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Twisted or shifted? Fluorescence measurements of late intermediates in transcription initiation by T7 RNA polymerase

Rosemary S. Turingan^{‡,£}, Karsten Theis[§], and Craig T. Martin^{*,‡,§}

[‡]Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003

[§]Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, Massachusetts 01003

Abstract

T7 RNA polymerase undergoes dramatic structural rearrangements in transitioning from initiation to elongation. Two models have been proposed for promoter-bound intermediates late in the transition: i) a subset of promoter interactions are maintained through completion of the protein conformational (twist) change and ii) concerted movement (shift) of all promoter-binding elements away from the growing DNA/RNA hybrid leads to an open intermediate, with large-scale domain rotations deferred until after promoter release. Fluorescence resonance energy transfer measurements provide very strong support for the latter.

Keywords

transcription; FRET; fluorescence; energy transfer; DNA

Initiation of transcription is an inherently complex process, requiring the conversion of the enzyme from a sequence-specific binding protein capable of unprimed synthesis of short transcripts, to a non-sequence specific complex efficiently extending RNA from a stable primer and directing the exit of the nascent RNA from the complex.

There are at least two well-characterized classes of DNA dependent RNA polymerases that appear to be evolutionarily unrelated. The multi-subunit family of RNA polymerases possesses dissociable specificity factors that allow reprogramming of the complex, while the simpler single subunit RNA polymerases contain promoter recognition and catalysis in a single subunit. Despite the lack of obvious structural relationship between these two classes of DNA-

*To whom correspondence should be addressed. Phone (413) 545-3299. Fax: (413) 545-4490. E-mail: cmartin@chem.umass.edu

£Current address: Network Biosystems, Woburn, Massachusetts 01801

¹Abbreviations:

FITC

fluorescein-5-isothiocyanate

FRET

fluorescence resonance energy transfer

SDS

sodium dodecyl sulfate

TAMRA

5-carboxytetramethylrhodamine

dependent RNA polymerases, they share substantial functional similarities, suggesting evolutionary constraints imposed by the transcription process.

To begin transcription, T7 RNA polymerase first recognizes and binds to its relatively short upstream promoter sequence, and upon binding, spontaneously forms a bent and open complex (1,2). This was recently confirmed by FRET (Fluorescence Resonance Energy Transfer) distance measurements between probes conjugated to DNA (2,3). Measurement of multiple distances between upstream and downstream DNA allows confirmation not only of the predicted bending of the DNA, but also of the predicted helical phasing and positioning of downstream DNA. These data have confirmed a structural model for the initiation complex first proposed by aligning downstream DNA from the elongation complex structure onto the initiation complex, where downstream DNA was not resolved (4).

The crystal structure of an initiating complex with a three base transcript clearly demonstrates retention of upstream promoter contacts (5), while biochemical assays indicate retention of promoter contacts on translocation through at least position +8 (6). Consistent with this view, fluorescence from base analogues in the DNA demonstrates that the initially melted 8 base bubble expands to at least 12-13 bases on translocation to position +8 (7). On further translocation, promoter contacts are lost and the bubble collapses back to about 8 base pairs, a size that presumably persists throughout subsequent elongation (8).

Crystal structures mimicking elongation complexes clear of the promoter reveals a very large rearrangement of the amino terminal third of the protein (compare Figures 1A and 1D). Promoter contacts are lost presumably due to the disruption of the binding interface during reorganization, accompanied by initial bubble collapse from the upstream edge and initial displacement of the 5' end of the RNA (4, 6, 9, 10)

As the RNA:DNA hybrid grows from 3 base pairs at initiation to about 8 base pairs in elongation, it is believed to “push” on the N-terminal platform, driving the structural change, while still maintaining promoter contacts (4,9). Two very different models describe how this rearrangement might occur. Noting that the template strand covalently connects the upstream, bound DNA duplex and the RNA:DNA hybrid, it was first proposed that the N-terminal platform executes both a translation and a left-handed rotation in response to growth of the hybrid, culminating in a late initiation complex that resembles the final elongation complex (9).

We have proposed an alternative path to the final elongation complex state (10,11). In this model, the N-terminal platform undergoes a primarily *translational* movement in response to hybrid growth. Only after release of the promoter contacts is the N-terminal platform then free to execute a 220° right-handed rotation to achieve the final elongation state. In the current study, we present FRET measurements that provide strong support for the alternative path.

MATERIALS AND METHODS

T7 RNA polymerase

His-tagged wild type T7 RNA polymerase was prepared from *Escherichia coli* (*E. coli*) strain BL21, carrying the plasmid pBH161 (kindly supplied by William McAllister), as previously described (6). The enzyme was purified and the concentration was determined ($\epsilon_{280} = 1.4 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) as described previously (12). Purity of the enzyme (>95%) was verified by SDS-denaturing polyacrylamide gel electrophoresis.

Oligonucleotides and FRET Experiment

Preparation of DNA constructs and FRET data analysis were carried out as in previous studies (3). In walking to position +8, DNA and enzyme (1:8) were incubated for 1-minute at 37 °C prior to addition of 5 μ L of GTP/ATP mix (0.48 M magnesium acetate, 3.2 mM GTP, 1.6 mM ATP and 0.32 M Hepes, pH 7.8). The final concentrations of GTP, ATP, magnesium acetate and Hepes were $\sim$ 100 μ M, $\sim$ 50 μ M, 15 mM and 10 mM, respectively in the final 160 μ L-reaction mixture. Samples were incubated for 3 minutes before taking fluorescence measurements.

RESULTS AND DISCUSSION

The two different models for late intermediates in transcription initiation make very different predictions regarding the nature of a complex poised at positions stepped away from the promoter, but prior to promoter release. To distinguish these models, we consider the DNA conformation in each. As illustrated in Figure 1B and 1C, for a complex walked to position +8, the predicted relationships of the upstream DNA to the downstream DNA are distinctly different. In the “shift promoter” +8 model, the N-terminal platform together with the upstream DNA has translated away from the C-terminal rigid domain, as proposed by Theis et al (11). In this model, all promoter interactions are maintained, while some interactions between the N-terminal and C-terminal parts of the protein are lost. In the “twist promoter” +8 model, the N-terminal platform has completed both the large rotation and the 20 Å translation, rotating the promoter with it as proposed for late intermediates by Tahirou et al (9). In this model, the protein conformation resembles that of the elongation complex.

In the current study, we use fluorescence resonance energy transfer (FRET) to distinguish these two competing structural models for the transition. As the polymerase continues to transcribe beyond position +3, the downstream DNA should be monotonically drawn in, showing FRET distance changes associated with that movement and with the accompanying rotation of the downstream helix, both easily modeled (3). Movement of the N-terminal platform on which the upstream DNA sits will also affect the distances in predictable ways. Prior to promoter release, a probe at position -5 will either translate a short distance away from the downstream duplex (Figure 1C) or will rotate, yielding a much larger separation (Figure 1B). Similarly a probe at position -17 will either translocate away from the downstream DNA, or rotate with the N-terminal platform *towards* the downstream DNA, offsetting somewhat the translation.

As in our earlier study (3), we have incorporated a dT analogue at the upstream -17 position of the nontemplate strand with a C6 linker terminating in a primary amine and labeled it with an acceptor probe 5-carboxytetramethylrhodamine (TAMRA). Complementary template DNA strands containing dT, dC or dA analogues with similar amino-terminated spacers at select positions +8, +10, +14 and +16 positions were synthesized and conjugated with fluorescein-5-isothiocyanate (FITC) as the donor. All constructs are fully duplex with the same sequence and length (to +17) and incorporation of the fluorophore does not measurably perturb transcription. In order to subtract the FITC contribution to the overall total emission, we also prepared standard duplex DNA constructs that contain the FITC-labeled template DNA hybridized to unlabeled nontemplate strand. Consistent with our previous FRET analysis, model distances are calculated from the point farthest out from the rigid component of the linkage (3).

As shown in Figure 2, for measurements from the upstream -17 position, the models predict different distances to some downstream positions, but similar distances to at least one (+8). The experimental data are somewhat ambiguous in distinguishing the models in that the measured distances to the +10 and +16 probes are consistent with the shift model, while the distance to +14 is more consistent with the twist model.

In order to resolve this ambiguity, a different set of constructs was prepared with TAMRA placed at position -5 (dC analogue) on the nontemplate strand and FITC at positions +6, +7, +8, +12 and +16 on downstream template strand (as shown by data presented in the Appendix, these modifications also do not measurably perturb transcription). In this case, the predictions of the models are very different and the distances derived from the shift model lie closer to the R_0 (45 Å) for this FRET pair. The data presented in Figure 2 show very clearly that the distance from the probe at position -5 to downstream duplex positions is not compatible with extensive rotation of the N-terminal platform. The distances instead support initial translocation with only minimal rotation as the more likely mechanism for the transition. The data are also inconsistent with the predicted shorter distances had the N-terminal platform remained structurally static as the hybrid grows to about 8 nucleotides.

CONCLUSIONS

In summary, these data support a model for the transition in which growth of the RNA:DNA hybrid drives an initial translation of the N-terminal platform away from the C-terminal rigid domain. We propose that the specificity loop remains associated with the platform, establishing the stability required to retain promoter contacts (6,7). As the polymerase translocates forward, the resulting translation of the N-terminal platform away from the C-terminal domain ultimately strains placement of the specificity loop, weakening promoter contacts, allowing release of the promoter DNA, which in turn allows the 220° right-handed rotation of the N-terminal platform previously proposed on the basis of topological considerations (11).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Models for initially transcribing complexes halted at position +8

Also shown are the initiation complex (1QLN), with modeled downstream DNA, and the elongation complex (1MSW). A sample distance from a base at position -5 to a base at position +12 is shown for each.

Figure 2. FRET distance measurements for complexes halted at +8 obtained from an average of 4 measurements (white bars) with standard deviations shown as error bars. Light gray bars show distances for the “twist promoter” model, while dark gray bars show distances for the “shift promoter” model.