Single-Molecule Analysis of RNA Polymerase Transcription

Lu Bai,[1] Thomas J. Santangelo,[2] and Michelle D. Wang[1]

[1]Department of Physics, Laboratory of Atomic and Solid State Physics, Cornell University, Ithaca, New York 14853; email: lb69@cornell.edu; mdw17@cornell.edu

[2]Department of Microbiology, Ohio State University, Columbus, Ohio 43210; email: santangelo.11@osu.edu

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Abstract

The kinetics and mechanisms of transcription are now being investigated by a repertoire of single-molecule techniques, including optical and magnetic tweezers, high-sensitivity fluorescence techniques, and atomic force microscopy. Single-molecule techniques complement traditional biochemical and crystallographic approaches, are capable of detecting the motions and dynamics of individual RNAP molecules and transcription complexes in real time, and make it possible to directly measure RNAP binding to and unwinding of template DNA, as well as RNAP translocation along the DNA during transcript synthesis.

Contents

INTRODUCTION

RNAP: RNA polymerase

Transcription is a crucial step in gene expression and its regulation. Transcription, the synthesis of an RNA transcript complementary to the template DNA, is carried out by DNA-dependent RNA polymerase (RNAP). RNAP is the subject of extensive regulation, dictated not only by interactions between RNAP and regulatory factors, but also by dynamics of the active site and domain movements associated with the transcription process, or cycle. During the past few decades all the phases of transcription have been studied extensively in order to elucidate the mechanism of transcription and its regulation. Before outlining the relatively new mechanical and kinetic details of the transcription cycle elucidated by single-molecule techniques, it is necessary to provide a brief outline of both RNAP structure and function and to summarize the transcription cycle in general.

RNAP Structure

RNAPs occur as both single- and multiple-subunit enzymes. RNAPs from bacteriophages and mitochondria are representative of the single-subunit family; bacterial, archaeal, and eukaryotic nuclear RNAPs constitute the multiple-subunit family. Although the single-subunit and multisubunit RNAPs do not likely share a common ancestor, the available biochemical and structural information from representatives from each family shows that these RNAPs share many characteristics (57). A great body of literature exists, but only those details pertinent to this review are presented here. To date, only two RNAPs, T7 RNAP (single-subunit) and *Escherichia coli* RNAP (multisubunit), have been utilized in single-molecule transcription studies. There is detailed structural information for T7 RNAP (9, 58); however, studies with *E. coli* RNAP typically rely on structural information gathered from other multisubunit RNAPs (10, 11, 38). This review focuses primarily on these two RNAPs.

A gross examination of the currently available structures of RNAP reveals that RNAPs from both families have a main internal channel that can accommodate an 8- to 9-bp RNA/DNA hybrid, a smaller, secondary channel or pore that likely serves as an entry channel for nucleotide triphosphates (NTPs), and an RNA exit channel. The active site is located in the junction of the main channel and secondary channels and contains at least one nucleotide binding site and a tightly bound Mg^{2+}. RNAPs from both families are two-metal ion-dependent enzymes and the second active-site Mg^{2+} is thought to be coordinated with the incoming NTP (56).

The Transcription Cycle

Transcription is traditionally divided into three sequential phases: initiation, elongation, and termination, although termination can be viewed as an alternate pathway branching from elongation (64). During initiation, RNAP recognizes a DNA sequence termed a promoter and melts, or separates, the two strands to form a single-stranded "DNA bubble." The single-subunit RNAPs are capable

of recognizing promoters without assistance, whereas multisubunit enzymes rely on separate proteins (typically σ-factors) for promoter recognition. T7 RNAP undergoes a global structural rearrangement following initiation to refold into an elongation complex (58). For multisubunit RNAPs, retention of the initiating σ-factor is not necessary for elongation and the dissociation of σ likely triggers a series of conformational changes during the transition to an elongation complex (35, 36, 38, 40).

The elongation stage of transcription involves movement of RNAP away from the promoter and production of a growing transcript. Elongation proceeds at different rates for the two RNAP families (in vivo: T7 RNAP at ~200 nt s^{-1}; *E. coli* RNAP at ~50 nt s^{-1}) (57). Each nucleotide addition is a competition among elongation, pausing (a transient conformational state incapable of elongation), arrest (a conformational state incapable of elongation without factor-assisted isomerization back to an active complex), and termination (transcript release and enzyme dissociation from the DNA template) (64). The polymerase moves with single-nucleotide steps along the DNA template during elongation, but it is also capable of reverse translocation in the absence of synthesis (termed backtracking) that leads to certain classes of pausing and arrest (4, 26, 27). Termination completes the transcription cycle, recycling RNAP for another promoter recognition event and round of synthesis.

The activity of each family of RNAP is distinct in many phases of transcription, although the transcription cycle is common to both families. Despite some differences during initiation, once an elongation complex is formed, NTP incorporation and the translocation of RNAP along the DNA template are necessary steps for both families of RNAP (13). Both enzymes incorporate individual NTPs into the growing, 3′ end of the transcript, have mechanisms to ensure incorporation of the correct NTP, and, in the unlikely event of misincorporation, mechanisms to remove the incorrectly incorporated nucleotide. Elongation complexes, as well as several intermediates during initiation, are extremely stable, making them good candidates for single-molecule studies.

Advantages and Disadvantages of Single-Molecule Experiments

Although structural studies have provided valuable insights into the structural organization of RNAP "frozen" at different stages of transcription, kinetic aspects of the structural transitions between these snapshots remain obscure. Biochemical studies have filled many gaps, but information regarding many conformational changes associated with each stage of the transcription cycle is still missing as are data on the dynamics of such movements. Many of these questions are better suited to be addressed by single-molecule techniques.

Compared with bulk studies, there are several advantages of single-molecule approaches. First and most importantly, properties measured in bulk studies represent ensemble averages of a population of molecules. Behaviors that are highly unsynchronized among different molecules, such as heterogeneity in population, transient intermediate states, and parallel reaction pathways, are difficult to quantitatively characterize. These problems, in principle, may be overcome by monitoring the motion of individual molecules in real time. Second, single-molecule techniques also provide tools for mechanically manipulating biomolecules, such as stretching and twisting DNA and protein molecules, thus allowing the researcher to manipulate equilibriums between competing reaction pathways in a defined manner.

Single-molecule techniques also have a number of drawbacks. In order to accurately determine kinetics and draw statistically meaningful conclusions, a large dataset of individual single-molecule measurements must be acquired and this can be time

consuming. In addition, single-molecule assays often introduce perturbations to the system under study (e.g., fluorophore labeling, surface attachment of molecules, and photodamage) that may complicate data interpretation. Finally, all single-molecule approaches are subject to some sort of measurement noise and sometimes require the use of altered reaction conditions (e.g., lowered NTP concentration) in order to achieve sufficient temporal and spatial resolution to probe fast kinetics.

In this article, we provide a rather detailed discussion on how single-molecule techniques have contributed to our better understanding of different phases of transcription. Because of space limitation, we do not focus on the operational principles of these techniques but instead on some of the important findings that have resulted from the use of these techniques.

IC: initiation complex

TEC: transcription initiation complex

INITIATION

Background

Initiation is the first phase of transcription, and historically, studies of transcription regulation have focused on initiation. Initiation requires a series of isomerizations, each of which has been biochemically characterized to some extent (33, 41). First, RNAP, either in combination with a σ-factor (e.g., *E. coli* RNAP) or alone (e.g., T7 RNAP), searches for and binds to a promoter sequence to form a so-called closed complex. The initial binding event is with double-stranded or closed DNA. Second, driven by the binding free energy, the complex isomerizes to an open complex by unwinding 10 to 15 base pairs of DNA surrounding the transcription start site. This initiation complex (IC) then undergoes a competition between NTP incorporation and short RNA oligo release, a phenomenon known as abortive initiation. Once the transcript length reaches ~12 nt, the complex enters the processive elongation phase (**Figure 1**).

The reaction pathway described above can be summarized as (41):

$$\mathrm{RNAP} + \mathrm{P} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{RNAP}\cdot\mathrm{P_c} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \mathrm{RNAP}\cdot\mathrm{P_o} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \mathrm{IC}_{\le 12} \overset{k_{+4}}{\rightarrow} \mathrm{TEC}, \quad 1.$$

where P represents the promoter, $\mathrm{RNAP}\cdot\mathrm{P_c}$ and $\mathrm{RNAP}\cdot\mathrm{P_o}$ represent the closed and open complexes, respectively, $\mathrm{IC}_{\le 12}$ represents abortive initiation complexes with transcript sizes generally ≤12 nt, and TEC represents the transcription elongation complex. $k_{\pm n}$ are the forward and reverse rate constants between the different states and thus determine the relative population distribution among the states. (This reaction pathway and subsequent pathways in this manuscript are simplified; typically more substates exist than are shown.) Different promoter sequences, DNA supercoiling configurations, and the association of regulatory factors such as other proteins and small molecules affect different $k_{\pm n}$ and thus modulate the overall initiation velocity and efficiency. Therefore characterization of the kinetic processes described in Equation 1 under different conditions is essential in order to elucidate the mechanism of initiation and its regulation.

Thus far single-molecule studies of initiation have allowed direct visualization of static intermediate ICs as well as the kinetic transition processes between the states, and measurement of some of the transition rate constants under various experimental conditions. These results are discussed in the following two sections.

Promoter Search

RNAP must accurately and efficiently locate promoters in spite of the large excess of nonpromoter DNA. It has been suggested that the efficiency of promoter recognition is enhanced by RNAP weakly binding to nonspecific DNA and rapidly transferring between different DNA segments (6). This transfer may include one-dimensional diffusion

along the DNA (sliding), microscopic dissociation/reassociation (hopping), and direct transfer between DNA segments. After consideration of this facilitated promoter targeting, the reaction pathway (1) should be expanded as (54):

$$\mathrm{RNAP} + \mathrm{DNA} \underset{k_{\mathrm{off}}}{\overset{k_{\mathrm{on}}}{\rightleftharpoons}} \mathrm{RNAP} \cdot \mathrm{DNA} \underset{k_{-1'}}{\overset{k_{+1'}}{\rightleftharpoons}} \mathrm{RNAP} \cdot \mathrm{P_c} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \mathrm{RNAP} \cdot \mathrm{P_o} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \mathrm{IC}_{\leq 12} \overset{k_{+4}}{\rightarrow} \mathrm{TEC}, \qquad 2.$$

where RNAP · DNA represents an RNAP and DNA complex. In this notation, the promoter search is considered in the step from RNAP · DNA to RNAP · P_c and is oversimplified as a single-step reaction. The proposed mechanisms for facilitated promoter searches are difficult to verify in bulk because binding, hopping, sliding, and transferring of RNAP may be transient events that are scattered along the DNA. Various single-molecule assays have been developed in order to directly detect these events.

The nonspecific binding of RNAP to DNA was first visualized using fluorescence microscopy by flowing fluorescently labeled *E. coli* RNAP through a bundle of stretched and oriented DNA molecules (23). While maintaining a constant flow rate, the interaction of an RNAP molecule with DNA was identified when its motion deviated from simple Brownian motion with drift. The observed motion of RNAP along the oriented DNA provided evidence for sliding as a

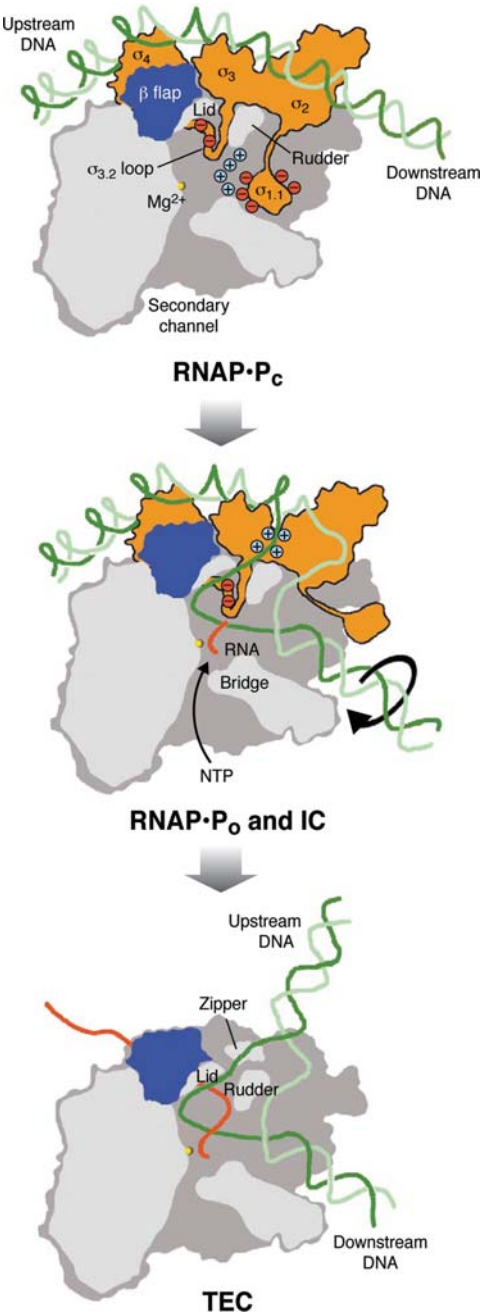

Figure 1

Structural transitions from closed complex (RNAP · P_c; *left panel*), to an open and abortive initiation complex (RNAP · P_o and IC; *center panel*), and to a transcription elongation complex (TEC; *right panel*). Shown are cross-sectional views of *Thermus aquaticus* RNAP holoenzyme (β flap, *blue*; σ, *orange*; rest of RNAP, *gray*; catalytic Mg^{2+}, *yellow sphere*), promoter DNA (template strand, *dark green*; nontemplate strand, *light green*), and the RNA transcript (*red*). Adapted and reprinted from *Current Opinion in Structural Biology*, Volume 13, Issue 1, Murakami KS and Darst SA, *Bacterial RNA polymerase: the whole story*, Pages 31–39, Figure 3, Copyright © 2003 Elsevier Science Ltd, with permission from Elsevier.

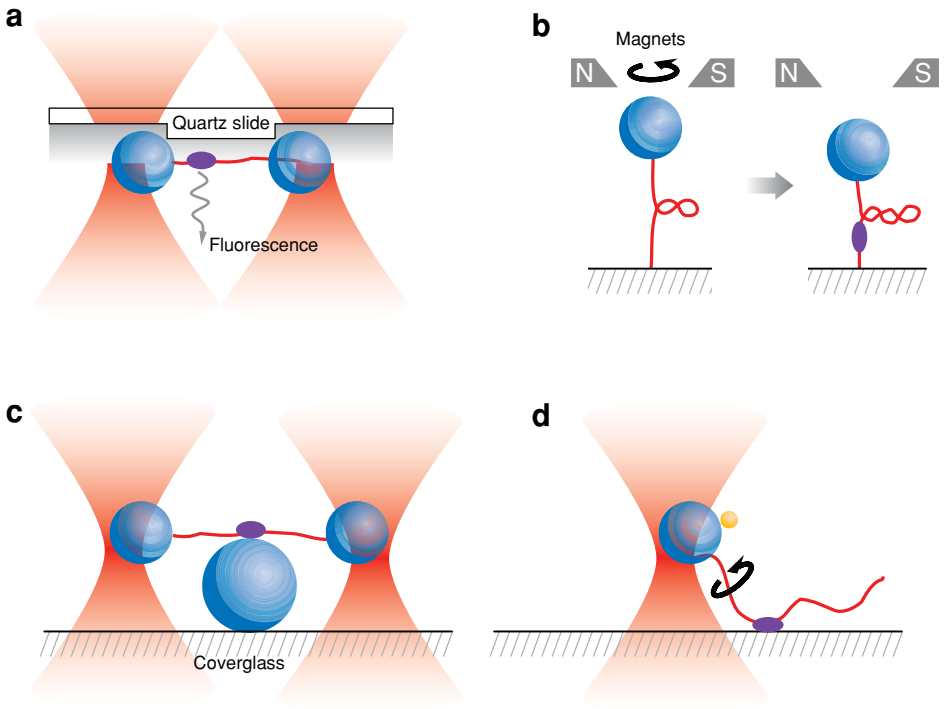

Figure 2

Cartoons of single-molecule experimental configurations used in initiation studies. Panels *a–d* represent the experimental design in Harada et al. (20) (TIRF and dual optical tweezers), Revyakin et al. (43) (magnetic tweezers), Skinner et al. (55) (dual optical tweezers), and Sakata-Sogawa et al. (49) (optical tweezers with rotation detection), respectively (for details see text). Focused laser beam, red; dielectric or magnetic bead, blue; DNA, red; RNAP, purple; small fluorescent bead, orange.

Total internal reflection fluorescence (TIRF): a technique that uses the evanescent field from light that is totally internally reflected at an interface to selectively excite fluorophores that are in very close proximity to the interface

Optical tweezers: an instrument that uses a tightly focused laser beam to trap and exert force on a microscopic dielectric particle

mechanism for promoter search. In a later experiment, the binding of *E. coli* RNAP to a single DNA molecule was observed by the combination of total internal reflection fluorescence (TIRF) with optical trapping (20) (**Figure 2*a***). A DNA molecule was held between two optical traps and individual RNAP molecules were fluorescently labeled. This experimental design decreased background fluorescence and aligned DNA for ease of visualization without the need for flow. The interaction between RNAP and DNA was found to be sequence dependent. RNAP bound more frequently to an AT-rich region than to a GC-rich region and dissociated more slowly at a promoter and promoter-like sequence. The measured association rate corresponds to $k_{on} \sim 10^3$ bp^{-1} M^{-1} s^{-1}. However, since this method only detected RNAP binding and did not differentiate among the three bound species (RNAP · DNA, RNAP · P_c, and RNAP · P_o), the measured dissociation rate of ~1 s^{-1} can only impose some constraints on k_{off}, $k_{\pm 1'}$, and $k_{\pm 2}$. Interestingly, a small fraction of RNAP exhibited random motion along the DNA before dissociation, which provided direct evidence for the linear diffusion of RNAP. The estimated diffusion coefficient of 10^{-10} cm^2 s^{-1} was 1 to 3 orders of magnitude smaller than those predicted by biochemical studies (54). The accuracy of the diffusion measurement was limited likely by the spatial resolution of the technique (~200 nm or ~600 bp of DNA), which might have exceeded the range of the linear diffusion.

Atomic force microscopy (AFM) is capable of a spatial resolution of ~10 nm. AFM first allowed observation of the binding of individual *E. coli* RNAP molecules to a DNA containing a promoter sequence in aqueous solution (17). Subsequent experiments using a promoterless DNA weakly adsorbed onto a surface demonstrated that *E. coli* RNAP could bind to DNA nonspecifically (18). By comparing sequential images of the same RNAP · DNA complexes and mapping the relative position of RNAP on the DNA as a function of time, RNAP was found to exhibit diffusion, hopping, or intersegment transfer along DNA (8, 18). The time resolution of these AFM studies (of the order of 10 s) made it difficult to measure fast kinetics. Also, the surface adsorption strongly affected RNAP's diffusion properties, and the average lifetime of the RNAP · DNA complex measured in the AFM study (~10 min) was much longer than values reported in other studies (20, 54).

The studies above measured the linear motion of RNAP during promoter search. Because of the double-helical structure of DNA, the translocation of RNAP may involve groove-tracking, which requires the rotation of RNAP around the helical axis of the DNA as it translocates. To determine if RNAP groove-tracks during the promoter search, an experimental setup utilizing a small fluorescent bead attached to a large bead was employed (21, 49) (**Figure 2*d***). DNA was multiply linked at one end to the large bead, which was then optically trapped. The tethered DNA was then dragged across a surface coated with immobilized *E. coli* RNAP molecules. The motion of the small fluorescent bead was analyzed to detect possible RNAP-driven rotation of the DNA. Many factors made these measurements difficult: large rotational Brownian motion of the large bead, low probability for the DNA to be bound by RNAP, bidirectionality of DNA rotation, and short lifetime of the one-dimensional diffusion. Nonetheless, it was argued that on average more coherent rotation of the bead was observed in the presence of RNAP, in support of groove-tracking (49).

Taken together, the experiments discussed above provide strong support for a diffusion-facilitated promoter search model. These studies, however, have some discrepancies in their quantitative values of the binding rate for nonspecific RNAP-DNA interactions, the diffusion coefficient of the RNAP along the DNA, and the lifetime of such diffusion. These values are important in order to understand the efficiency and rate of promoter recognition in vivo and should be further examined in the future. In principle the single-molecule techniques established during these studies could also be configured to study mechanisms of other proteins targeting specific DNA sequences.

Open Complex Formation

After RNAP locates a promoter, it isomerizes to form a relatively stable binary complex with a transcription bubble. On strong promoters, open complexes are much more energetically favorable, and the closed-to-open complex transition is essentially irreversible (41). AFM imaging of individual static open complexes of *E. coli* RNAP · σ^{70}-factor showed that the DNA was severely bent by 55° to 88° and its apparent contour length was reduced by ~90 bp, which was interpreted as DNA wrapping around RNAP (42, 47).

Single-molecule techniques have been utilized to follow the formation of open complex. Revyakin et al. (43) specifically probed the transition between closed and open complexes of *E. coli* RNAP by employing a magnetic tweezers setup in which a small change in DNA supercoiling associated with the formation of open complex was amplified as a large end-to-end distance change in the DNA extension (**Figure 2*b***). These studies were capable of monitoring a number of kinetic parameters of promoter formation under a variety of supercoiling states and with different promoter sequences. When NTPs were not present, transcript synthesis was blocked and

Atomic force microscopy (AFM): a scanning probe microscopy that uses the interaction force between a probe tip and a sample to generate high-resolution topographical images of the surface of the sample

σ^{70}-factor: a major σ factor in *E. coli* that directs RNAP to promoters with −10 and −35 elements

Magnetic tweezers: an instrument that uses a magnetic field to generate both force and torque on a microscopic magnetic particle

only a subset of states in Equation 2 were accessible:

$$\mathrm{RNAP} + \mathrm{DNA} \underset{k_{\mathrm{off}}}{\overset{k_{\mathrm{on}}}{\rightleftharpoons}} \mathrm{RNAP} \cdot \mathrm{DNA} \underset{k_{-1'}}{\overset{k_{+1'}}{\rightleftharpoons}} \mathrm{RNAP} \cdot \mathrm{P_c} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \mathrm{RNAP} \cdot \mathrm{P_o}. \quad 3.$$

The lifetime of the unwound (open complex) state directly yielded k_{-2}. For a strong promoter with a negatively supercoiled DNA, k_{-2} was so small that the open complex formation was effectively irreversible. With a strong consensus promoter and positively supercoiled DNA, k_{-2} was $\sim 0.03\ \mathrm{s}^{-1}$. Under the assumptions that RNAP binding and closed promoter formation were in rapid equilibrium and that $\mathrm{RNAP} \cdot \mathrm{P_c}$ was a much more stable complex than $\mathrm{RNAP} \cdot \mathrm{DNA}$, analysis of the time interval between unwinding events yielded $k_{+2} = 0.3\ \mathrm{s}^{-1}$ and the effective equilibrium binding constant $K_B = k_{\mathrm{on}} k_{+1'} / k_{\mathrm{off}} k_{-1'} = 10^7\ \mathrm{M}^{-1}$. Using this analysis, factors such as ppGpp and the initiating nucleotide were shown to alter the stability of the open complex.

In the study above, upon open complex formation, the DNA extension changes for negative and positive supercoiled DNA were not completely symmetric. This is consistent with the idea that DNA wraps around or is bent by RNAP as proposed by Rivetti et al. (47). This was also supported by results from Harada et al. (20), who found that tension in the DNA decreased the association rate and increased the dissociation rate of RNAP binding.

To our knowledge, there has been only one single-molecule study performed on initiation with T7 RNAP (55). In this experiment, a DNA molecule suspended between two optically trapped beads was held near a surface-immobilized bead sparsely coated with RNAP (**Figure 2*c***). By oscillating one bead with the optical trap, RNAP-DNA binding events were detected when the motion of the two trapped beads became decoupled. Nonspecific binding of RNAP was considered too fast to be detectable so only Equation 1 needed to be considered. The measured RNAP dissociation rate of $2.9\ \mathrm{s}^{-1}$ places some constraints on possible values of k_{-1} and $k_{\pm 2}$. Because the measurements were carried out in the presence of NTPs, there was some probability ($\sim$1% in this study) for RNAP to start elongation, which was detected as a large unidirectional motion of the downstream bead. The transition rate from initiation to elongation ($\sim 0.4\ \mathrm{s}^{-1}$) was significantly slower than the dissociation rate of a bound RNAP complex, consistent with a model in which abortive initiation limits the rate of initiation on strong promoters.

ELONGATION

Background

As the nascent transcript RNA reaches $\sim$13 nt the transcription complex escapes the promoter and enters the elongation phase. During elongation, RNAP, DNA, and RNA form a stable tertiary complex, the TEC, and RNAP moves processively along the DNA template while incorporating complementary NTPs onto the 3′ end of the RNA. An NTP incorporation cycle is composed of multiple reaction steps, including RNAP translocation from the pre- to posttranslocational state, NTP binding, NTP hydrolysis, PPi release, and possible conformational changes of RNAP (13, 28, 45, 63). An AFM-based study showed that DNA contour length was decreased in an elongation complex, consistent with the notion that DNA either is bent by or wraps around the RNAP in elongation complexes. However, the extent of contour length change measured for the elongation complexes was less than that of corresponding open promoter complexes (46).

One of the most fundamental questions in transcription is how RNAP's chemical catalysis is coupled to its mechanical translocation. A number of experimental results from *E. coli* RNAP support a thermal ratchet mechanism in which RNAP can slide back and forth on the DNA template activated by thermal energy and the incorporation of the next nucleotide

biases the polymerase forward by one base pair (5, 15, 22, 26, 27, 67). An example of a thermal ratchet model (5, 15) is shown below in which $TEC_{N,\ pre(post)}$ represents the TEC with transcript size N at the pre-(post)translocational mode:

$$\mathrm{TEC}_{N,\,\mathrm{pre}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{TEC}_{N,\,\mathrm{post}} \underset{k_{-2}}{\overset{\mathrm{NTP}\ k_2}{\rightleftharpoons}} \mathrm{TEC}_{N,\,\mathrm{post}}\cdot\mathrm{NTP}$$

$$\underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{TEC}_{N+1,\,\mathrm{pre}}\cdot\mathrm{PPi} \underset{k_{-4}}{\overset{k_4\ \mathrm{PP_i}}{\rightleftharpoons}} \mathrm{TEC}_{N+1,\,\mathrm{pre}} \qquad 4.$$

However, more recent findings from crystallographic data of T7 RNAP are challenging this view (28, 59, 77). Examination of the RNAP structures of elongation intermediates suggests that PP_i release promotes RNAP to undergo a conformational change that induces forward translocation of the polymerase by a single base pair. This conformation-driven translocation was thought to be tightly coupled, with one translocation step for each NTP incorporation cycle. This supported a power-stroke mechanism in which the chemical energy derived from the NTP condensation reaction directly drives the forward translocation of the RNAP along the DNA template. However, it is possible that single-subunit and multisubunit RNAPs utilize distinct mechanisms. More direct kinetic studies using single-molecule and biochemical techniques in conjunction with theoretical studies (5, 15, 60) are beginning to differentiate between these mechanisms of mechanochemical coupling.

Unlike traditional molecular motors (e.g., kinesin and myosin), RNAP moves along a varying substrate because of the varying template DNA sequence. Consequently, transcription does not proceed at a uniform rate and the motion of RNAP is DNA sequence dependent. In particular, RNAP tends to dwell transiently at certain template positions known as pause sites (45, 63, 64). Numerous pause sequences have been shown, or are suspected, to provide regulatory functions such as allowing transcription factors to bind and thereby modify gene expression (31, 48). Other pause sequences that have been detected in vitro have no known biological function but nonetheless reflect the intrinsic sequence dependence of RNAP motion. Biochemical assays have led to the suggestion that transcription pausing results from misalignment of the RNA 3′ end with the RNAP active site (4, 26, 27). Although a large number of pause-inducing sequences are known (30), no consensus sequences have been identified. Recent theoretical work might make it possible to predict some of the pause sites for a given DNA sequence (5).

Many of the pauses studied in traditional bulk biochemical assays are only prominent at low NTP concentrations, which is consistent with a competitive kinetic model in which pausing is an alternative pathway branching from active elongation (64, 65). In this model, the reaction shown in Equation 4 may be rewritten as:

$$\mathrm{TEC}_{N,\,\mathrm{pre}} \underset{k_{-1,\,-2,\,-3,\,-4}}{\overset{k_{1,2,3,4}}{\rightleftharpoons\ \cdots\ \rightleftharpoons}} \mathrm{TEC}_{N+1,\,\mathrm{pre}} \quad \text{Main pathway}$$

$$k_5 \Big\downarrow\Big\uparrow k_{-5}$$

$$\text{Branched pathway} \qquad 5.$$

On the basis of this mechanism, pausing could be caused by a slow rate in the main pathway (e.g., under low NTP concentration) or by a relatively fast rate into the nonproductive branched pathway with a slow rate of returning to the main pathway (e.g., at a pause induced by misalignment of the 3′ end of the RNA). The two pathways are kinetically competitive: a slow rate in the main pathway also increases the probability for RNAP to enter the branched pathway.

Similar to initiation, the rates in the reaction pathway above are sensitive to both internal conditions (e.g., RNAP species, DNA sequence, and RNAP mutation strains) and external conditions (e.g., NTP substrate concentration, temperature, buffer composition, and protein factors) (44). Many single-molecule assays have been carried out to further quantify the reaction pathways under different conditions.

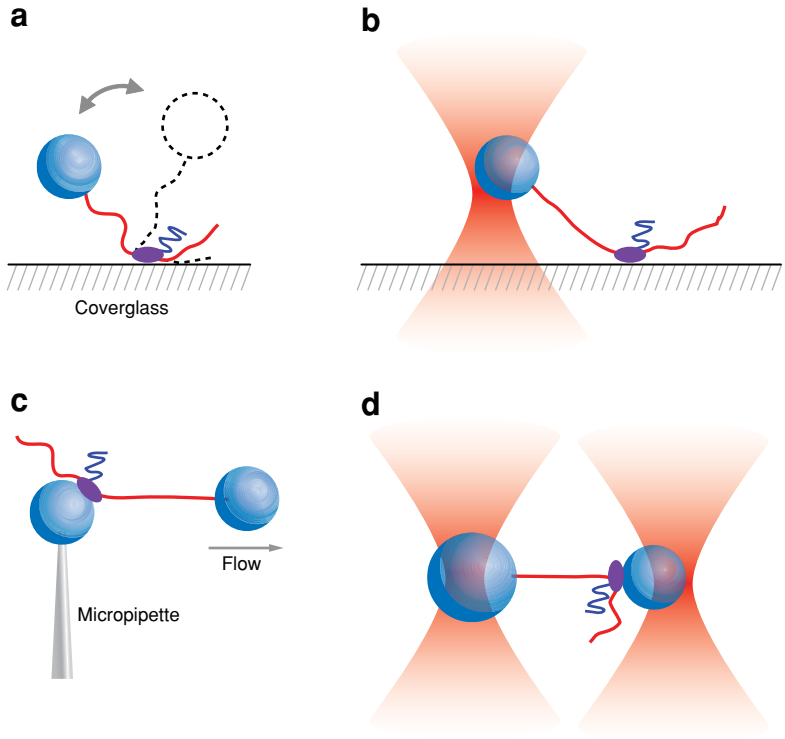

Figure 3

Cartoons of single-molecule experimental configurations used in elongation studies using (*a*) tethered particle motion, (*b*) optical tweezers, (*c*) flow-control video microscopy, and (*d*) dual optical tweezers (for details see text). Focused laser beam, red; dielectric bead, blue; DNA, red; RNAP, purple; RNA, blue.

TPM: tethered particle motion

Below we first discuss general single-molecule approaches for elongation studies, followed by experimental results on active elongation and pausing kinetics.

General Approaches

The initial observations of elongation activity at the single-molecule level came from tethered particle motion (TPM) studies (51, 75) (**Figure 3*a***). In TPM experiments, an RNAP molecule is immobilized onto the surface of a microscope coverglass, and a small particle is tethered to the RNAP by the DNA template that is being transcribed. The tethered particle has a confined Brownian motion with a range indicative of the length of the DNA tether. Therefore the motion of the RNAP along the DNA template can be monitored by the range of the motion of the constrained particle. The TPM method minimally perturbs RNAP activity, and multiple polymerase molecules can be monitored simultaneously via video microscopy to allow high data throughput. However, the large Brownian motion of the tethered bead limits the spatial and temporal resolutions for detecting RNAP motion.

Subsequent studies improved resolution by reducing the Brownian noise of the bead via an external force using optical tweezers (2, 39, 52, 53, 60, 68, 76) (**Figure 3*b***) or a constant flow (12, 14) (**Figure 3*c***). In these studies, an RNAP molecule was attached to a surface (of a microscope coverglass or a bead) while one end of the DNA that was transcribed was attached to another surface in such a way that a bead was tethered by the DNA. Translocation of the polymerase was then monitored via the movement of the bead and the higher resolution of these studies revealed some detailed behaviors of individual RNAP molecules. Movement of an individual RNAP molecule was often interrupted by numerous pauses, even on templates with no known strong regulatory pause sites at

saturating NTP concentrations. In these studies, active elongation was differentiated from pausing by setting a threshold to either the instantaneous velocity (12, 14, 39, 68) or the dwell time at each base (2, 53). The threshold needed to be set according to the resolution and data filtering of a given measurement and was thus experiment specific. Transient pausing of short duration (<1 s) often could not be directly resolved, and this inevitably affected the statistics of active elongation velocity and pausing probability. Improved instrumentation and analysis now allow for more precise resolution of RNAP location, including the identification of sequence-dependent pause sites (53), and resolution of backtracking events associated with certain pauses (52).

A single-molecule magnetic tweezers approach also allowed the investigation of the rotational behaviors of RNAP (21). As with the one-dimensional diffusion of RNAP during a promoter search, elongating RNAP is also expected to track the groove of the DNA double helix and rotate relative to the DNA. Using a setup similar to that shown in **Figure 2*d*** except with the bead being pulled up by a single magnet, Harada et al. (21) observed processive unidirectional rotation of a magnetic bead multiply linked to one end of a DNA that was transcribed by a surfaced-immobilized *E. coli* RNAP. These experiments, together with those from initiation studies, are providing increasing evidence that RNAP is a groove-tracking motor molecule.

Active Elongation Kinetics

Compared with bulk methods, single-molecule measurements can more readily separate active elongation from pausing. However, the measured average active elongation velocity of *E. coli* RNAP varied from study to study (10 to 20 bp s^{-1} at room temperature and 1 mM NTPs) even under apparently similar experimental conditions (e.g., temperature, buffer, NTP concentration) (2, 12, 14, 39, 52, 53, 68). The high end of this range is comparable to the rate measured in bulk studies (15 to 20 bp s^{-1}, even including pauses), but the low end is significantly slower. Slight temperature differences may contribute to the inconsistencies because transcription rate was found to have a strong dependence on temperature (1). However, it is unlikely that temperature differences alone can account for the high variability of transcription rates in single-molecule studies. Discrepancies in velocity reported by different labs are due presumably to differences in experimental details and data analysis and need to be carefully resolved.

Single-molecule studies also brought out two other aspects of active elongation. First, Davenport et al. (12) reported a bimodal distribution for active elongation velocity, even for the same RNAP molecule, and suggested that a single RNAP molecule could switch from a more competent to a less competent elongation state. However, other studies did not observe such switching behavior (2, 39, 53, 61). Instead the instantaneous velocity during active elongation was well fit with a single Gaussian distribution (2, 39). Second, it is still controversial whether an RNAP population is homogeneous. While some studies supported uniform kinetics of RNAP among different molecules (2, 53), others provided evidence for a heterogeneous population in which the active elongation velocity varies from molecule to molecule (39, 61). The spread in the velocity, typically 5 to 7 bp s^{-1}, was thought to be larger than the variation arising from the stochastic nature of each NTP incorporation step or experimental conditions, and thus reflected the intrinsic heterogeneity among RNAP molecules. Interestingly, heterogeneous reaction rates within a single molecule or among different molecules of the same population have been reported for other enzymes (19, 32, 70, 71). Single-molecule studies have brought these issues to light and provide a direct way to resolve them.

Optical tweezers (**Figure 3*b***) and the flow technique (**Figure 3*c***) have allowed the application of an assisting or resisting force to

Backtracked pause: transcription pause induced by the reverse translocation of RNAP in the absence of synthesis

the motion of a single RNAP molecule. *E. coli* RNAP is a powerful motor, capable of generating ~25 pN of force (39, 68, 76). The measured force-velocity relation shows that transcription velocity remains nearly constant over a wide range of resisting and assisting forces (+25 pN to –35 pN) under saturating NTP concentration (12, 14, 39, 68, 76). A recent study with T7 RNAP also found a similar force-velocity relation (60). These results indicate that translocation does not limit the rate of transcription at saturating NTP concentration.

Single-molecule experiments are also beginning to elucidate the mechano-chemical coupling mechanism of transcription. Thomen et al. (60) examined the relation of elongation velocity of T7 RNAP and NTP concentration under various forces. Their results were consistent with a thermal ratchet model first proposed by Guajardo & Sousa (15). Extending this simple model, Bai et al. (5) formulated a sequence-dependent thermal ratchet model for transcription by *E. coli* RNAP by constructing a quantitative sequence-dependent transcription energy landscape and performing a full kinetic analysis based on thermodynamic calculations (72). The predictions of this model were consistent with a number of biochemical and single-molecule measurements. Nevertheless, the power-stroke mechanism of transcription can not be ruled out and differentiation between the two models requires future effort.

Pausing Kinetics

Single-molecule studies have provided much information on transcription pausing. In a single-molecule measurement, a transcription pause is characterized by its template sequence, probability of occurrence, and duration. On templates with no known strong pause sites, optical tweezers studies have shown that observed pauses were dominated by those with short durations (<10 s) with 1 mM NTPs (2, 39). The average duration of these pauses was a few seconds, which would have been too short to be detected in conventional bulk transcription studies, for which pauses must be of both significant duration and high enough probability for a significant percentage of the population to be observed at the pause site. The distance between pauses followed a single exponential distribution with a characteristic distance of ~250 bp (2), and this distribution suggested that the pauses either occurred stochastically in a sequence-independent manner, or were sequence dependent but distributed randomly, or occurred at very low probability. An examination of pausing probability versus template position revealed a small but statistically significant variation along the template, suggesting that the pausing should be at least partially sequence dependent (39). A small fraction of pauses detected also had a long duration (>10 s) (2, 39), and long pauses with a duration of 20 to 100 s have also been identified by using a flow force setup (12, 14). These pauses occurred with much lower frequency (~every 5000 bp) and seemed to have a strong sequence dependence. The application of a force had little effect on the short pause kinetics (39) but reduced the long pause efficiency if applied in the assisting direction (14). The exact mechanisms of both short and long pauses and their possible sequence dependence are yet to be fully understood.

The studies above based their statistics on the pauses observed over the whole template. Pausing kinetics at a specific template position, and the effect of assisting or repressive loads, was directly examined by Shundrovsky et al. (53). By using runoff transcription as a well-defined position marker for alignment, the position of RNAP on the DNA template was determined to ~5 bp precision, thus allowing definitive confirmation of sequence-dependent pausing. This resolution represented an ~20-fold enhancement over previous methods of detection (2, 12, 14, 39, 52, 68, 76). At a well-known backtracked pause, ΔtR2, the pause duration decreased significantly with increasing assisting force,

providing further evidence for backtracking at this pause site.

Single-molecule studies have also been effective in elucidating the mechanism of a small inhibitor of transcription, the cyclic peptide microcin J25 (3). In the presence of microcin, RNAP paused more frequently but the active elongation velocity was not affected. This finding, in combination with bulk biochemical studies that show that microcin interacts with RNAP in the secondary channel (3, 37), supports a model in which microcin inhibits transcription by binding to the secondary channel of RNAP and thereby blocks the entrance of NTP substrate.

Single-molecule studies have also provided direct evidence for RNAP backtracking during transcription pausing. Backtracked pauses are typically of long duration, and therefore their characterization by single-molecule measurements is often plagued by instrument drift. Shaevitz et al. (52) circumvented this problem by using a dual trap (**Figure 3*d***) so that the long-lived backtracked pauses could be observed with near base pair resolution. The mean backtracking distance was ~5 bp and the pause duration was of the order of 1 min. Consistent with biochemical studies, the addition of GreA and GreB factors, which were known to rescue a backtracked complex by stimulating cleavage of the 3′ end of the RNA (7), decreased the backtracking pause probability.

TERMINATION

During termination, RNAP dissociates from the DNA and releases the transcript. Termination can be triggered by specific DNA sequences (intrinsic termination) or mediated by protein factors (45). An intrinsic terminator sequence encodes an RNA that can form a stem-loop hairpin structure preceding a U-rich segment, and in some way, this sequence (or rather the terminator structure) destabilizes the TEC. The nucleotide sequences in the hairpin and the U-rich region, as well as the spacing between them, strongly affect the termination efficiency. The exact mechanism for intrinsic termination is still under continued investigation. Several models have been proposed, including extensive forward-tracking of RNAP (50, 73), conformational change of RNAP due to the interaction with the RNA hairpin (62, 69), and melting of the RNA/DNA hybrid without physical movement of RNAP (16, 25). Similar to backtracking pause, termination may be an alternative branch pathway competing with active elongation (34, 66).

The termination phase of transcription is the least examined by single-molecule analysis. Only one study has investigated termination, specifically intrinsic termination (74). Using a TPM method (**Figure 3*a***), this study was aimed at differentiating these models by elucidating the reaction pathway leading to termination. This study showed that RNAP molecules that terminated at the intrinsic terminator paused immediately before dissociation, but those molecules that did not terminate lacked an equivalent pause at the terminator sequence. This study suggested that pausing was a necessary intermediate step before dissociation. However, bulk studies have indicated intrinsic termination as a fast process and the TPM data analysis could be complicated by the potential for released DNA to form an RNAP-DNA binary complex indistinguishable from the pausing complex (24). Also, termination on different terminators could have different properties. More studies on various terminators are needed to generalize the conclusion on the termination pathway.

CONCLUSIONS AND PERSPECTIVES

In the past 25 years, single-molecule techniques have been applied to study different phases of transcription carried out by prokaryotic RNAPs. By monitoring individual transcription complexes in real time, researchers study processes that are highly unsynchronized among molecules in a large population

FRET: fluorescence resonance energy transfer

and address questions relating to population heterogeneity. AFM imaging has provided information about the conformation and conformational changes of single-transcription complexes. Other assays using TPM, optical tweezers, magnetic tweezers, and fluorescence techniques have directly detected kinetic processes including RNAP binding, RNAP diffusion during promoter search, transition between closed-to-open IC, and RNAP translocation along DNA. In particular, branched reaction pathways during elongation, which lead to active elongation or pausing of the TEC, could be differentiated and analyzed separately in single-molecule assays. Furthermore, by stretching and twisting DNA, the effect of DNA supercoiling on the transition between closed-to-open IC, and the force dependence of elongation velocity, could be measured. These measurements are difficult, if not impossible, to realize in bulk assays. These studies have begun to shed light on the mechanism of transcription as well as its regulation.

The techniques discussed here typically require extensive signal averaging or filtering over tenths of a second or seconds in order to achieve near nanometer resolution. So far the translocational motion of RNAP during a single-nucleotide addition cycle has not yet been resolved, and it is not clear whether RNAP oscillates between pre- and posttranslocational states or whether translocation occurs only once per NTP incorporation cycle. Also, the dynamics of the conformational changes of a transcription complex upon the transition from initiation to elongation phase and during each elongation step (38, 58) has not been addressed at the single-molecule level. Fluorescence resonance energy transfer (FRET) is sensitive to nanometer-distance changes at a microsecond timescale and has been applied to study structural change during transcription in bulk experiments (35, 36). Recent research has demonstrated that by using alternating laser excitation (ALEX), single-molecule FRET is capable of accurately measuring the distance between the donor and acceptor in a transcription complex (29). An exciting possibility would be to combine the fluorescence and single-molecule mechanical techniques to correlate the short-distance structural changes with the long-distance movement.

SUMMARY POINTS

1. Single-molecule methods, including TPM, optical tweezers, magnetic tweezers, AFM, and fluorescence techniques (TIRF and FRET), have been applied to study transcription.
2. Single-molecule studies of transcription initiation have directly detected important reaction intermediates and measured reaction rates.
3. Single-molecule studies of elongation and termination have revealed kinetics of both active elongation and pausing.
4. By stretching and twisting DNA, the effects of DNA tension and supercoiling on transcription have been studied.

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