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Evidence for RNA template-directed elongation

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Abstract. In vitro cryptic transcription product is often observed when using T7 RNA polymerase. We obtained a *ca*. 35 mer of cryptic RNA by-product, which was originally designed to be 23 mer by *in vitro* run-off transcription. Biochemical research and structural analysis indicated that the cryptic by-product was synthesized through the process of aberrant extension by the T7 RNA polymerase. This extension could have occurred through two pathways. One pathway could have been an aberrant termination of transcription, which met a conventional prolonged extension without precise transcription termination, and the other could have been a re-extension of nascent RNA by binding with T7 RNA polymerase.

INTRODUCTION

In vitro RNA synthesis is important for RNA engineering. In general, there are two ways for preparing synthetic RNA; one is organic synthesis and the other is enzymatic RNA transcription using recombinant phage derived-RNA polymerase such as T7 RNA polymerase (1). Especially, *in vitro* RNA transcription using recombinant RNA polymerase is a convenient and economical way for preparing synthetic RNA because the RNA polymerase is available from several suppliers and can be easily prepared in a laboratory.

Contrary to the convenience of the availability of the recombinant RNA polymerase mentioned above, several reports indicated some problems when using the RNA polymerase. For example, N+1 nucleotide addition (2) aberrant extension at the 3' end of the run-off transcript (3) or unexpected transcription termination resulting in a premature, nascent RNA transcript (2) were often observed. This abortive transcription could be dependent on the transcription conditions and sequence of the transcript; however the precise mechanism is still controversial among researchers. Therefore, describing the correlation between the sequence and secondary structure of the aberrant elongation product will be helpful for clarifying the precise mechanism of the aberrant elongation.

RNA secondary structures can be predicted by several RNA secondary structure prediction programs; such as the centroid fold program (4), the Mfold program (5) and the Vsfold program (6). The predicted secondary structure model was conventionally verified with a nuclease mapping experiment (7). In addition, the secondary structures of RNAs can be analyzed by using nuclear magnetic resonance (NMR), because observation of imino proton signals indicates the presence of hydrogen bonding such as not only the A–U and G–C of the Watson-Crick base pairings, but also G–U of the non-Watson Crick base parings (8).

In this study, we obtained two RNA transcripts: one is a nascent 23 mer of RNA and the other is *ca*. 35 mer of cryptic elongated by-product made by run-off transcription. To understand the production mechanism of the cryptic run-off transcription by-products driven by the T7 RNA polymerase, we analyzed the elongated RNA as a model of cryptic elongated transcripts.

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MATERIALS AND METHODS

RNA preparation

RNA **1A** (5′-GGUUAGGGUUAGACAAAAAAUGU-3′) and RNA **1B** (5′-GGUUAGGGUUAGACAAAAAAUGUCUAACCCUAACC-3′) were prepared by run-off transcription or were chemically synthesized (JBios, Japan). For the *in vitro* transcription, a double-stranded synthetic DNA template containing T7 promoter was prepared. The RNA polymerase used in this study was commercially purchasable Thermo T7 (TT7) RNA polymerase (TOYOBO, Japan). The transcription mixture consisted of 40 mM Tris-HCl (pH 8.0), 50 mM NaCl, 8 mM $MgCl_2$, 5 mM DTT, and 1 mM ribonucleotides. For the purification of RNA **1A** or **1B**, transcripts were electrophoresed in a 7 M urea containing a 16% denaturing gel. The precise migration band of RNA **1A** or **1B** was monitored by UV shadowing and excised. The excised gel was crushed and soaked with an elution solution (0.3 M NaOAc, 0.1% SDS and 0.1 mM EDTA). Eluted RNA **1A** or **1B** was concentrated by a conventional ethanol precipitation.

NMR measurements

Electro-purified RNA **1A** was dissolved in 20 mM sodium phosphate (pH 6.5) buffer in 5% D_2O (9). The imino proton spectrum and TOCSY spectrum were measured using a AVANCE 600 spectrometer (Bruker). Spectra were recorded at either a 10°C or 30°C probe temperature.

Electro mobility shift assay (EMSA)

Organic synthesized RNA **1A** and RNA **1B**, or electro-purified tRNA transcripts were used for EMSA. Each RNA (0.1 μg) was mixed with or without TT7 RNA polymerase (150 units) or BSA (3 μg) and incubated at 37°C for 30 min. Complex formation of RNA **1A** with TT7 RNA polymerase or BSA was monitored by 10% Native PAGE.

RNA extension assay

TT7 RNA polymerase-induced extension reaction was performed under the same reaction buffer conditions for *in vitro* transcription as described above. Chemically synthesized RNA **1A** (2 μg) was incubated with TT7 RNA polymerase (50 units) in the presence or absence of 1 mM of ribonucleotides. At various duration times (see figure), aliquots were recovered and mixed with a stop solution (7 M urea, 0.1 mM EDTA) and analyzed by 16% denaturing PAGE.

RESULTS AND DISCUSSION

We performed 1H-NMR to obtain secondary structural information of a model of a ca. 35 nt-long cryptic elongated transcript (RNA 1B) that was originally designed to be 23 nt long (RNA 1A). The 1H-NMR data showed that electro-purified RNA 1B formed a stem-loop structure, which did not contain any non-Watson Crick base pairings (data not shown). According to the secondary structure prediction program, the Mfold program (5), the elongated RNA formed a blunt-ended stem-loop structure (Fig. 1), which agreed with the 1D imino proton spectrum (Fig. 2) and TOCSY spectrum (data not shown). Therefore, we hypothesized that the RNA extension was carried out through RNA template-directed RNA elongation.

To clarify the hypothesis of RNA template-directed RNA elongation, we prepared 23-nt of chemically synthesized nascent RNA (RNA 1A) as a control RNA transcript without any aberrant elongation, which formed a stem-loop structure at the 3′ end with a 5′ overhang (Fig. 1). To our surprise, we found that T7 RNA polymerase

bound to the chemically synthesized 23 mer of RNA 1A and 35 mer of stem-loop structured RNA 1B and in vitro prepared tRNA, while BSA did not (Fig. 3). This result indicated that T7 RNA polymerase bound to some structured RNA. In addition, in the presence of T7 RNA polymerase, RNA template-directed elongation was initiated by adding ribonucleotides and the RNA extension was almost completed within 30 min (Fig. 4).

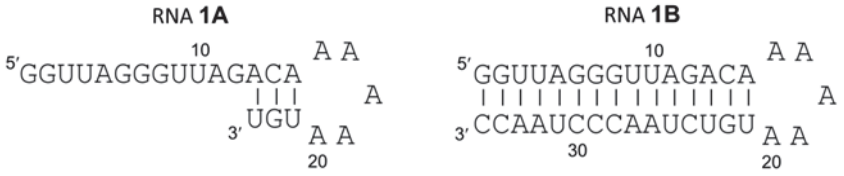

FIGURE 1. Predicted secondary structure of RNA 1A and RNA 1B. Predicted secondary structures by the Mfold program are shown. Vertical lines indicate hydrogen bonding.

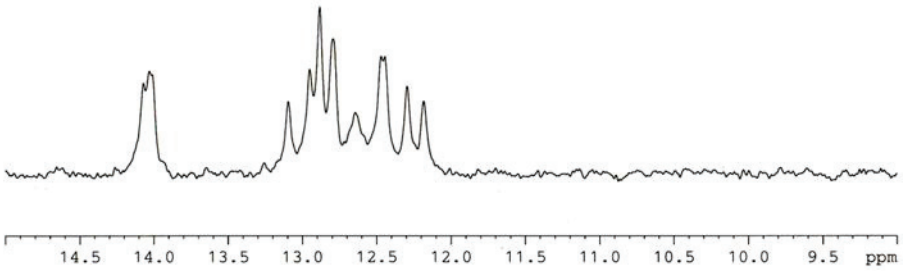

FIGURE 2. Imino proton spectrum for elongated RNA transcript. The spectrum was recorded at 10°C.

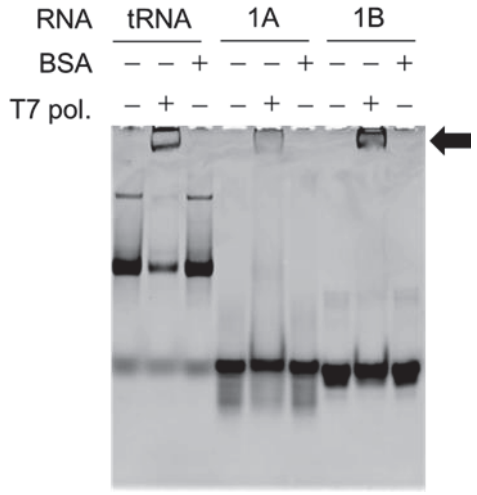

FIGURE 3. T7 RNA polymerase bound to RNA. In vitro transcribed tRNA, organic synthesized RNA 1A (1A) and RNA 1B (1B) are incubated with TT7 RNA polymerase (T7 pol.) or bovine serum albumin (BSA), respectively. After non-denaturing gel electrophoresis, ethidium bromide staining gel was monitored with the Fluorescent Image analyzer FLA-9000 (Fujifilm). The arrow shows the migration of the RNA- TT7 RNA polymerase complex.

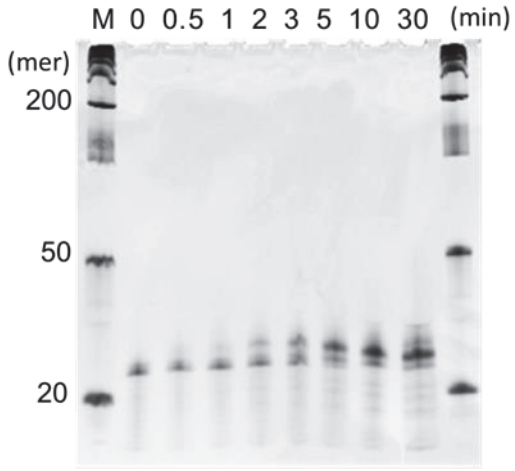

FIGURE 4. RNA-directed extension by TT7 RNA polymerase. Organic synthesized RNA 1A was incubated with TT7 RNA polymerase in the presence of 1 mM of ribonucleotides. During each reaction's duration, aliquots were recovered and were monitored by 16% denaturing PAGE. Ethidium bromide-stained gel images were obtained by an FLA-9000 (Fujifilm). "M" indicates the RNA seize marker.

Therefore, we concluded that the aberrant transcription elongation proceeded at the 3′ end of the 23-nt nascent RNA transcript with a stem-loop structure using the 5′ overhanging strand as a template strand and the end

result was a 35-nt blunt-ended stem-loop structure (Fig. 5). We cannot rule out the possibility of prolonged aberrant extension bypassing T7 RNA drop-offs and rebinding, because the elongation reaction was rapid enough for the completion of unexpected aberrant extension by T7 RNA polymerase (Fig. 4). Further study will be necessary for a more complete understanding of the aberrant extension of T7 RNA polymerase; however these findings offer novel insight into preparing RNA by using recombinant RNA polymerases in future.

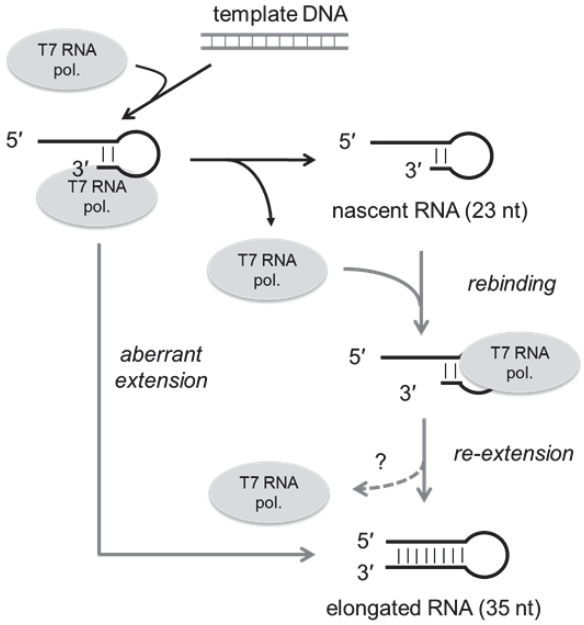

FIGURE 5. Two possible pathways for aberrant extension. Black and gray arrows indicate normal RNA transcription pathways and elongated by-product synthesis pathways, respectively.

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