), the correctly-sized RNA product (correct), and the incorrectly-sized longer RNA products (incorrect); and the ratios of incorrect/whole, correct/whole, correct/incorrect were determined, as shown in Fig. 2(c). The relative amount of correctly-sized RNA product was enhanced, as the amount of T7 DNA helicase in the reaction increased. This result implies that more molecules of T7 helicase are involved in disrupting T7 RNAP-DNA template interaction at the end of DNA template as the relative amount of T7 helicase increases in the RNA synthesis reaction.

To further investigate whether the reduction of aberrant run-off RNA products is caused by the action of T7 helicase, we performed several *in vitro* transcription reactions with three different duplex DNA templates. When the forked DNA template was used for RNA synthesis, decrease of aberrant run-off RNA was observed (Fig. 3). Interestingly, the second DNA template containing only one tail at the 5'-end, which is not a proper substrate for T7 helicase, did show the same result as shown by the forked DNA substrate. This can be explained by that as T7 RNAP recognizes its promoter sequence and forms an open promoter complex, the duplex strands are temporarily unwound and generate ssDNA overhang at the 3'-end. Thus, two tails of ssDNA are readily formed, which can be eligible for T7 helicase to unwind. However, the partial duplex DNA template without 5'-ssDNA tail for T7 helicase binding at the direction of T7 RNAP progression did not show the same effect as shown by the forked DNA template. Therefore, duplex DNA unwinding and translocation activity by T7 helicase is truly responsible to reduce the production of aberrant run-off RNA products during the *in vitro* transcription with T7 RNAP.

In conclusion, combining T7 helicase in T7 RNAP reaction reduced the amount of aberrant run-off RNA products, increasing the relative yield of the correctly-sized RNA product. With short DNA template, T7 RNAP tends to synthesize a few more sequences at the end of the RNA product while it stalls at the 3'-terminal of DNA template. If the T7 DNA helicase is present in the reaction, the helicase translocates along the DNA template during unwinding of the duplex DNA, and eventually disrupts T7 RNAP-DNA interaction (Fig. 4). The displacement of T7 RNAP from the DNA template will facilitate the production of correctly-sized RNA products. In support of our model, helicase has been recognized as a versatile molecular motor that can readily displace proteins bound to DNA, disrupting protein-nucleic acid interactions.[20,21] To our knowledge, this is the first report to use DNA helicase in the RNA synthesis by T7 RNAP to reduce aberrant run-off RNA products. Despite its

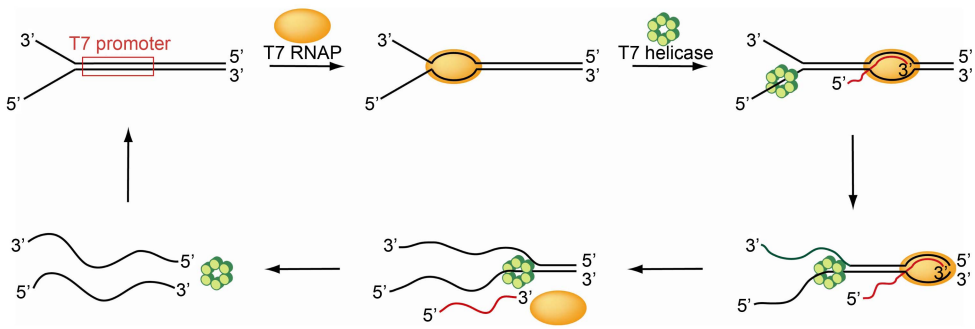

Figure 4. Schematic diagram of *in vitro* transcription of the forked DNA template by T7 RNAP in the presence of T7 DNA helicase; See the text for details.

usefulness in the *in vitro* transcription, this unique helicase-combined RNA synthesis system further needs to be investigated to enhance overall amount of RNA products in order to be adapted as a practical method.

Experimental Section

Enzymes. T7 RNA polymerase was purified from the *Escherichia coli* strain BL21 containing the plasmid pAR1219 with a method of ammonium sulfate fractionation and ion-exchange column chromatography as previously described.[22] T7 gp4A helicase is an M64L mutant of T7 helicase-primase protein. This protein was overexpressed in *E. coli* and purified as described elsewhere.[23] Concentration of T7 RNAP and T7 DNA helicase was determined from its absorbance at 280 nm in 8 M urea by using an extinction coefficient of 1.4×10^5 $M^{-1}cm^{-1}$ and 76,100 $M^{-1}cm^{-1}$, respectively.

Oligonucleotides. Template DNAs used in this study were prepared by annealing two DNA oligonucleotides; two oligoneucleotides were mixed at a 1:1 ratio in a buffer (25 mM Tris-HCl (pH 6.8), 25 mM NaCl) and heated at 95 °C for 5 min and slowly cooled to room temperature for annealing. Oligonucleotides shown in the figures were synthesized chemically and purchased (Cosmo genetech, Seoul, Korea). Nucleotide sequences are shown in Figure 1(a), and all of ssDNA tails are oligothymidylates. Sequence of T7 promoter annealing oligonucleotide is 5'-GGACTAATACGACTCACTATA-3' (T7 promoter sequence underlined.).

***In vitro* Transcription.** RNA synthesis reaction was performed in a buffer containing 50 mM Tris-HCl (pH 6.8), 15 mM $MgCl_2$, 2 mM spermidine, 5 mM dithiothreitol (DTT), and 2 mM of NTPs with proper DNA template (0.4 μM) and T7 RNA polymerase (0.5 μM) at 37 °C for 3 h. Where indicated, T7 DNA helicase (as an equivalent to T7 RNAP) and dTTP (2 mM) were included in the standard RNA synthesis reaction for a DNA helicase-combined *in vitro* transcription reaction. The reaction was quenched with the quenching buffer (8 M Urea, 30 mM EDTA, 10% glycerol with proper dye for size marker). The RNA products were analyzed by electrophoresis in denaturing polyacrylamide gels containing 8 M urea, and were quantified using the Gel-Pro analyzer software (Media Cybernetics, Bethesda, MD).

DNA Unwinding Assay. A solution containing the transcription buffer without $MgCl_2$ (50 mM Tris-HCl (pH 6.8), 2 mM spermidine, 5 mM DTT), 5 mM EDTA, 2 mM dTTP, 5 nM of ^{32}P-labeled duplex DNA substrate (dsDNA), and 1.0 μM of T7 helicase was rapidly mixed with an equal volume of the same buffer containing 2 mM dTTP, 30 mM $MgCl_2$, and 3 μM trap DNA(unlabeled 5'-strand ssDNA).[18] The reaction mixture was incubated at room temperature and quenched with quenching buffer (100 mM EDTA, 0.4 % (v/v) sodium dodecylsulfate). Unwound DNAs were detected by EMSA (Electrophoretic Mobility Shift Assay) using Native-PAGE.[16,18]

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