ChemComm

Cite this: *Chem. Commun.*, 2012, **48**, 5121–5123

www.rsc.org/chemcomm

COMMUNICATION

The yields of transcripts for a RNA polymerase regulated by hairpin structures in nascent RNAs†

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Received 6th March 2012, Accepted 30th March 2012
DOI: 10.1039/c2cc31657a

The yields of transcripts capable of forming RNA hairpins with different stem lengths at elongation phase were determined in a T7 RNA polymerase. The amount of RNA transcripts decreased with stem length, demonstrating that transcriptional efficiency is regulated by secondary structure formation in the nascent RNA.

Transcriptional regulation is one of the central mechanisms for control of gene expression. For example, it is well known that on/off and termination switches play critical roles in biological processes.[1] Structural formations of DNA templates and nascent RNAs have been studied as one of the attractive regulation factors for transcription.[2] G-quadruplex formation by DNA templates in promoter regions[2a,b] and formation of higher-order structures in nascent RNAs in the termination region regulate the amount and length of transcripts.[2c–f] Most characterized structures impact initiation or/and termination. There are recent reports demonstrating that the transcription can be regulated at elongation phase.[3] However, there is limited information regarding the impact of structure in guanine-rich and trinucleotide-repeat sequences on transcriptional regulations.[4] The nascent RNA can form various types of secondary and tertiary structures during the transcription process.[5] A hairpin structure is one of the most simple and frequent secondary structures observed in RNAs. Formation of hairpins containing 5 to 7 base pair (bp) stems caused the elongation complex to pause.[6] A model study on the elongation complex of RNA polymerase suggested that the RNA exit tunnel accommodates 4–6 nucleotides (nt) of RNA in an extended conformation.[7] Therefore, it was proposed that hairpins with 5 bp stems would affect the elongation complex; however, an experimental analysis of how hairpin structures in the nascent RNA affect transcriptional efficiency had not been performed. Herein, we studied the effect of stem length of nascent RNA hairpins on transcriptional yield.

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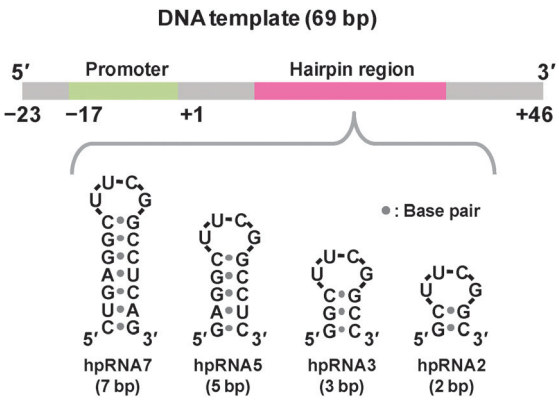
† Electronic supplementary information (ESI) available: Experimental methods, CD spectra, UV melting curves, and gel electrophoresis. See DOI: 10.1039/c2cc31657a



Scheme 1 Design of template DNAs used in this study.

We chose T7 RNA polymerase (T7 RNAP) as a model RNA polymerase, because T7 RNAP is a single-subunit enzyme.[8] The DNA templates used in this study are shown in Scheme 1. The lengths of the DNA templates were 69 bp, which include a 6 bp extended sequence at the 5′-end, a 17 bp T7 promoter sequence and a 46 bp transcribed sequence. The sequences in the transcribed region were designed to form hairpin structures once the elongation phase of the transcription reaction was begun. Templates were designed to yield transcripts that can form hairpin structures containing 7 bp, 5 bp, 3 bp or 2 bp stems, each with a 4-nucleotide loop (Scheme 1). The hairpin with a 2 bp stem should be stable energetically under physiological conditions.[9] The sequences of DNA templates (DNA7, DNA5, DNA3 and DNA2) are shown in Table 1. The secondary structures of the transcribed RNA sequences were predicted using Mfold.[10] In the region from the 5′-end to the hairpin sequence, only the desired hairpin structures were predicted to form (Fig. S1, ESI†). As it has been reported that transcriptional efficiency depends on the sequence and the duplex stability of the DNA template,[11] we unified the proportion of bases and avoided the sequence bias.

The transcription reactions were performed using 1 U μL^{-1} T7 RNAP and 0.2 μM DNA template at 37 °C in a buffer containing 40 mM Tris–HCl (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 200 μM rNTPs and 20 units RNase inhibitor, similar to experimental conditions reported previously.[12] The yields of RNA transcripts at different reaction times were determined by analysis of denaturing polyacrylamide gel electrophoresis (PAGE).

Table 1 Sequences of template DNAs used in this study

Template DNAs	Sequences[a,b]
DNA7	5′-GATCACTAATACGACTCACTATA**GGGATGATATAGTGCCTGAGGCTTCGGCCTCAGTTTCAGACAGTAG**-3′
DNA5	5′-GATCACTAATACGACTCACTATA**GGGATGGTATACTGGCTGAGGCTTCGGCCTCTTTGAGACAACAATG**-3′
DNA3	5′-GATCACTAATACGACTCACTATA**GGGATGCTAATAGTGATACGGCTTCGGCCTTTAGACAGTGACGTGC**-3′
DNA2	5′-GATCACTAATACGACTCACTATA**GGGATGATACTAGTGCTTCGGCTTTCAGGACACGGTGACCTGATAG**-3′

[a] Bold fonts indicate a transcribed region. [b] Underlines indicate a hairpin region.

The gels were stained by SYBR Gold. We obtained a saturation time of 240 min under these reaction conditions.

The structures of DNA templates are known to affect the transcriptional process.[4a,b] DNA templates have the potential to form cruciform structures in the region of the hairpin sequences in our system. To investigate the structure of the DNA templates, we first performed circular dichroism (CD) experiments in a buffer containing 40 mM Tris–HCl (pH 8.0), 100 mM NaCl and 10 mM $MgCl_2$ at 37 °C (Fig. S2A, ESI†). All of the CD spectra showed the same peak positions and intensities. In addition, the melting curves of all DNA templates were obtained in UV melting experiments monitored at 260 nm in this buffer (Fig. S2B, ESI†), indicating that all of UV melting curves are the same. In native PAGE (Fig. S3, ESI†), all of the DNA templates exhibited one band at a similar migration time. Third, a restriction enzyme *Hae*III was used to cleave the 5′-GGCC-3′ sequence in the DNA template; cleavage should only occur if the DNA forms a duplex rather than a cruciform. DNA7, DNA5, and DNA3 have a 5′-GGCC-3′ sequence in the loop of hairpin region. The cleavage reaction with *Hae*III was performed for 30 min by the addition of 10 units of *Hae*III to the transcription solutions. Analysis by denaturing PAGE (Fig. S4, ESI†) revealed a band of around 50 nt in DNA7, DNA5 and DNA3 samples corresponding to the expected fragment from the 5′-end. This result indicated that these DNA templates form a duplex structure in the hairpin sequence regions. DNA2 does not have a *Hae*III cleavage site so was not evaluated. Taken together, CD, native PAGE and the cleavage assay indicate that each of the DNA templates forms duplex structures, rather than cruciform or hairpins. In other words, DNA template structure should not impact the transcriptional efficiencies measured in this study.

In the case of nascent RNAs, the local structural formation in the whole-length RNAs has a potential to impact the transcriptional process. To evaluate the structures of the RNA transcripts made from each template, we analyzed the short RNA strands (hpRNA7, hpRNA5, hpRNA3 and hpRNA2) shown in Scheme 1. CD spectra showed a positive peak at 260 nm and negative peaks at 240 nm and 210 nm in a buffer containing 40 mM Tris–HCl (pH 8.0) and 100 mM NaCl at 37 °C, indicating that the RNA strands formed hairpin structures (Fig. 1A). The peak intensities decreased in the following order: hpRNA7 > hpRNA5 > hpRNA3 > hpRNA2; this supports the hypothesis that the number of base pairs decreased as expected from our design. In addition, UV melting experiments at 260 nm in a buffer containing 40 mM Tris–HCl (pH 8.0) and 0.1 mM NaCl indicated that the melting temperature of the hairpin structures decreased in the same order: hpRNA7 > hpRNA5 > hpRNA3 > hpRNA2 (Fig. 1B). The thermodynamic stability of hpRNA7, hpRNA5, hpRNA3 and

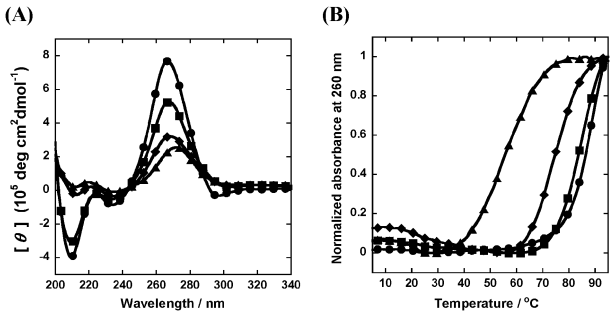


Fig. 1 (A) CD spectra of 10 μM hpRNA7 (circles), hpRNA5 (squares), hpRNA3 (diamonds), and hpRNA2 (triangles) at 37 °C in a solution containing 40 mM Tris–HCl (pH 8.0), 100 mM NaCl and 10 mM $MgCl_2$. (B) Normalized UV melting curves of 10 μM hpRNA7 (circles), hpRNA5 (squares), hpRNA3 (diamonds) and hpRNA2 (triangles) at 260 nm in a solution containing 40 mM Tris–HCl (pH 8.0) and 0.1 mM NaCl.

hpRNA2 structures was estimated to be -9.1 ± 0.4, -7.2 ± 0.3, -4.6 ± 0.1 and -1.6 ± 0.1 kcal mol^{-1}, respectively. The results suggested that the RNA hairpins formed in transcription reactions will have a range of stabilities.

The transcriptional efficiencies were inferred from analysis of samples of RNA transcription reactions taken at 240 min on 7 M urea PAGE. All of the RNA transcripts were of the same length as indicated by similar migration on PAGE (Fig. 2A); thus no shorter RNA fragments were produced by the arrest of T7 RNAP. Interestingly, the amount of the RNA transcripts increased significantly in the following order: DNA2 > DNA3 > DNA5 > DNA7 (Fig. 2B). We examined the correlation between the transcriptional efficiencies from DNA templates and the thermodynamic stabilities ($-\Delta G^{\circ}_{37}$ at 0.1 mM NaCl) of the hairpin structures. The plot showed that the transcriptional efficiencies decreased as the predicted stabilities of RNA hairpins increased (Fig. 3).

Our results suggest that nascent RNA structure affects elongation efficiency. The differences in yields from DNA templates used in this study are due to the stability differences in the hairpin structures formed by the nascent RNAs. Previous studies reported that a hairpin structure in an RNA transcript causes the RNA polymerase to pause.[6b,7b,13] The paused state can affect the total transcription rate, resulting in a decrement in yield of RNA products.[14] Nudler, and Datta and von Hippel proposed that the aberrant paused state was induced by the disruption of the DNA scaffold triggered by the hairpin formation in the nascent RNA.[6b,13] This may destabilize the elongation complex. The T7 RNAP binds to nascent RNA mainly through the N-terminal domain and a specificity loop.[15] These domains also bind the promoter region of the DNA template.[16] Therefore, the destabilization induced by a disruption

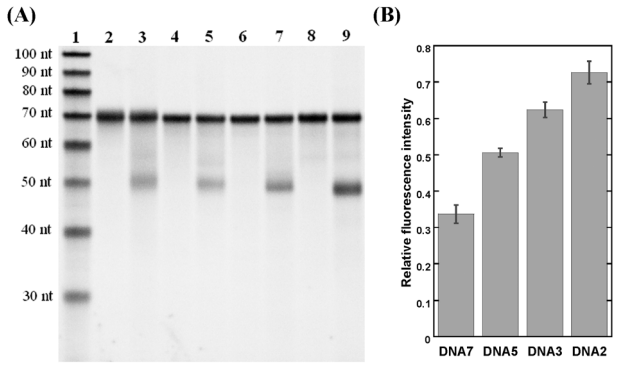


Fig. 2 (A) Denaturing 12% PAGE containing 7 M urea analysis of transcription reactions of DNA7 (lanes 2 and 3), DNA5 (lanes 4 and 5), DNA3 (lanes 6 and 7) and DNA2 (lanes 8 and 9). The samples in the absence of T7 RNAP were loaded in lanes 2, 4, 6 and 8. The samples in the presence of T7 RNAP were loaded in lanes 3, 5, 7 and 9. The 10 nt ladder was loaded in lane 1. The gel was stained with SYBR Gold. (B) The yields of RNA transcripts. The error bars were obtained from at least four measurements. The relative fluorescence intensity is the fluorescence intensity of RNA divided by the fluorescence intensity of DNA.

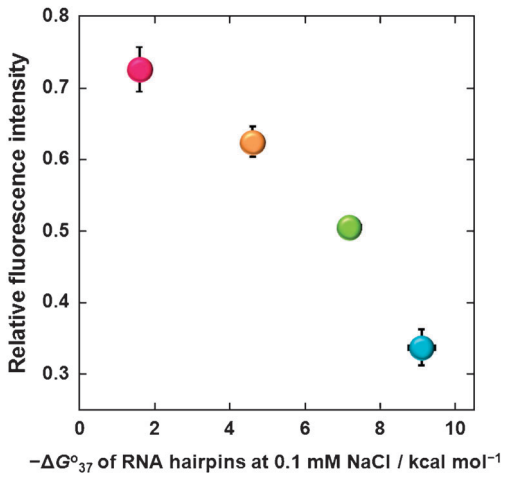


Fig. 3 Plot of transcriptional efficiency (the relative fluorescence intensity of the product) *versus* the thermodynamic stabilities of RNA hairpins for DNA2 (red), DNA3 (orange), DNA5 (green) and DNA7 (blue). The error bars were obtained from four measurements.

of the interaction between a nascent RNA and a RNA polymerase probably causes the inactivation of the transcription cycle. It has been reported that a conformational change in T7 RNAP can affect the re-initiation efficiency.[17] Thus, hairpin structures formed by the nascent RNA likely affect the processivity of T7 RNAP.

A recent paper proposes that coding regions of RNA in yeast genome form more stable secondary structures than do untranslated regions.[18] Structures in the coding regions of an mRNA do affect gene expression levels.[19] Although there are reports of regulation of transcriptional efficiency during the elongation phase,[3] so far there has been no demonstration of a causal relationship between the gene regulation and the transcriptional efficiency. The present work provides evidence of a general correlation between transcriptional yield and stability of transcript structure and suggests that transcription reaction efficiencies may be controlled by design of transcript structures for *in vitro* applications.[8b,20]

In conclusion, the present study demonstrated that the transcriptional efficiencies of T7 RNAP decreased as the stem length of RNA hairpins in the transcripts increased. The results suggest that the transcriptional efficiencies of the RNA polymerase are regulated by the structural stability of nascent RNAs. Our finding indicates that the structural stability of the nascent RNA encodes a previously unexplored level of information used for regulation of transcription.

This work was supported in part by Grants in-Aid for Scientific Research and the "Core research" project (2009–2014) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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