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Tricia M. Coleman
University of Southern Mississippi

Guocan Wang
University of Southern Mississippi

Faqing Huang
University of Southern Mississippi, Faqing.Huang@usm.edu

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Superior 5' homogeneity of RNA from ATP-initiated transcription under the T7 ϕ 2.5 promoter

Tricia M. Coleman, Guocan Wang and Faqing Huang*

Department of Chemistry and Biochemistry, University of Southern Mississippi, Hattiesburg, MS 39406-5043, USA

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ABSTRACT

Transcription from the commonly used GTP-initiating T7 class III promoter ϕ 6.5 frequently produces heterogeneous RNA at both 3' and 5' ends. We demonstrate here that RNA transcripts from the T7 class II promoter ϕ 2.5 have superior 5' homogeneity over those from the ϕ 6.5 promoter, with comparable total RNA yields. The overall homogeneity of RNA transcripts is improved to different degrees depending on RNA sequences, although transcription under ϕ 2.5 does not affect the 3' heterogeneity of RNA. In combination with 3' RNA trimming by DNazymes or ribozymes, this ATP-initiated transcription system based on the T7 ϕ 2.5 promoter can provide excellent quality of RNA for applications requiring a high degree of RNA size homogeneity.

INTRODUCTION

In vitro transcription by T7 RNA polymerase (1) has become a standard procedure for the preparation of RNA in a variety of applications. The commonly used *in vitro* transcription system is derived from the T7 consensus promoter (same as the class III ϕ 6.5 promoter) (2). Transcription under the promoter is initiated by GTP (1,2), and usually requires two or more consecutive guanosines (2) at the first few nucleotide positions of RNA for efficient transcription. Repeated abortive initiation may result in substantial concentrations of oligomers of guanosine. Subsequently, initiation by both GTP and its oligomers may produce significant 5' heterogeneity of RNA (3–5). While standard RNA purification methods such as gel electrophoresis can be used to fractionate different sizes of RNA, it is difficult to remove 5' RNA heterogeneity (4). We have recently developed an adenosine-initiating transcription system (6–8) based on the T7 class II promoter ϕ 2.5 (2). In the present work, we compare the two T7 promoters and demonstrate that the T7 class II promoter produces superior 5' homogeneity of RNA over the most commonly used T7 class III promoter. Such high quality RNA provided by *in vitro* transcription under the T7 ϕ 2.5 promoter may be ideal for

applications such as structural investigation by NMR (9–15) and X-ray crystallography (16–18).

MATERIALS AND METHODS

RNA preparation

Three DNA sequences were prepared using either the T7 class III promoter (ϕ 6.5) or the class II promoter (ϕ 2.5) for a total of six separate DNA template molecules (Fig. 1 shows the top strand of each). Duplex DNA was prepared for each sequence by PCR amplification of two separate synthetic DNA oligomers (IDT). All DNA oligomers were purified by 8% denaturing PAGE prior to use. To ensure that the concentration for each template was the same, the concentration of each oligo was determined by UV and the same PCR mixture (excluding sequence-specific DNA oligomers) was used to prepare all dsDNA templates.

Transcription was carried out under the following standard conditions: 1 mM each of ATP, UTP, GTP and CTP (Roche), 40 mM Tris (pH 8.0), 6 mM MgCl₂, 2 mM spermidine, 0.01% Triton X-100, 5 mM DTT, and 5 U/ μ l T7 RNA polymerase. Double strand DNA concentration was 0.3 μ M for all transcriptions. As an internal label, [α -³²P]ATP (NEN) was added to the transcription mixture. All transcription reactions were made from the same stock solution of the above components to ensure identical conditions, and were allowed to proceed for 4 h at 37°C before product analysis by 8% low resolution denaturing PAGE. Transcription yields were determined by quantitation of the RNA bands by phosphorimaging followed by analysis by Molecular Analyst software (Bio-Rad).

DNazyme trimming of 3' end of RNA transcripts

For both the CA2 and CA0 sequences, a DNazyme with the sequence TTGCATGCCAGGCTAGCTACAACGAGCAA-GCGCTC was used to specifically cleave off a portion of the 3' heterogeneous ends of the RNA transcripts, yielding a 3' homogenous 34-nt RNA for the N band. Any 5' heterogeneity would therefore appear as 35- and 36-nt bands for the 5' N+1 and N+2 RNA transcripts. Similarly, the 35n RNA sequences were treated with a separate DNazyme (TGATCGGCT-AGGCTAGCTACAACGAGGCTGGCCGC) (7) to produce a 3' homogenous 25-nt RNA for the N band. For DNazyme treatment, RNA transcripts (1 μ M) were incubated with 1 μ M

*To whom correspondence should be addressed. Tel: +1 601 266 4371; Fax: +1 601 266 6075; Email: faqing.huang@usm.edu

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

G ϕ 6.5-CA2 CAGTAATACGACTCACTATAGGGCCATAGCTCAGCTGGGAGAGCGCTTGCATGGCATGCAAGAGGTGAGCGGTTCCGATCCCCTTAGGTCCACCA
 A ϕ 2.5-CA2 CAGTAATACGACTCACTATAGGGCCATAGCTCAGCTGGGAGAGCGCTTGCATGGCATGCAAGAGGTGAGCGGTTCCGATCCCCTTAGGTCCACCA

G ϕ 6.5-CA0 CAGTAATACGACTCACTATAGGGGGTATAGCTCAGCTGGGAGAGCGCTTGCATGGCATGCAAGAGGTGAGCGGTTCCGATCCCCTTAGGTCCACCA
 A ϕ 2.5-CA0 CAGTAATACGACTCACTATAGGGGTATAGCTCAGCTGGGAGAGCGCTTGCATGGCATGCAAGAGGTGAGCGGTTCCGATCCCCTTAGGTCCACCA

G ϕ 6.5-35n CAGTAATACGACTCACTATAGGGGAAGCGGGCATGCGGCCAGCCATAGCCGATCA
 A ϕ 2.5-35n CAGTAATACGACTCACTATAGGGGAAGCGGGCATGCGGCCAGCCATAGCCGATCA

Figure 1. DNA sequences used in transcription. Only the sense strands (top strands) are shown. The T7 promoter sequences are underlined and the initiating nucleotides are in bold. The grouped pair sequences are the same except for the promoter sequences and the initiating nucleotides. CA0 is *Escherichia coli* tRNA^{Ala}, and CA2 is the GC4-69 mutant of CA0 described previously (4). 35n is a RNA sequence used previously in our work (7,8).

DNAzymes in 50 mM MgCl₂ and 50 mM Tris (pH 8.0) for 1 h at 37°C, ethanol-precipitated and analyzed by 8% single nucleotide resolution denaturing PAGE.

RESULTS AND DISCUSSION

In vitro transcription usually produces heterogeneous sizes of RNA, mainly due to the non-templated addition of nucleotides at the 3' end (7,8). In addition, RNA transcripts may also possess significant 5' heterogeneity (3–5). Based on the mechanism of 5' RNA heterogeneity generation by repeated initiation and abortive initiation, we reasoned that the adenosine-initiating T7 ϕ 2.5 promoter (with the RNA sequence of 5' AG...) (2,6–8) would produce a high degree of 5' RNA homogeneity, in contrast to RNA transcripts prepared under the guanosine-initiating T7 ϕ 6.5 promoter. In addition, our previous experiments have suggested that transcription under either the T7 ϕ 2.5 promoter or ϕ 6.5 promoter yields a similar amount of total RNA transcripts (6–8). Experiments below compare both the total RNA yields and 5' RNA heterogeneity of different RNA sequences under the two T7 promoters.

The sense strand (top strand) DNA sequences (including the T7 promoter sequences and RNA-coding sequences) are listed in Figure 1. The RNA sequences labeled as CA2 and CA0 are identical to those described previously (4) for a direct comparison. The two sequences differ by a single nucleotide (G or C) at the fourth nucleotide position of RNA. In addition, a 35-nucleotide RNA sequence (35n), which was used in our previous work (7,8), was included to assess sequence variation effects. For all three RNA sequences, both the T7 ϕ 6.5 promoter and the ϕ 2.5 promoter were used to initiate transcriptions with GTP or ATP, respectively.

Total RNA yields of the six individual sequences (Fig. 1) under identical transcription conditions are shown in Figure 2 by a low resolution PAGE analysis (15 × 13 cm gel). To ensure equal concentrations of DNA templates, synthetic oligodeoxyribonucleotide sequences were gel-purified and quantitated by UV spectroscopy. The resulting DNA oligomers were then used for full-length double stranded DNA (dsDNA) template construction by PCR (one cycle). Relative dsDNA concentrations of each pair were checked by non-denaturing PAGE before use in transcription. For the three RNA sequences (CA2, CA0 and 35n), the average of four independent experiments showed that the ϕ 2.5 promoter had an efficiency of 94 ± 19% that of the ϕ 6.5 promoter. Considering the variations of the experiments, we conclude



Figure 2. Comparison of total transcription yields of RNA for the six sequences listed in Figure 1. For each RNA sequence, four independent experiments indicate that guanosine- and adenosine-initiated transcriptions lead to similar total RNA yields. The 8% denaturing gel was run at a low resolution (15 × 13 cm). Therefore, each transcription produced a single RNA band that contained heterogeneous RNA transcripts of similar sizes.

that the two T7 promoters have similar transcription initiation efficiencies, consistent with our previous results (6,7).

To examine the 5' heterogeneity of RNA transcripts from the two different promoters, the commonly occurring 3' RNA heterogeneity must first be eliminated. Otherwise, RNA size fractionation by PAGE cannot resolve the 3' and 5' RNA heterogeneities. RNA 3' end trimming can be easily achieved through site-specific cleavage by the DNAzyme 10-23 (19), which was used in our earlier work (7). As shown in Figure 3A, there are nine different RNA species from transcription (assuming N, N+1 and N+2 at both 3' and 5' ends), with RNA sizes ranging from N to N+4. A DNAzyme can be designed to cleave the RNA at the site indicated by the arrow. After the DNAzyme treatment, all 3' RNA heterogeneities are removed, reducing the original nine different RNA species to three 5' heterogeneous sizes: 5' N, 5' N+1 and 5' N+2, which can then be easily resolved by high resolution PAGE (single-nucleotide resolution).

Figure 3B shows high-resolution gels (42 × 34 cm) before and after DNAzyme cleavage. Before the DNAzyme treatment, differences between the two promoters look small but clearly visible (Fig. 3B, top panel) (comparing lane 1 with 2, 3 with 4 and 5 with 6). It can be seen that the T7 ϕ 6.5 promoter generates more RNA bands than the ϕ 2.5 promoter does. Transcription under the ϕ 2.5 promoter reduces the overall heterogeneity by 17, 30 and 10%, respectively, for the CA2, CA0 and 35nt RNA sequences relative to their corresponding G-initiated transcripts. The differences between the two promoters become more apparent after a small portion of the 3' end was removed by a DNAzyme (Fig. 3B bottom panel). Comparing the two gels, it is evident that the ϕ 2.5 promoter produces little 5' heterogeneous RNA and the 3' RNA heterogeneity contributes to the overall RNA size heterogeneity under the ϕ 2.5 promoter. The degree of 3' RNA

heterogeneity depends on RNA sequences (7,8). On the other hand, the gels reveal that the $\phi 6.5$ promoter generates significant amount of both 3' and 5' heterogeneous RNA transcripts. Therefore, the combination of the two different heterogeneities at both ends can result in a high degree of overall RNA size heterogeneity.

Radioactivity profiles of the gel (bottom panel in Fig. 3B) can better illustrate different 5' heterogeneities produced by the two promoters (Fig. 3C). As demonstrated previously (4), the degree of 5' RNA heterogeneity under the GTP-initiating $\phi 6.5$ promoter is sequence-dependent. For CA2 and 35n RNA transcription under the $\phi 6.5$ promoter, 80–86% of total RNA transcripts are N band RNA, while 12–15% RNA has an additional 5' G (5' N+1 band) and 3–5% total RNA transcripts carry additional 5' GG (5' N+2 band). The degree of 5' RNA

heterogeneity of CA0 transcripts is significantly higher than those of CA2 and 35n under identical transcription conditions, with the 5' N+1 and 5' N+2 bands reaching 29 and 8%, respectively. These experiments not only confirm the previous findings on 5' heterogeneous RNA transcription under the GTP-initiating $\phi 6.5$ promoter, but also agree quantitatively with those results (4).

Combined with our previous experiments (6–8), the present finding has demonstrated distinct advantages of the class II $\phi 2.5$ promoter (A-initiation) over the commonly used class III $\phi 6.5$ promoter (G-initiation). First, transcription under the $\phi 2.5$ promoter produces 5' homogeneous RNA transcripts, while the $\phi 6.5$ promoter can lead to a significant degree of 5' RNA heterogeneity (4,5). Although both systems may add non-templated nucleotides at the 3' end of RNA depending on RNA sequences, such 3' RNA heterogeneity can be easily eliminated by either DNAzymes (7,19) or ribozymes (20–23). The 3' RNA heterogeneity can also be dramatically reduced by the introduction of C2' methoxy groups ($-\text{OCH}_3$) on the last two nucleotides of DNA templates (24,25). As a result, highly homogeneous RNA sizes can be prepared by *in vitro* transcription under the $\phi 2.5$ promoter, followed by 3' end RNA trimming. On the other hand, it is difficult to eliminate the 5' RNA heterogeneity. Such heterogeneity can lower RNA functional activities (4) and may cause problems in RNA structure, folding and mechanism investigation such as by NMR (9–15) and X-ray crystallography (16–18), where high purity of RNA is essential. Second, numerous adenosine analogs can be incorporated onto the 5' ends of RNA transcripts. Adenosine itself may be used to prepare adenosine-initiated RNA with free 5' hydroxyl groups, allowing easy ^{32}P -labeling of the 5' end by polynucleotide kinase. AMP can also be used for transcription initiation, and the resulting RNA transcripts may be directly used in RNA ligation by T4 RNA ligase. Further, two kinds of extensive modifications (at the either 5' or N6 sites of adenosine) can be introduced without abolishing transcription initiation. A variety of functional groups including coenzymes, fluorophores and biotin can be used to label RNA at the specific 5' end (7,8,26), providing functionalized RNA for biophysical,

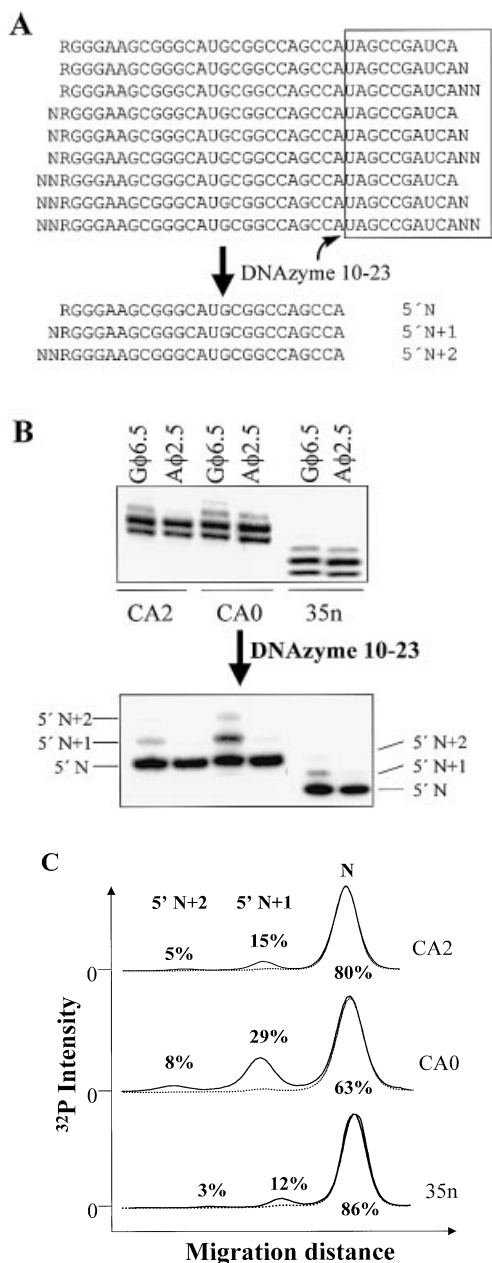


Figure 3. RNA size heterogeneity analysis by 8% high resolution PAGE (42×34 cm) and phosphorimaging of different RNA sequences prepared from the T7 $\phi 2.5$ and $\phi 6.5$ promoters. (A) Combination of RNA heterogeneities at both 3' and 5' ends and elimination of the 3' heterogeneity by site-specific DNAzyme cleavage, which reduces the nine differently-sized RNA species to three 5' heterogeneous RNA bands. (B) Resolution of RNA bands by single nucleotide resolution PAGE before and after DNAzyme treatment. The top panel shows the original RNA size heterogeneities (both 3' and 5' ends) directly after transcription. The bottom panel indicates 5' RNA heterogeneity of different sequences. It is noticeable that the $\phi 2.5$ promoter produces less heterogeneity even before the DNAzyme cleavage. DNAzyme trimming greatly improves the detection of 5' RNA heterogeneity and reveals the superiority of the $\phi 2.5$ promoter over the $\phi 6.5$ promoter. (C) Radioactivity profiles of different sequences. The major RNA bands at the right side of the figures are N band RNAs, while the two smaller peaks to the left are associated with the 5' N+1 and 5' N+2 bands, respectively. The solid line in each figure represents the result from the $\phi 6.5$ promoter with GTP-initiated transcription, and the dotted line shows the RNA from the $\phi 2.5$ promoter with ATP-initiated transcription. The percentages of radioactivity for different RNA bands prepared under the $\phi 6.5$ promoters are indicated on the figure. For all ATP-initiated transcriptions (dotted lines), the N band RNA is $94 \pm 2\%$.

biochemical and biomedical applications. Third, an option for the 5' end nucleotide (either A or G) of RNA is provided with the choice of T7 ϕ 2.5 and ϕ 6.5 promoters. In addition, the two transcription systems may use the same conditions, and give similar total RNA yields. Based on the above discussion, we recommend the use of the T7 ϕ 2.5 promoter over the commonly used ϕ 6.5 promoter for RNA transcription, if experiments are compatible with the 5' end switch from a guanosine to an adenosine.

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