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

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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'-moieties 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.

Graphical Abstract

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Keywords

Transcription; Ribosomal RNA; RNA polymerase I; Transcription elongation; Gene expression

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 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].

Materials and Methods

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 $\text{mg}\cdot\text{ml}^{-1}$ bovine serum albumin (BSA)); prepared from concentrated stocks immediately prior to each experiment.

Proteins

Pol I was purified from *Saccharomyces cerevisiae* expressing as described previously [18]. For ease of purification, the cells expressed a FLAG-his₆-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.

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).

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].

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 μ M 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].

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.

Figures 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 is plotted normalized 11-mer RNA phosphor counts (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 figures 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] dependence (decay phases overlay across the [ATP] range).

The time courses in figures 1A and B are virtually identical in their shape indicating that if present, the difference(s) between Pol I-catalyzed nucleotide incorporation onto a 5'-OH or 5'-PPP RNA are subtle. We have reported that the observed rate constant governing the rising phase of Pol I nucleotide incorporation time courses displays a hyperbolic dependence on [ATP]. Further, we recently presented a strategy that enables quantitative definition of this hyperbolic rate dependence by a direct global fit of nucleotide incorporation time courses collected as a function of [ATP]. We have applied this approach here using EQs.1 and 2.

$$[11-mer](t) = \alpha \left(e^{-k_{decay}t} - e^{-k_{rise}([ATP])t} \right) + C \quad 1$$

$$k_{rise}([ATP]) = \frac{k_{max} [ATP]}{K_{1/2} + [ATP]} \quad 2$$

In EQ. 1 k_{decay} is a single global parameter that describes the decay kinetics of all the time courses in a given data set (i.e., 5'-OH or 5'-PPP) and α and C are local amplitude parameter and a global scaling factors, respectively. EQ.2 defines the [ATP]-dependence of k_{rise} in EQ.1 by two parameters: k_{max} which defines the k_{rise} value at saturating [ATP], and $K_{1/2}$ which defines the [ATP] at which $k_{rise} = k_{max}/2$. The solid lines in figures 1A and 1B display the results of the global fit to EQs. 1 and 2. For visualization, Figure 1C displays evaluations of EQ. 2 at the best fit k_{max} and $K_{1/2}$ parameter values obtained from the 5'-OH and 5'-PPP data sets (blue and red solid lines respectively). In Figure 1C, the broken blue and red lines represent the 68% confidence interval of the 5'-OH and 5'-PPP fit, respectively. Figure 1D displays the k_{decay} values obtained from the global fitting analysis. Finally, the parameter values obtained from the global fit are collected in table 1.

To obtain confidence intervals on the fitted parameter values, grid searching was performed as described [15] and the results from the grid search are displayed in figures 2A, B, and C for the parameters k_{max} , $K_{1/2}$, and k_{decay} , respectively (circles). In addition, the 68% confidence interval of the fit is plotted as a broken line in figure 2. Consistent with the analyses presented in figure 1C, although the best fit values for k_{max} and $K_{1/2}$ are different with the two RNA substrates, the confidence intervals from the fit display strong overlap indicating no statistical difference in these two kinetic parameters. In contrast, the k_{decay} values obtained from the 5'-OH and 5'-PPP data set are different beyond the 68% confidence interval of the fit. Taken together, the data in figure 2 indicate that although there is little if any effect of the 5'-moiety of the nascent RNA on k_{max} and $K_{1/2}$, there is a measureable effect on k_{decay} . Importantly, the effect on k_{decay} demonstrates that the 5'-end of the nascent RNA influences hydrolysis of the nascent RNA. This finding has potential implications on several steps in transcription, including the transition from initiation to elongation.

Conclusions

Structural studies with prokaryotic and eukaryotic RNA polymerases have demonstrated that at least 10 bases of nascent RNA are in direct contact with the RNA polymerase [16, 17, 19]. Since most synthetic RNA:DNA templates employ RNA substrates with unnatural 5'-hydroxyl moieties, it stands to reason that these modifications may influence enzyme function. We tested whether 5'-hydroxyl versus 5'-triphosphate RNA substrates influenced Pol I function and found no significant effect on nucleotide addition; however, cleavage of a dinucleotide from the 3' end was more rapid with the 5-triphosphate. The Murakami group solved a series of high resolution crystal structures of the prokaryotic *Thermus thermophilus* RNA polymerase with de novo synthesized RNA primers, which carry a 5'-triphosphate. Interestingly, they found that these complexes, unlike previously solved structures with 5'-hydroxyl substrates, favored a pre-translocated state, not a state competent for nucleotide incorporation [17]. If the negative charge of the phosphate groups somehow favors a

backtracked conformation, this would be consistent with our observed increased rate of dinucleotide cleavage with the triphosphate substrate.

During the transition from transcription initiation to elongation, all RNA polymerases will encounter short RNA hybrids with 5'-triphosphates in the active center. It is well documented that the transition to processive elongation is not always efficient and can result in abortive initiation [ie. release of short RNA products and re-initiation of de novo synthesis; [20, 21]]. Although Pol I abortive product formation has not been characterized extensively, it is clear that the transition from initiation to elongation by Pol I must be efficient in vivo, based on the observed dense packing of polymerases on the ribosomal DNA [22]. Since abortive transcription presents a barrier to promoter escape and the 5'-end of the nascent RNA appears to directly influence the polymerase's backtracking and hydrolytic activity, there is a need to further investigate the interaction between the newly synthesized RNA 5'-end and the polymerase.

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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.

Figure 1. Kinetics of Pol I-catalyzed extension of 5'-OH and 5'-PPP RNA

A) Time courses of Pol I-catalyzed single nucleotide extension of 5'-OH RNA collected at 7 substrate [ATP] (circles). Solid lines represent global fits to EQs. 1 and 2. B) Time courses of Pol I-catalyzed single nucleotide extension of 5'-PPP RNA collected at 7 substrate [ATP] (circles). Solid lines represent global fits to EQs. 1 and 2. C) Evaluations of EQ. 2 at the best fit parameter values obtained from the global fits in panels A and B. Solid red lines correspond to the parameter values obtained from the fit of the 5'-PPP data. Solid blue lines correspond to the parameter values obtained from the fit of the 5'-OH data. Broken lines of the corresponding color represent the 68% confidence interval from the global fits displayed in panels A and B. D) Observed rate constants of the decay phases (k_{decay}) obtained from the global fits displayed in panels A and B.

Figure 2. Grid search evaluation of global fitting

A) F-statistics plotted as a function of k_{max} values from the global fits displayed in figures 1A and 1B (blue and red circles respectively). Broken line represents the f-critical value at the 68 % confidence interval. B) F-statistics plotted as a function of $K_{1/2}$ values from the global fits displayed in figures 1A and 1B (blue and red circles respectively). Broken line represents the f-critical value at the 68 % confidence interval. C) F-statistics plotted as a function of k_{decay} values from the global fits displayed in figures 1A and 1B (blue and red circles respectively). Broken line represents the f-critical value at the 68 % confidence interval.

Table 1

Parameter values and associated 68% confidence intervals

RNA	k_{max} (s ⁻¹)	$K_{1/2}$ (μM)	k_{decay} (s ⁻¹)
5'-OH	102 [73,147]	75 [40,140]	0.25 [0.19,0.33]
5'-PPP	113 [92,144]	56 [39,80]	0.47 [0.41,0.55]