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Quantifying the influence of 5′-RNA modifications on RNA polymerase I activity

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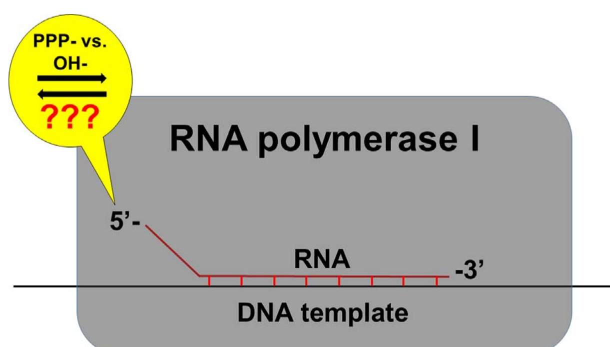
HIGHLIGHTS

- Most synthetic RNA oligonucleotides carry a 5′-hydroxyl.
- De novo synthesis of RNA by RNA polymerase results in a 5′-triphosphate.
- These data demonstrate that 5′-moieties do not affect nucleotide addition kinetics.
- The 5′-end of the RNA substrate may affect polymerase backtracking or abortive initiation.

GRAPHICAL ABSTRACT



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ABSTRACT

For ensemble and single-molecule analyses of transcription, the use of synthetic transcription elongation complexes has been a versatile and powerful tool. However, structural analyses demonstrate that short RNA substrates, often employed in these assays, would occupy space within the RNA polymerase. Most commercial RNA oligonucleotides do not carry a 5′-triphosphate as would be present on a natural, de novo synthesized RNA. To examine the effects of 5′-moities on transcription kinetics, we measured nucleotide addition and 3′-dinucleotide cleavage by eukaryotic RNA polymerase I using 5′-hydroxyl and 5′-triphosphate RNA substrates. We found that 5′ modifications had no discernable effect on the kinetics of nucleotide addition; however, we observed clear, but modest, effects on the rate of backtracking and/or dinucleotide cleavage. These data suggest that the 5′-end may influence RNA polymerase translocation, consistent with previous prokaryotic studies, and these findings may have implications on kinetic barriers that confront RNA polymerases during the transition from initiation to elongation.

1. Introduction

Transcription of DNA sequence into RNA is the first step in gene expression and has been characterized as a key target for regulation. As with any polymerization reaction, transcription necessarily includes: 1) an initiation step, where the RNA polymerase binds and locally unwinds the DNA and synthesizes a short RNA de novo; 2) an elongation phase, when the RNA polymerase leaves the promoter and extends the nascent RNA, synthesizing a new molecule complementary to the template DNA strand; 3) and a termination step, when the RNA

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molecule is released from the polymerase and the enzyme dissociates from the DNA template. The efficiency of each step in this process can be manipulated and is therefore a potential target for regulation of gene expression [example reviews, see [1–3]].

Our lab and several others have shown that transcription elongation efficiency can be influenced by several factors including template sequence or trans-acting transcription factors [4–7]. It is also clear in both prokaryotic and eukaryotic systems that alterations in transcription elongation efficiency can influence processing of the nascent RNAs [8–10]. As a consequence, there is a need to develop quantitative methods for analyzing and characterizing transcription elongation kinetics under various experimental conditions.

One of the most versatile experimental strategies for characterizing transcription elongation kinetics is assembly of transcription elongation complexes from synthetic oligonucleotide substrates and purified RNA polymerase [11–13]. These synthetic transcription elongation complexes are generally assembled by hybridizing an RNA oligonucleotide to a template DNA oligonucleotide. This RNA:DNA hybrid is then incubated with purified RNA polymerase. Finally, the non-template DNA oligonucleotide is annealed to the complex. There are many variations on this method, including altered substrate length, complementarity, and base composition. Given the simplicity of the reagents and the generality of application to various RNA polymerases, this method is versatile.

It has been shown that eukaryotic ribosome biosynthesis is at least partially orchestrated by the elongation rate of RNA polymerase I (Pol I) [14]. In order to characterize the kinetic mechanism of nucleotide addition by Pol I, we established methods for assembling synthetic transcription elongation complexes using Pol I purified from the model eukaryote *Saccharomyces cerevisiae* (brewer's yeast) [15]. As part of our experimental strategy, we use an RNA oligonucleotide that is nine nucleotides long; this RNA is extended by one nucleotide during radioactive labelling of the RNA, prior to kinetic analysis of nucleotide addition. Using this approach, we observed nucleotide addition as well as robust hydrolysis of a dinucleotide from the 3′-end of the nascent RNA by Pol I. Based on these data sets, we proposed a kinetic mechanism for Pol I nucleotide addition and hydrolysis.

In our work and that of several others, a short RNA:DNA hybrid serves as the substrate for transcription. However, structural data demonstrate that the entire length of this nascent RNA will reside within the polymerase during the reaction [16]. Furthermore, commercial oligonucleotides typically carry a hydroxyl group on the 5′-position. This is a substrate configuration that would almost never be presented to an RNA polymerase in vivo, since the enzymes synthesize RNA de novo, using nucleoside 5′-triphosphate substrates. Recent work from the Murakami lab asked whether the triphosphate group on the 5′-end of the RNA primer can have an effect, and they observed several structural consequences [17].

Here we asked whether 5′-hydroxyl versus 5′-triphosphate RNA substrates influenced the kinetics of nucleotide addition or RNA hydrolysis under our reaction conditions. We found that although there were qualitative effects on complex assembly in vitro, the rate of nucleotide addition was not significantly impacted by the 5′-modification of the RNA. Interestingly, we did observe a ~2-fold effect of the 5′-end of the RNA on hydrolysis activity by Pol I. Our findings are consistent, at least in part, with predictions from the structural analyses by the Murakami lab that used prokaryotic RNAP [17].

2. Materials and methods

2.1. Buffers

All buffers except those used for electrophoresis were filtered through 0.22 μm Millipore express plus vacuum-driven filters (EMD Millipore, Billerica, MD) unless specified otherwise.

Nucleotide incorporation reactions were run in reaction buffer: (40 mM KCl, 20 mM Tris-Acetate (OAc) pH 7.9 at 25 °C, 2 mM dithiothreitol, 0.2 $mg \cdot ml^{-1}$ bovine serum albumin (BSA)); prepared from concentrated stocks immediately prior to each experiment.

2.2. Proteins

Pol I was purified from *Saccharomyces cerevisiae* as described previously [18]. For ease of purification, the cells expressed a FLAG-his_6-tagged version of A190 as the sole source of that subunit. Pol I is stored in 0.55 M K-OAc, 10 mM K-HEPES, 0.5 mM $MgCl_2$, 45% (v/v) glycerol pH 7.8; at − 20 °C.

2.3. Nucleotides, nucleic acids, heparin, BSA

Preparation of nucleotides, nucleic acids, heparin, and BSA was described in detail previously [15]. 5′-triphosphorylated RNA was purchased from TriLink Bio Technologies (San Diego, CA). All other nucleic acids were purchased from Integrated DNA Technologies (Cedar Rapids, IA).

2.4. Quenched flow time courses

Quenched flow time courses of single nucleotide incorporation reactions and electrophoresis and quantification of gels were performed as described previously [15].

2.5. Data analysis

Time course data were normalized in the following manner. Background phosphor counts from a region immediately above the 11-mer band were subtracted from 11-mer signal. This background subtracted 11-mer signal was normalized by dividing by the maximum background subtracted 11-mer signal in each time course. Time courses at each [ATP] were collected in duplicate and the normalized data were averaged. Error on each measurement was calculated as the standard deviation of these two measurements. In the 10 μM ATP data set the maximum value of each time course fell on the same time point, generating an undefined standard deviation. In weighted fitting, the weight applied to this point was the average standard deviation of all time points at all [ATP]. All curve fitting was accomplished using custom written Matlab scripts based on the Matlab function Lsqnonlin. Grid searching was accomplished as described previously [15]. The 68% confidence intervals displayed in Fig. 1C were calculated as described previously [23].

3. Results and discussion

We recently reported a transient-state kinetic assay to monitor Pol I–catalyzed single nucleotide extension of a 10-mer RNA [15]. To evaluate the effects of the RNA 5′-end on Pol I–catalyzed nucleoside monophosphate (NMP) incorporation, we monitored single NMP extension reactions of 5′-OH and 5′-PPP RNA oligonucleotides.

Figs. 1A and B display time courses of single NMP incorporation reactions catalyzed by Pol I complexes assembled using 5′-OH and 5-PPP RNA oligonucleotides, respectively. Each time course was collected at seven [ATP] and in each panel normalized 11-mer RNA phosphor counts are plotted (see materials and methods) as a function of time (circles). As described previously, 11-mer time courses are biphasic with amplitudes of opposite sign. The fast rising phase corresponds to Pol I–catalyzed NMP incorporation and the slower decay phase corresponds to Pol I–catalyzed phosphodiester bond hydrolysis [15]. Consistent with our previous work, we observe that Pol I's nuclease activity liberates a dinucleotide fragment from the 3′-end of both 5′-OH and 5′-PPP RNA. Finally, the time courses in Figs. 1A and B appear to respond similarly to [ATP]. The rising phases speed up (traces shift left on the time axis) as [ATP] is increased and the decay phases exhibit no [ATP]

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