Bioorganic & Medicinal Chemistry Letters 18 (2008) 4562–4565

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Fluorescence generation from tandem repeats of a malachite green RNA aptamer using rolling circle transcription

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ARTICLE INFO

Article history:
Received 11 June 2008
Revised 8 July 2008
Accepted 10 July 2008
Available online 15 July 2008

Keywords:
Fluorescence
Nucleic acid
Amplification
RNA
Polymerase

ABSTRACT

We demonstrate a generation of tandem repeats of a malachite green (MG) RNA aptamer using rolling circle transcription. To keep the higher-order structure of each aptamer on long RNA, we designed a sequence of circular DNA with a 14-base linker. T7 RNA polymerase was superior to *Escherichia coli* RNA polymerase in the specific transcription of the MG RNA aptamer. Finally, the generation of the fluorescence signal was confirmed from aptamer repeats with MG.

Aptamers can be created by the in vitro selection technique.[1,2] A large number of oligonucleotide aptamers for small-molecule targets, including organic dyes, porphyrins, other nucleotides and free nucleobases, amino acids, cofactors, basic antibiotics, and transition-state analogs, have been isolated in the past decade.[3–5] The tandem repeats of RNA aptamers were recently found to inhibit the active domain of a protein corresponding to a drug. Nishikawa et al. constructed a vector that produced tandem repeats of the GP9-II RNA aptamer, which bound specifically with the nonstructural protein 3 protease domain of hepatitis C virus and demonstrated the aptamer's efficient protease inhibition activity in HeLa cells.[6] The structured RNA with repeated sequences plays important roles in living organisms.[7–10] For example, gene expression is regulated by the mechanism called riboswitch, which comprises repeated binding domains for pyrophosphate[8] or thiamine glysine[9,10] targets. These suggest that aptamer tandem repeats are an interesting target to create functional RNA molecules. For example, a tandem repeated aptamer might achieve multiple abilities beyond those of a single aptamer.

Creating tandem repeats of an aptamer requires an efficient tool for DNA or RNA elongation. Rolling circle amplification (RCA) is a technique in which a circular oligonucleotide sequence serves as a template to create a complementary single-stranded DNA (ssDNA) chain that contains periodic repeats of the sequence coded by the circular oligonucleotide.[11–17] Recently, Cheglakov et al. created linear DNA chains containing DNA aptamers for the proteins thrombin and thrombin and lysozyme together.[17] Rolling circle transcription (RCT) is one of the most general methods for generating RNA repeats.[18–24] Small single-stranded circular oligonucleotides can be used as efficient templates for initiation and elongation of RNA sequences in the RCT reaction, which can occur in the absence of added primers, RNA promoter sequences, or a duplex structure. The synthetic circular DNAs are considerably smaller than the enzyme itself, yet they serve as highly efficient templates for the production of repeating RNA sequences that are thousands of nucleotides in length. Kool and coworkers reported that ribozyme repeats can be synthesized by RCT.[19] However, there are no reports on the production of RNA aptamer tandem repeats using the RCT reaction.

Malachite green (MG) normally has extremely low quantum yields for fluorescence due to easy vibrational de-excitation, but viscous or cold environments are known to encourage some fluorescence by restricting such vibrations. Babendure et al. show that the MG aptamer indeed increases the fluorescence of MG and close relatives 2360-fold.[25]

We report that tandem repeats of an RNA aptamer can be generated by the RCT reaction. A sequence of MG RNA aptamer[25–30] was used for transcription. We demonstrate that a generation of tandem RNA repeats can be elucidated by gel electrophoresis and the fluorescence signal appearing from the multiple MG aptamer.

The designed circular DNA encoded two different motifs: an MG RNA aptamer and a linker sequence. As shown in Figure 1, when circular DNA was treated with RNA polymerase, continuous RNA elongation known as RCT proceeded to yield the long RNA strand repeating aptamer and linker motif. Each aptamer on multiple re-

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doi:10.1016/j.bmcl.2008.07.040

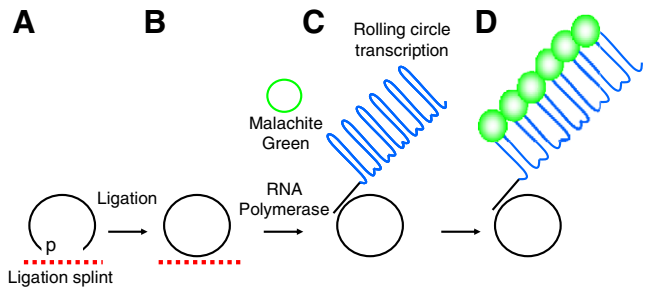

Figure 1. Generation of the tandem repeats of the malachite green (MG) RNA aptamer by rolling circle transcription. (A) Precircularized DNA containing the complementary strand of the MG RNA aptamer was hybridized with ligation splints. The 5′-end of the precircularized DNA was phosphorylated. 5′-Phosphorylation is denoted by 'p' in the figure. (B) Circularized DNA was conformed by ligation. (C) The MG RNA aptamer was amplified by rolling circle transcription. (D) MGs were combined with the amplified MG RNA aptamers.

peats is expected to bind with MGs and emit multiple fluorescence signals. The oligonucleotide sequence used for preparing the DNA nanocircle is listed in Table 1. The MG RNA aptamer (Fig. 2A) containing the linker sequence was 52 bases in length, and the structure calculated by *m*-fold[31] was characterized by two loops with 6 bases and 16 bases, and two stems with 6 bases and 5 bases, respectively. The design of the linker seemed to be important because a wrong linker sequence disturbed the folding aptamer repeat on the long RNA strand. To design the best linker sequence, we used *m*-fold to calculate the two-dimensional structure of a repeated sequence-encoding aptamer and linker. After testing various sequences, the linker with 14 bases was selected because it could fold 10 repeated aptamers or more (Fig. 2B and C).

Circular DNA was synthesized out using the conventional method.[19] Phosphorylated precircularized DNA at the 5′-terminal containing the complementary strand of MG RNA aptamer was hybridized with splint DNA, and the precircularized DNAs were then ligated by T4 ligase (Fig. 1A). The ligation reaction gave three products by gel electrophoresis analysis, and each product was isolated. To confirm the circularity, these were treated with the S1 nuclease method as shown in Figure S1. The fastest band was the desired circular DNA (lanes 3 and 4) because treatment with the S1 nuclease gave precircularized DNA. Similarly, the middle band (lanes 5 and 6) was a linear dimer and the slowest band (lanes 7 and 8) shows the circular dimer. The purified circular DNA was obtained in yields of 40.2%.

Although previous work has shown that certain synthetic circular oligodeoxynucleotides can be transcribed by T7 or *Escherichia coli* RNA polymerases,[18] a number of cases are not transcribed efficiently by either enzyme,[32] apparently because of undefined sec-

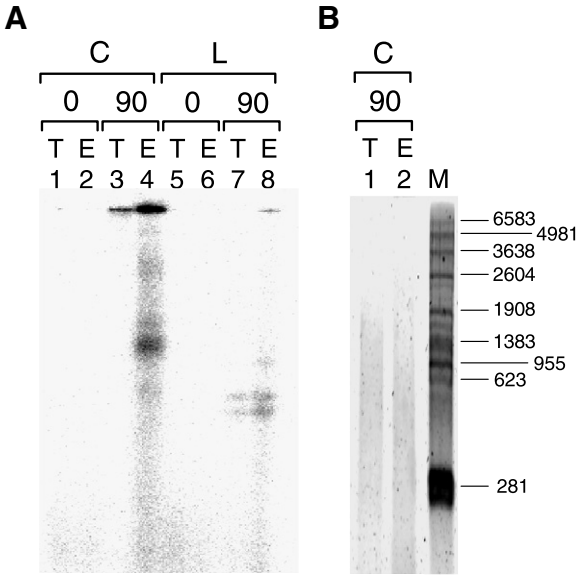

Figure 3. Transcription of linear and circular ssDNAs encoding the MG aptamer, which shows the appearance of long RNAs and shorter, specific bands. (A) Autoradiogram from 10% denaturing polyacrylamide gel analysis of RCT products. (B) Fluorescent image from 1.75% denaturing agarose gel analysis of RCT products with long marker RNA staining with SYBR Green II. Topology of template DNA is denoted by L (linear) and C (circular); the enzyme is denoted by E (*Escherichia coli* RNA polymerase) and T (T7 RNA polymerase).

Table 1
Aptamer and oligonucleotide sequences used in this study

	Sequence (5′–3′)[b]	Length (mer)
MG RNA aptamer	GGAUCCCGACUGGCGAGAGCCAGGUAACGAAUGGAUCC	38
Transcribed product	(GGUACGUGGAUCCCGACUGGCGAGAGCCAGGUAACGAAUGGAUCCUGUGGCC)*n*	(52)*n*
Precircularized DNA[a]	pGGCCACAGGATCCATTCGTTACCTGGCTCTCGCCAGTCGGGATCCACGTACC	52
Splint DNA	CCTGTGGCCGGTACGTGG	18

[a] The MG aptamer sequences and their complementary sequences are underlined.
[b] The 5′-end was phosphorylated.

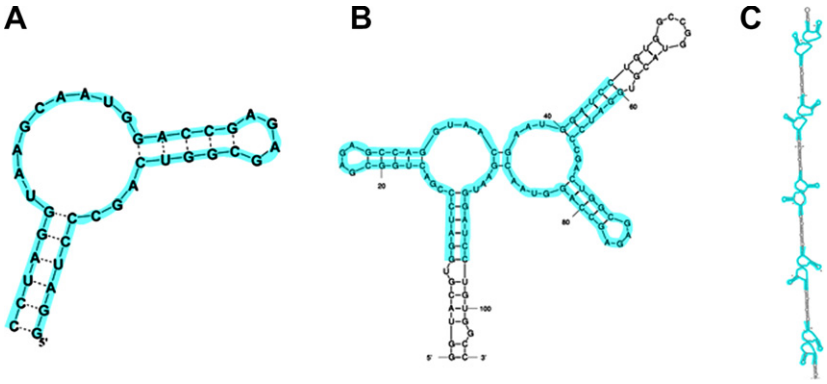

Figure 2. (A) Structure of the malachite green (MG) aptamer. (B) Two repeats of the MG aptamer containing the linker sequence. (C) Ten repeats of the MG aptamer containing the linker sequence. Light blue lines indicate the MG aptamer sequences.

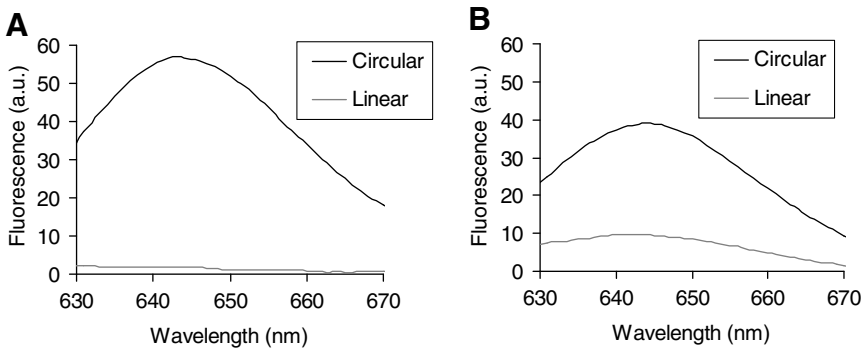

Figure 4. Fluorescence spectra of MG in the presence of RCT products from circular and linear DNA templates with T7 RNA polymerase (A) or *Escherichia coli* RNA polymerase (B). After the RCT reaction, MG solution was added at a final concentration of 30 μM. The fluorescence spectrum was measured at an excitation wavelength of 610 nm.

ondary structure preferences for initiation. Therefore, to see whether it is possible to produce MG RNA aptamer motifs by this strategy, we used both RNA polymerases for the RCT reaction.

Transcription was carried out on circular DNA and linear precursor DNA as a negative control using both T7 and *Escherichia coli* RNA polymerases. We tested the circular forms and the full-length linear precursor DNA as a template for the RCT reaction to evaluate the effect of circularity on the product. Both PAGE and agarose gel analysis showed that the circular template was transcribed well by both polymerases (Fig. 3). Because the products were too long to be run on 10% PAGE, the bands of transcripts were observed at the top of the gel (Fig. 3A). *Escherichia coli* RNA polymerase gave more abundantly transcribed products than did T7 RNA polymerase. The linear DNA produced only shorter RNAs using T7 RNA polymerase, presumably by nonspecific initiation (Fig. 3A, lane 7). In contrast, *Escherichia coli* RNA polymerase produced a few longer RNAs even from linear DNA (Fig. 3A, lane 8). These results indicate that, in the RCT reaction, T7 RNA polymerase had better specificity but inferior transcription ability compared with *Escherichia coli* RNA polymerase. As shown in Figure 3B, the products from both polymerases from denaturing agarose gel electrophoresis with long size markers in the case of T7 RNA polymerases fall largely in the range 200–2000 bases, which corresponds to 4–40 turns around a 52-mer circular template (Fig. 3B, lane 1). *Escherichia coli* RNA polymerase produced shorter products with 1500 bases or less corresponding to 30 turns or less (Fig. 3B, lane 2). Because a circle acts as a template for many repeating units, these results suggest it can be used as a catalytic template for RNA synthesis.

Next, the RCT product was incubated in the 30 μM of MG solution to test the fluorescence response. The resulting fluorescence spectra are shown in Figure 4. For each enzyme, the circular template produced greater fluorescence intensity than did the linear template. The signal-to-background (S/B) ratio was calculated as the ratio of the fluorescence intensity of the MG-RCT product solution from the circular template (signal) to that from the linear template (background) at a wavelength of 643 nm. The S/B ratio was 30.2 for the T7 RNA polymerase and 4.0 for the *Escherichia coli* RNA polymerase. The lower S/B ratio of *Escherichia coli* RNA polymerase was caused by a few longer products from the linear template, as shown in Figure 3A (lane 8). The short RNA aptamer bound with MG and yielded undesired background fluorescence. In contrast, the T7 RNA polymerase produced the long RNA product only from the circular template and never yielded long RNA products from the linear template. Therefore, the T7 RNA polymerase was better for the RCT-based sensor for generating tandem repeats of the MG RNA aptamer and for emitting the fluorescence signal without undesired signals.

To our knowledge, this is the first demonstration of the generation of tandem repeats of an RNA aptamer except for ribozyme.[19] Cheglakov et al. generated tandem repeats of a DNA aptamer using RCA.[17] In this report, we could not carry out quantitative analysis in MG binding or fluorescence intensity, because of undefined molecular size of aptamer repeats. Previous report[25] showed that the fluorescence from MG could be detected from the 4 μM of MG aptamer monomer with 0.32 μM of MG. We could detect MG fluorescence starting from 1 μM of circular DNA template. The results indicated that RCT approach is comparable or superior to previous approach in detection limit. Our technique has potential in nucleic acid sensing, especially by combining this method with chemical ligation[33,34] instead of enzymatic ligation, which enables nucleic acid detection in one step. We have demonstrated that *Escherichia coli* RNA polymerase can be used in RCT to produce MG RNA aptamers. Our method could be applied in the automatic detection of RNA species in *Escherichia coli* cells using a process that requires only the addition of MG and precircularized DNA into the cells.

Acknowledgments

H.A. was financially supported by the Riken Directors' Fund, MEXT (Ministry of Education, Culture, Sports, Science, and Technology), and NEDO (New Energy and Industrial Technology Development Organization).

Supplementary data

Protocols for synthesizing circular RNA, gel analysis, and fluorescence analysis and the nuclease studies showing circular structure. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.07.040. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.07.040.

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