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Fluorescent Methods to Study Transcription Initiation and Transition into Elongation

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Abstract

The DNA-dependent RNA polymerases induce specific conformational changes in the promoter DNA during transcription initiation. Fluorescence spectroscopy sensitively monitors these DNA conformational changes in real time and at equilibrium providing powerful ways to estimate interactions in transcriptional complexes and to assess how transcription is regulated by the promoter DNA sequence, transcription factors, and small ligands. Ensemble fluorescence methods described here probe the individual steps of promoter binding, bending, opening, and transition into the elongation using T7 phage and mitochondrial transcriptional systems as examples.

Keywords

Fluorescence; Transcription initiation; RNA polymerase; Kinetics; 2-Aminopurine; Anisotropy; FRET

6.1. Introduction

The past few years have witnessed enormous growth in the understanding of the mechanism and regulation of DNA transcription through numerous structural snapshots of the RNA polymerase (RNAP) complexes from various organisms and through genome-wide gene expression studies and mutational studies [1–11]. Nevertheless, most of the quantitative research to elucidate the detailed mechanism of the transcriptional pathway has been conducted using kinetic approaches that monitor RNA synthesis by high-resolution radiometric gel assays [12] or intermediates by real-time fluorescent assays [13–15, 2, 16].

The focus of this chapter is to discuss how ensemble fluorescence measurements can be combined with transient-state kinetic methods to probe and quantify the various steps of transcription initiation and transition into elongation, the two stages of transcription that critically regulate the efficiency of RNA synthesis.

DNA-dependent RNA polymerases are motor enzymes that read the sequence of the DNA and copy the information into RNA molecules using ribonucleotide triphosphates (NTP) as substrates in a process called transcription. Transcription occurs in distinct stages including initiation, elongation, and termination. During initiation, the RNAP binds to a specific promoter DNA to form a closed and open complex in which a short stretch of DNA near the +1 transcription start site is melted to expose the template for initiation of RNA synthesis (Fig. 6.1). The initiating NTPs bind in the active site of the RNAP and base pair with the melted +1 and +2 template bases to form the initially transcribing complex. Phosphodiester bond formation between the two NTPs results in the synthesis of the first 2-mer RNA product, which is then extended to longer RNAs by sequential addition of NTPs coupled to DNA unwinding. During this phase, short RNAs from 2 to 12 mer dissociate into solution as abortive products. After 8–12 mer RNA synthesis, the RNAP is able to clear the promoter and make the transition into the elongation phase, where full-length RNA is made by processive translocation, DNA unwinding, and RNA synthesis.

This chapter is designed to follow the individual steps of transcription initiation (Fig. 6.1) and to empower researchers entering the field of transcription to ask targeted questions about particular steps and employ appropriate fluorescence techniques to address them. We also describe how these methods can be used to study the conformational changes in DNA and to determine the exact stage when the RNAP transitions into elongation. We use studies of the well-characterized single-subunit T7 phage RNAP and the homologous yeast mitochondrial (mt) RNAP to elaborate on how fluorescence methods can be combined with transient-state kinetics to elucidate the mechanism of transcription initiation. We give a general overview of the theory behind each fluorescence technique, explain the rationale in designing the experiment, and expound on how to quantitatively analyze the data. The methods described here are general and applicable to studies of other RNA polymerases.

6.2 Promoter Binding

Transcription begins with RNAP binding to the promoter DNA. In T7 phage, the single-subunit T7 RNAP recognizes the consensus –17 to +6 promoter sequence without requiring any transcription factors. In yeast (*Saccharomyces cerevisiae*) mitochondria, transcription is carried out by a single-subunit class of enzymes related to T7 RNAP, but the core Rpo41 polymerase requires the transcription factor Mtf1 for specific transcription [17]. T7 RNAP discriminates against the non-promoter sequences at the initial binding step, showing $\sim 10^5$ -fold tighter affinity for promoter relative to non-promoter sequences [18]. In contrast, Rpo41–Mtf1 shows little discrimination between promoter and non-promoter sequences at the binding step [19]. Most of the discrimination occurs at the subsequent steps of DNA bending and melting. Thus, initial binding, bending, and melting of the promoter DNA by RNAP are the primary steps of initiation subject to regulation by inhibitors or other environmental cues [20–22, 19].

The DNA binding step can be studied using fluorescence methods that rely on signals from intrinsic protein fluorescence or fluorophore-labeled protein or DNA. Intrinsic protein fluorescence (excitation at 280–295 nm and emission at 340 nm) can be used to quantify protein–DNA interactions, but it depends on the number and placement of tryptophan and tyrosine residues and on a quantifiable signal upon DNA binding [23]. If there is no intrinsic protein fluorescence change, then fluorescence anisotropy changes with fluorophore-labeled DNAs is a robust method for quantifying protein–DNA interactions.

6.2.1 Fluorescence Anisotropy

Fluorescence anisotropy-based titrations allow one to monitor protein–DNA interactions with a high precision, in real-time, and under true-equilibrium conditions and are a preferred technique over the traditional filter-binding or electrophoretic mobility shift assays [24]. The basic principle of fluorescence anisotropy is that when a group of fluorescently labeled molecules are excited with polarized light, the molecules with their excitation dipoles oriented along the incident light will get excited and further emit polarized light depending on the location, size, and movement of the fluorophore [25]. A fluorescently labeled DNA will freely tumble in solution and depolarize the emitted light. However, when the DNA is bound by the large RNAP, tumbling will be reduced and emission will retain more polarization, resulting in an increase in anisotropy (Fig. 6.2a). Fluorescence anisotropy change serves as a signal to carry out both equilibrium and kinetic studies of RNAP–DNA complexes.

6.2.2 Fluorophore-Labeled DNA Substrates

Fluorophore-labeled oligodeoxynucleotides can be purchased with a wide selection of fluorophores which absorb and fluoresce above the absorbance maxima of the DNA and protein (>400 nm), thus minimizing background fluorescence and inner-filter effects. The parameters that need to be considered for designing the DNA substrates are as follows: (1) the preferred length of the DNA is the minimal promoter length that binds RNAP with 1:1 stoichiometry, (2) the sequence at the DNA ends such as GC bp that quench the fluorescence of the fluorophore or stack the fluorophore [26], (3) the extinction coefficient and quantum yield of the fluorophore, (4) the length of the linker between the fluorophore and the DNA, and (5) the distance between the fluorophore and the RNAP binding site. The fluorophore is positioned away from the RNAP binding site to avoid direct interactions [27].

Labeled oligodeoxynucleotides are purified by denaturing PAGE under dark conditions [14]. The concentration of single-stranded (ss) DNA is calculated from its absorbance at 260 nm and the extinction coefficient including the fluorophore. During annealing to prepare the double-stranded (ds) DNA, the unlabeled strand should be kept in slight excess over the labeled strand (1.1: 1 ratio) to ensure that there is no free labeled single strand that could contribute to higher background fluorescence. Alternatively, the dsDNA is purified or the correct annealing ratio is determined by titrating the two strands, resolving dsDNA from the ssDNA by native PAGE [18, 14]. When using fluorescently labeled oligonucleotides, it is important to check using competition methods [28] that the fluorophore does not greatly perturb the interactions of the DNA with the RNAP.

6.2.3 Fluorescence Anisotropy to Measure the Equilibrium Dissociation Constant (K_d) of the RNAP–DNA Complex

For measuring the K_d or the equilibrium dissociation constant of the RNAP–DNA complex using fluorescence anisotropy, the fluorophore-labeled DNA is titrated with increasing concentration of the RNAP. The fluorescence measurement is carried out using a spectrofluorometer with polarizers. A test run over RNAP concentration from nM to μ M will provide a rough idea of the K_d . After this preliminary estimation, the DNA concentration is kept close to the K_d value, and RNAP is increased from tenfold lower to tenfold higher than the DNA concentration with enough data points covering the transition phase to saturation. If the goal is to determine the stoichiometry of the complex, then the DNA concentration is kept ten times higher than the K_d value. After each addition of the RNAP, the solution in the cuvette is mixed well (with a magnetic stir bar or manual mixing by pipette) and incubated at a constant temperature for a predetermined time interval to reach equilibrium before exciting with sample with the excitation wavelength of the fluorophore. For all fluorescence measurements, we recommend using the highest grade spectroscopic reagents. Additionally, distilled water stored in plastic containers should be avoided for making buffers, as the plastic material tends to leach fluorescent species over time.

The titration of tetramethylrhodamine (TAMRA)-labeled promoter DNA with increasing concentration of Rpo41 and Rpo41-Mtf1 is shown (Fig. 6.2b) [19]. The sample was excited at 555 nm, and fluorescence intensities ($I_{\text{parallel}(\parallel)}$ and $I_{\text{perpendicular}(\perp)}$) were measured at 580 nm. The observed anisotropy, $r_{\text{obs}} = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$, is a function of anisotropies of both free DNA (r_f) and the RNAP-bound DNA (r_b) (Eq. 6.1):

$$r_{\text{obs}} = r_b f_b + r_f (1 - f_b) \quad (6.1)$$

where f_b is the fraction of the total DNA ($[D_t]$) in the RNAP–DNA complex. In case the fluorescence intensity of the DNA-labeled dye is quenched by a factor Q , the anisotropy is corrected using Eq. (6.2):

$$r_{\text{obs}} = \frac{Q r_b f_b + r_f (1 - f_b)}{1 - f_b (1 - Q)} \quad (6.2)$$

Formation of the RNAP–DNA complex will increase the anisotropy of labeled DNA without significantly changing the total fluorescence intensity ($I_{\text{total}} = I_{\parallel} + 2I_{\perp}$). However, if fluorescence intensity changes are greater than $\sim 10\%$, then the anisotropy is corrected (Eq. 6.2). Alternatively, both anisotropy and emission intensity data are simultaneously fit using SPECTRABIND [29]. If the intensity change is significant, then fluorescence intensity itself can be used to determine the K_d value.

The corrected anisotropy is plotted as a function of total RNAP concentration ($[E_t]$) to obtain the K_d values. The data are fit to the quadratic equation (Eq. 6.3) using the least squares method with softwares such as SigmaPlot (Systat software, Inc. San Jose, CA) [22]:

$$f_b = \frac{K_d + [E_t] + [D_t] - \sqrt{(K_d + [E_t] + [D_t])^2 - 4[E_t][D_t]}}{2[D_t]} \quad (6.3)$$

Anisotropy-based titrations showed that Rpo41-promoter DNA complex has a K_d of 58 nM, which indicated that Rpo41 itself binds to the promoter DNA with a high affinity. However, in the presence of its transcription factor Mtf1, promoter binding became ~300-fold tighter (K_d of 0.2 nM) [19]. The two K_d values were measured using 2–8 nM 5'-end TAMRA-labeled promoter DNA titrated with increasing protein concentration. Similar titrations determined that Rpo41–Mtf1 binds non-promoter DNA with only a fivefold weaker affinity ($K_d \sim 1$ nM) than the promoter DNA. This is in contrast to T7 RNAP, where competition assays showed that a non-promoter DNA binds ~10⁵-fold more weakly than the promoter DNA [18]. Thus, the two RNAPs use different mechanisms for choosing promoter over non-promoter DNAs during transcription initiation.

6.2.4 Kinetic Studies of RNAP–DNA Complex Formation

Fluorescence anisotropy changes can be monitored in real time to determine the dynamics of the RNAP–DNA complex. The *on-* and *off-*rates show how fast RNAP binds and falls off the DNA, and studies with transcription factors determine how the stability of the complexes is modulated by other factors. Such measurements demonstrated that the high affinity of Rpo41–Mtf1 for the promoter DNA is because Mtf1 decreases the *off-rate* and not due to an increase in *on-rate*. The *on-rate* and *off-rate* can also be used to determine K_d values (*off-rate/on-rate*) of extremely tight RNAP–DNA complexes such as T7 RNAP with pre-melted promoter (K_d of 5 pM).

6.2.4.1 Measurement of the On-Rate of RNAP–DNA Complex—To measure the *on-rate* of RNAP–DNA complex, fluorescence anisotropy is recorded after mixing fluorescently labeled promoter DNA with RNAP in a stopped-flow instrument (Fig. 6.2c). Here, automated motor-driven syringes help rapid mixing of RNAP with DNA at a constant temperature with continuous fluorescence emission measurement at a particular wavelength. The anisotropy changes are measured as a function of time after mixing at constant DNA and various RNAP concentrations under pseudo-first-order conditions (where the RNAP concentration is always in tenfold excess over the labeled DNA). Multiple time traces (at least 7–8 shots) are averaged for each RNAP concentration and the averaged observed anisotropy (r_{obs}) versus time is fit to the exponential equation (Eq. 6.4) to obtain the observed rate constant (k_{obs}):

$$r_{\text{obs}} = r_f + Ae^{-k_{\text{obs}}t} \quad (6.4)$$

where r_f is the anisotropy value at the start, A is the change in anisotropy, and t is time. Representative time traces of increase in anisotropy of TAMRA-labeled promoter DNA upon addition of Rpo41 and Rpo41–Mtf1 are shown (Fig. 6.2d) [19]. The observed rates increase linearly with increase in Rpo41 and Rpo41–Mtf1 concentrations (Fig. 6.2e), indicating that binding of RNAP to promoter DNA is a single-step process (Scheme 6.1).

Therefore, the dependency can be fit to a linear equation (Eq. 6.5) to obtain the *on-rate* (k_1) and *off-rate* (k_{-1}):

$$k_{\text{obs}} = k_{-1} + k_1 [E] \quad (6.5)$$

where the slope provides the *on-rate* and the y-intercept is the *off-rate*. If the *off-rate* is slow, then it is best determined more directly from chase experiments (Sect. 6.4.2.2). Such measurements with Rpo41 and Rpo41–Mtf1 showed that each binds to the promoter DNA with similar *on-rates* ($2\text{--}2.5 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$), which indicates that the transcription factor Mtf1 does not affect the kinetics of complex formation [19]. Thus, the lower K_d of Rpo41–Mtf1–DNA complex must be due to slower *off-rate*.

Similar anisotropy binding experiments showed that T7 RNAP forms a closed complex with its promoter DNA with an *on-rate* of $1.9 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and an open complex with a pre-melted promoter with similar *on-rate* of $3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ [30]. Thus, the lower K_d of T7 RNAP–pre-melted promoter complex is due to its slower *off-rate*. Once both *on* and *off-rates* are obtained, then the ratio provides an independent measure of the equilibrium dissociation constant (K_d) for the RNAP–DNA complex, which should match the K_d from the equilibrium titrations (Sect. 6.2.3).

6.2.4.2 Measurement of the Off-Rate of RNAP–DNA Complex—The *off-rate* of the RNAP–DNA complex is directly measured using a chase experiment, where a preformed complex of RNAP and fluorescently labeled DNA is mixed with a large molar excess (10- to 20-fold) of the same unlabeled DNA (Fig. 6.2f). When the labeled DNA dissociates from the RNAP, it is diluted in the pool of excess of unlabeled DNA. The fluorescence anisotropy of the free labeled DNA is lower, and therefore a decrease in anisotropy is observed over time with a rate equal to the *off-rate*. A TAMRA-labeled promoter DNA complexed with Rpo41 (Fig. 6.2g) or Rpo41–Mtf1 (Fig. 6.2h) was chased with excess of unlabeled promoter DNA [19]. The exponential decrease in anisotropy with time is fit to Eq. (6.4) to obtain the *off-rate* from the k_{obs} .

The *off-rate* of the promoter DNA from Rpo41 is 16 s^{-1} and from Rpo41–Mtf1 is 0.02 s^{-1} . Hence, Mtf1 stabilizes the ternary complex by decreasing the *off-rate* by 800-fold without significantly affecting the *on-rate*, as shown above. Similar methods were used to measure the *off-rates* of T7 RNAP complexed with duplexed or pre-melted promoter. T7 RNAP has a very high affinity for the pre-melted promoter such that the K_d could not be measured by equilibrium binding assays. Therefore, the K_d of the pre-melted promoter complex was determined from the ratio of the *off-rate/on-rate* [18]. The *off-rate* of the duplex promoter from T7 RNAP is 0.8 s^{-1} and of the pre-melted promoter is 0.0015 s^{-1} [30]. Thus, the ratio of the *off-rate/on-rate* provided a K_d of 5 pM for the pre-melted promoter–T7 RNAP complex and 4 nM for the duplex promoter complex. The kinetic experiments show that the tighter binding of the pre-melted promoter is not due to its faster *on-rate* on T7 RNAP (1.6-fold faster than duplexed promoter), but due to its slower *off-rate* (530-fold slower).

6.3 Promoter Bending

RNA polymerases bend the promoter DNA during open complex formation, and the bending angle changes as the RNA gets elongated during the initiation stage and when the RNAP makes the transition into elongation. DNA conformational changes like bending can be detected using structural methods such as X-ray crystallography [31-33], nuclear magnetic resonance [34], electron microscopy [35], atomic force microscopy [36, 37], electrophoretic mobility assays like circular permutation assay [38–40] and helical phasing assay [39, 41, 40], ring closure methods [39, 42, 40], and fluorescence methods including Förster resonance energy transfer (FRET). FRET methods are increasingly used in transcription for monitoring DNA bending changes and for monitoring movements of the RNAP relative to the DNA [19, 43, 30, 22, 44]. With the recent advances in single-molecule FRET [45–48], these events can be monitored in real time on single molecules. Both ensemble and single-molecule FRET have been successfully used to study bending and unbending dynamics of T7 RNAP and yeast mt RNAP promoter complexes [22, 19, 49, 30].

6.3.1 Förster Resonance Energy Transfer

FRET relies on two-fluorophore system, donor, and acceptor, chosen carefully such that there is spectral overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor (Fig. 6.3a). On exciting the donor, energy is transferred through dipole–dipole coupling to the acceptor that emits at a higher emission wavelength. The FRET efficiency (E_{FRET}) depends on the distance between the fluorophores (Eq. 6.9) and therefore serves as a molecular ruler with a range of ~ 10 – 100 Å, ideal for monitoring protein-DNA conformational changes and DNA bending angles in complexes. The E_{FRET} also serves as a signal to measure the K_d of RNAP–DNA complexes and the kinetics of DNA bending and unbending.

6.3.2 DNA Substrates for FRET Studies

FRET measurements require DNAs that are doubly labeled with donor and acceptor fluorophores. Commercial DNA synthesis facilities covalently link the fluorophore through a linker to the 5'-end or the 3'-end of the ssDNA or to the dT base within DNA. A donor and acceptor pair must be chosen, and several are available with Förster distance (R_0) ranging from 50 to 85 Å. The R_0 is the distance (r) between the fluorophores when E_{FRET} is 50 %. For example, the R_0 of Fluorescein–TAMRA or Cy3–Cy5 is ~ 55 Å, TAMRA–Alexa647 or Alexa488–Alexa546 is ~ 65 Å, and Alexa594–Alexa647 is 85 Å. Thus, depending on the size of the consensus promoter, appropriate pairs and attachment sites on the duplex DNA are chosen to maximize the FRET signal from DNA bending. The FRET pairs TAMRA–Alexa647 and Alexa488–Alexa546 were used in the studies of T7 and mt RNAP [22, 30, 47, 50, 19, 26]. One can also label the DNA with an intrinsic 2-aminopurine probe (2-AP) (Sect. 6.4.2) making it a triply labeled DNA, which can monitor both DNA bending and opening [22].

The fluorophore-labeled ssDNAs are purified by denaturing PAGE that separates labeled and unlabeled molecules. It is important to accurately determine concentration of each strand based on extinction coefficient of the DNA as well as the fluorophore [51]. The two

labeled strands are then annealed in a 1:1 ratio and PAGE purified (Sect. 6.2.2). In addition to the doubly labeled dsDNA, one also needs donor-only and acceptor-only control dsDNAs, where only one strand is fluorescently labeled, while the complementary strand is unlabeled.

6.3.3 FRET Studies for Monitoring DNA Bending

6.3.3.1 (Ratio)_A Method—The (ratio)_A method is an accurate way to measure the E_{FRET} [52, 22]. In this method, the acceptor emission after donor excitation is divided by the acceptor emission when it is directly excited, and the resulting ratio is used to determine the E_{FRET} . Thus, the experiment includes measuring three different steady-state fluorescence spectra: (a) acceptor emission in doubly labeled DNA after donor excitation $F_{\text{DA}}(\text{ex}_D, \text{em}_A)$, (b) acceptor emission in doubly labeled DNA when the acceptor is directly excited $F_{\text{DA}}(\text{ex}_A, \text{em}_A)$, and (c) donor emission in donor-only DNA excited at its own excitation wavelength $F_D(\text{ex}_D, \text{em}_D)$. The value of (ratio)_A is calculated from Eq. (6.6):

$$(\text{ratio})_A = \frac{F_{\text{DA}}(\text{ex}_D, \text{em}_A) - \alpha \cdot F_D(\text{ex}_D, \text{em}_A)}{F_{\text{DA}}(\text{ex}_A, \text{em}_A)} \quad (6.6)$$

where α is a normalization factor of donor fluorescence in the absence and presence of FRET, i.e., $F_{\text{DA}}(\text{ex}_D, \text{em}_D)/F_D(\text{ex}_D, \text{em}_D)$. Depending on the FRET fluorophore pairs used and their spectral overlap, the donor excitation wavelength slit is between 1 and 2 nm to minimize direct excitation of the acceptor. E_{FRET} is linearly dependent on the measured (ratio)_A by Eq. (6.7):

$$(\text{ratio})_A = E_{\text{FRET}} d^+ \frac{\varepsilon_{\text{ex D}}^D}{\varepsilon_{\text{ex A}}^A} + \frac{\varepsilon_{\text{ex D}}^A}{\varepsilon_{\text{ex A}}^A} \quad (6.7)$$

where d^+ is the labeling ratio with the donor dye, $\varepsilon_D(\text{ex}_D)$ and $\varepsilon_A(\text{ex}_A)$ are the extinction coefficients of the donor and acceptor at their respective excitation wavelengths, and $\varepsilon_A(\text{ex}_D)$ is the extinction coefficient of the acceptor at the donor's excitation wavelength. Ideally, labeled DNA should be PAGE purified from the unlabeled DNA, resulting in d^+ as 1.

Since the observed E_{FRET} depends on the concentration of the complex, the doubly labeled DNA is titrated with increasing amounts of RNAP to determine the maximal E_{FRET} . The binding fraction of DNA is related to the measured E_{FRET} by Eq. (6.8):

$$f_b = \frac{E - E_0}{E_b - E_0} \quad (6.8)$$

where E_0 and E_b are starting and ending E_{FRET} values, respectively, in the titration of DNA with increasing RNAP. One can also obtain the RNAP promoter K_d by plotting the observed E_{FRET} as a function of RNAP concentration and fitting it to a quadratic equation (Eq. 6.3).

In studies of mt RNAP, the promoter DNAs were 20 bp in length (−12 to +8) with TAMRA and Alexa647 attached to the two 5' ends. Representative traces of change in E_{FRET} of promoter (Fig. 6.3b) and non-promoter DNA (Fig. 6.3c) with increasing concentrations of Rpo41 and Rpo41–Mtf1 are shown [19]. The E_{FRET} of free DNAs is ~ 0.3 which increased to ~ 0.45 upon addition of Rpo41 (Fig. 6.3b, c, empty triangles). This indicates that Rpo41 induces a slight bend in both the promoter and non-promoter DNAs. In contrast, similar experiments with Rpo41–Mtf1 resulted in a higher E_{FRET} of ~ 0.74 with the promoter DNA, but not with the non-promoter DNA (Fig. 6.3b, c respectively, circles). This indicates that Rpo41–Mtf1 recognizes the promoter sequence and induces a selective severe bending in the promoter DNA. The non-promoter sequence actually showed a final decrease in E_{FRET} , which indicates DNA unbending. The titrations also provide K_d values, which are consistent with the K_d values obtained from the fluorescence anisotropy-based titrations (Sect. 6.2.3) [19].

FRET studies of mt RNAP promoter under single-molecule conditions are consistent with the ensemble FRET studies and show that Mtf1 is needed to bend the promoter [49]. The advantage of single-molecule studies is separation of populations and measurement of dynamics of bending/unbending of single molecules in real time. Single-molecule time traces showed that Rpo41 alone can bend the promoter DNA to the same degree as Rpo41–Mtf1, however, with a very low frequency. Thus, the low E_{FRET} of the Rpo41-promoter complex observed in ensemble studies is not due to a small bending angle, but a consequence of the rare bending events [49, 17]. The single-molecule FRET time traces measured the DNA bending rates; k_{bending} by Rpo41 alone is 0.0052 s^{-1} and by Rpo41–Mtf1 is 2.8 s^{-1} . The $k_{\text{unbending}}$ by Rpo41 is 3.0 s^{-1} and by Rpo41–Mtf1 is 0.066 s^{-1} [49].

The donor and acceptor fluorophores were placed in the 31-bp T7 promoter at −22 and +9 positions. Due to the greater separation between the donor and acceptor, the E_{FRET} of free DNA is much lower (0.02). However, in the presence of T7 RNAP, the E_{FRET} increases to 0.06 and further to 0.12 in the presence of the initiating GTP, which indicates that GTP aids in promoter bending [22]. Similarly, the E_{FRET} of free pre-melted promoter (bubble of 6 bp) is low (0.02), and upon addition of T7 RNAP, E_{FRET} increases to 0.12 and addition of GTP has little effect. This indicates that GTP binding drives the T7 RNAP complex toward the bent open state that resembles the pre-melted promoter.

6.3.3.2 Measurement of the Donor–Acceptor Distance—The end-to-end distance of a linear doubly labeled DNA can be calculated from the observed E_{FRET} (Eq. 6.9) assuming helical geometry of the B-type duplex DNA in aqueous solution [52]. FRET efficiency is inversely proportional to the sixth power of the distance between the donor and the acceptor:

$$R = R_0 \left[\frac{1 - E_{\text{FRET}}}{E_{\text{FRET}}} \right]^{\frac{1}{6}} \quad (6.9)$$

where R_0 is the Förster radius of the donor-acceptor pair (D–A) at which 50 % of E_{FRET} occurs and R is the actual D–A distance measured from E_{FRET} . As indicated above, when Rpo41 binds to promoter or non-promoter DNA, the E_{FRET} of the DNA increases from 0.3

to 0.45, which indicates that the D–A distance decreases by ~ 8 Å [19]. On the other hand, when Mtf1 is added to the complex of Rpo41 and promoter DNA, the E_{FRET} increases from 0.45 to 0.74, which indicates that the D–A distance decreases by an additional 13 Å, resulting in a total shortening of ~ 21 Å compared to free DNA. The E_{FRET} of T7 promoter with fluorophores at –22 and +9 indicate D–A distance of 113 Å, which decreases to 99 Å upon binding T7 RNAP and 86 Å upon further addition of GTP.

6.3.3.3 Measurement of the DNA Bending Angle—Increase in E_{FRET} in the end-labeled promoter DNA upon RNAP binding is indicative of DNA bending. The bending angle is calculated from the measured distance between the donor–acceptor pair using simple trigonometry. If the DNA is relatively small, one can assume the simplest “single-kink” model, where RNAP binds the promoter and bends it at a single point. In such cases, the bending angle α can be calculated as shown in Eq. (6.10):

$$R^2 = L_1^2 + L_2^2 + 2L_1L_2\cos\alpha \quad (6.10)$$

where R is the measured D–A distance, and L_1 and L_2 are the DNA lengths upstream and downstream of the bending center. Bending angles can vary depending on what model one assumes, for instance, single-kink, two-kink, or smooth bend [53], as well as the positions of these kinks, which can be distinguished only through additional experiments such as crystallography.

Based on the calculated end-to-end distances and assuming a bend centered at the –1 position in the promoter DNA (+1 transcription start site), it was calculated that Rpo41 bends the DNA by $\sim 52^\circ$ [19]. On the other hand, Rpo41–Mtf1 bends the promoter DNA by $\sim 90^\circ$. Similarly, T7 RNAP bends the promoter by 80 – 90° in the presence of GTP [22, 32]. These values are consistent and close to ~ 55 – 80° bending angle of the *Escherichia coli* RNAP initiation open complex [54, 55].

6.4 Promoter Opening

Melting of the duplex promoter is a key step wherein a specific region of the promoter is unwound by the RNAP, so the template strand becomes accessible for binding to the incoming NTPs. Many footprinting methods have been developed over the years to map the open complex [56–59] using DNase I, potassium permanganate, diethylpyrocarbonate, or hydroxyl radicals, each with its own set of merits and demerits. Fluorescence footprinting using 2-AP has gained popularity in recent times, being less time consuming and relatively easy to carry out compared to other gel-based techniques, and has been successfully used to map open complexes with single nucleotide resolution, as well as to study opening–closing dynamics in real-time [23, 60, 61, 49, 43, 62]. In both T7 RNAP and yeast mt RNAP complexes, the open DNA region is mapped to –4 to +2 region using 2-AP fluorescence assays described below [22, 19, 63].

6.4.1 2-Aminopurine Fluorescence

2-AP acts as a fluorescent adenine analogue that can form Watson–Crick-type base pairs with thymine (Fig. 6.4a). Since promoters contain many A:T base pairs in the region that is melted by RNAPs, promoters can be modified with 2-AP to spectroscopically monitor open complex formation [43, 23, 64]. The fluorescence intensity of 2-AP is highly sensitive to base-stacking interactions in the DNA helix [65, 66]. The 2-AP fluorescence emission intensity varies depending on the identity of the neighboring bases, as well as external factors like pH and temperature [67, 68, 66]. Compared to the fluorescence of free 2-AP nucleoside, the fluorescence of 2-AP (excitation at 315 nm and emission at 370 nm) in the duplex DNA is quenched and partly recovered when the DNA is melted on addition of RNAP and 2-AP is in the ssDNA region (Fig. 6.4b). If the base is flipped and unstacked, then there is a much larger 2-AP fluorescence increase.

6.4.2 Equilibrium Studies of Open Complex Formation

6.4.2.1 Mapping of the Open Complex—To use fluorescence footprinting to map the melted DNA region, chemically synthesized promoter DNA is prepared where the adenines are systematically substituted with 2-AP, one at a time (Fig. 6.4c). The duplex promoter DNA contains only one 2-AP at a known position. The steady-state fluorescence measurements are carried out on a spectrofluorometer. The 2-AP modified promoter and RNAP are thoroughly mixed and incubated at desired temperature and for a predetermined time interval to reach equilibrium, before exciting at 315 nm. A control experiment using RNAP with unmodified DNA is necessary, because of the spectral overlap between RNAP and 2-AP fluorescence. After subtracting fluorescence intensity contributions from the RNAP, the corrected 2-AP fluorescence intensities at 370 nm are compared for different positions. Such fluorescence footprinting showed that the promoter DNA in the open complexes of T7 RNAP and yeast mt RNAP are melted from -4 to $+2$, with respect to the start site at $+1$ [61, 43, 69]. In T7 RNAP, this result was confirmed by crystallography studies of the open complex [69]. The greatest increase in fluorescence was observed at the -4 position indicative of flipping and unstacking of the -4 base from the neighboring base (Fig. 6.4d). This was confirmed by crystal structures of T7 RNAP that showed that the promoter template is bent between positions -4 and -5 , and the -4 template base is unstacked from -3 base in the open complex. Even with mt promoter, highest fluorescence is observed at the -4 position, indicating a similar bend between positions -4 and -5 in the template. In case of mt RNAP, both Rpo41 and Mtf1 are required to observe 2-AP fluorescence increase in the promoter. Thus, the inability of Rpo41 to bend the promoter correlates with its inability to melt the promoter. This temporal coupling of bending and opening steps during open complex formation was verified using triply labeled promoter with FRET probes and 2-AP for the T7 RNAP complex [22, 30].

6.4.2.2 Equilibrium Binding of Promoter by 2-AP Fluorescence Changes—One can also use 2-AP fluorescence change as a signal to measure the K_d and stoichiometry of the promoter-RNAP complex. Typically, RNAP is titrated with increasing concentrations of 2-AP modified promoter [23, 70]. Since 2-AP at -4 undergoes the largest increase in fluorescence, a single 2-AP at the -4 position can provide the signal for these experiments. To determine the stoichiometry of the complex, concentrations are kept well above their K_d

values. To determine the K_d values, concentrations are kept close to the K_d values. Light absorption and emission by RNAP itself can be significant, because 2-AP excitation (315 nm) and emission (370 nm) are close to the tryptophan and tyrosine absorption (280–290 nm) and emission (340 nm). Therefore, it is critical to correct the observed 2-AP fluorescence for inner-filter effects in addition to volume changes using Eq. (6.11):

$$F_c = F_{\text{obs}} \times \left(\frac{v_f}{v_0} \right) \times 10^{0.5(\text{Abs}_{\text{ex}} + \text{Abs}_{\text{em}})} \quad (6.11)$$

where F_c is corrected fluorescence, F_{obs} is the observed fluorescence intensity, v_f is the final volume of the solution, v_0 is the initial volume, Abs_{ex} is the absorbance of the RNAP–DNA solution at the 2-AP excitation wavelength of 315 nm, and Abs_{em} is the absorbance of the same solution at the 2-AP emission wavelength of 370 nm. The concentration of the total RNAP and DNA at each point in titration can be determined by Eqs. (6.12) and (6.13):

$$[E]_i = \frac{v_0}{v_{if}} [E]_0 \quad (6.12)$$

$$[D]_i = \frac{v_0}{v_{if}} [D]_0 \quad (6.13)$$

where $[E]_i$ and $[D]_i$ are the concentrations of T7 RNAP and DNA, respectively, up to point i in titration, $[E]_0$ and $[D]_0$ are the initial concentrations, v_{if} is the volume of the solution up to point i in the titration, and v_0 is the initial volume.

By conducting multiple titrations, where Mtf1 or 2-AP modified promoter or Rpo41 were titrated against each other (Fig. 6.4e), it was determined that a 20 bp mt RNAP promoter forms a 1:1:1 complex with Rpo41 and Mtf1 [43]. Similar experiments with longer promoters showed multiple Rpo41–Mtf1 per DNA.

6.4.3 Kinetics of Open Complex Formation

Transcription can be regulated at the promoter opening step, where the extent and/or the rate of opening is modulated by transcription factors and small ligands. To determine the rate of promoter opening, the 2-AP fluorescence change is monitored as a function of time after mixing the promoter with RNAP in a stopped-flow instrument (Fig. 6.4f). The experiment is conducted with increasing concentrations of RNAP under pseudo-first-order conditions, and multiple time traces (at least 7–8 shots) are averaged at each RNAP concentration, and the data are fit to a single exponential equation:

$$y = A \times (1 - e^{-k_{\text{obs}} t}) + C \quad (6.14)$$

where y is observed fluorescence, A is fluorescence change, t is time, k_{obs} is the observed rate, and C is y-intercept.

In T7 RNAP, the observed rate of 2-AP fluorescence increase was not dependent on the position of the probe within the -4 to -1 region, indicating that the entire DNA region is melted in a coordinated fashion [61]. The observed rates increased in a hyperbolic manner with increasing T7 RNAP concentration (Fig. 6.4g) [71], which supported a two-step mechanism of promoter binding (Scheme 6.2) with an initial closed complex (ED_c) followed by open complex (ED_o):

Only one kinetic phase was observed in the 2-AP stopped-flow studies, which indicates that the closed complex intermediate is in rapid equilibrium with free RNAP and promoter, and the observed rate is a function of the equilibrium constant of the closed complex (K_d) and the sum of promoter opening and closing (k_{max}) (Eq. 6.15):

$$k_{obs} = C + \frac{k_{max}[E]}{K_d + [E]} \quad (6.15)$$

If $E + D \rightarrow ED_c$ is not in rapid equilibrium and one observes multiple phases in the binding kinetic trace, the data are computationally fit to the two-step model using KinTek Explorer Pro Software (KinTek Corp., Austin, TX). Data fitting showed that $k_{max} = 180 \text{ s}^{-1}$ and K_d of the closed complex was $1 \text{ }\mu\text{M}$. In the presence of GTP, k_{max} decreased to 32 s^{-1} . Global fitting of the two sets of data, in absence and presence of GTP, yielded the promoter opening rate as $\sim 30 \text{ s}^{-1}$ and closing rate as $\sim 150 \text{ s}^{-1}$ [71].

6.5 Initial Transcription and Transition into Elongation

Once the open complex is formed, the initiating $+1$ and $+2$ NTPs bind at the RNAP's active site to base pair with the melted template bases and to catalytically react to form the first 2-mer RNA product. After RNA of certain length is synthesized, the RNAP transitions into the elongation mode by losing contacts with the promoter region [72, 73], where it couples processive RNA synthesis to downstream DNA unwinding. Along with transcription initiation, the transition from initiation to processive elongation is a critical step that regulates the synthesis of full-length RNA [21, 25, 26]. This transition from promoter-specific initiation to promoter-independent elongation is a multistep process, accompanied by conformational changes in both RNAP and DNA [20, 50, 33, 74]. It is extremely difficult to capture these intermediates in real time because of the transient and heterogeneous nature of the subpopulations. Hence, methods were developed to stall the RNAP at different positions by addition of limited NTPs and studying the associated DNA conformational changes using FRET and 2-AP assays, as described below.

6.5.1 FRET Studies to Monitor DNA Conformational Changes

FRET measurements on stalled complexes have provided snapshots of the conformational changes in RNAP–DNA complexes as reaction proceeds from IC2 to IC7 (IC: initiation complex) and as RNAP makes the transition from initiation to elongation (EC10: elongation complex at position 10) (Fig. 6.5a) [47]. Ensemble FRET studies of T7 RNAP carried out with T7 promoter labeled with TAMRA at -4 , and Alexa647 at $+18$ positions ($R_0 \sim 67 \text{ }\text{\AA}$) showed a progressive increase in E_{FRET} from a low value with free DNA to a maximum

value with RNAP-bound IC7 and then a decrease from IC7 to EC10. These results indicate progressive bending of the promoter DNA as the RNA:DNA hybrid grows from 2 to 7 bp and then DNA unbending upon transition into elongation. This conclusion was confirmed by single-molecule FRET measurements carried out with Cy3–Cy5-labeled T7 promoter (–4 and +17 positions) that showed increase in E_{FRET} with increasing lengths of RNA:DNA hybrid. Additionally, the single-molecule FRET studies were able to separate the IC population from the EC population that started appearing after IC7 [50].

The bending–unbending dynamics of DNA were monitored in real time by following the changes in E_{FRET} in stopped-flow assays. In these experiments, T7 RNAP was preincubated with T7 promoter labeled with TAMRA at –22 and Alexa647 at +18, and E_{FRET} change was measured as a function of time after adding a limited set of NTPs to walk RNAP to a particular position on the template DNA (Fig. 6.5b) [50]. At the start, a rapid increase in E_{FRET} in all the IC5–12 complexes was observed, indicative of DNA bending during initiation (Fig. 6.5c). However after IC7, there was a late phase of E_{FRET} decrease, consistent with DNA unbending. Both stopped-flow and single-molecule E_{FRET} time courses of T7 RNAP, walking from +8 to +12 positions, provided rates of transition from initiation to elongation, which revealed efficient transition after 12-mer RNA synthesis [50].

6.5.2 Measurement of Initial Bubble Collapse

When RNAP makes the transition from initiation to elongation, the initially melted promoter region from –4 to +2 closes (bubble collapse), and the bubble propagates to downstream region. The initial bubble collapse process was monitored in real time using a stopped-flow setup (Sec. 6.5.1) by following the decrease in 2-AP fluorescence. The T7 promoter was labeled with 2-AP at the –4 position, and T7 RNAP was walked to different positions on the template DNA using limited NTPs (Fig. 6.5b). Up to the synthesis of 7-mer RNA, only an increase in 2-AP fluorescence was observed as GTP drives open complex formation (Fig. 6.5c) [72]. However, once the RNA length reached 8-mer stage, the initial increase in 2-AP fluorescence was followed by a phase of decreasing fluorescence, which indicated collapsing of the initial bubble. Interestingly, the observed rate of fluorescence decrease became progressively faster as RNA transcript length increased from 8 to 12-mer, which indicates that the bubble collapse process becomes more probable or efficient as the RNA length increases. This conclusion from real-time 2-AP studies is consistent with the FRET studies and shows that initial bubble closing and DNA unbending are temporally coupled (Fig. 6.5c).

6.6 Concluding Remarks

This chapter describes several ensemble fluorescence-based methods to measure the individual steps of transcription initiation, including binding of RNAP to the promoter, bending of the promoter, open complex formation, and binding of the initiating nucleotides, as well as the transition from initiation to elongation. A combination of fluorescence stopped-flow kinetics and equilibrium binding assays, along with radiometric quenched-flow assays [14, 75, 13] that are not described here, provides a complete description of the transcription initiation pathway. Having a detailed understanding of the steps and quantified rate and equilibrium constants allows the investigator to ask how variations in promoter

sequence, presence or absence of transcription factors, small ligands, or external conditions regulate transcription and which particular step is affected. Even if the individual steps are affected to a small extent, the cumulative effect on the final amount of RNA product can be significant. The findings from the ensemble studies provide the basis to conduct studies at the single-molecule level to tease out populations. These biochemical studies together with high-resolution structures are comprehensive means to obtain the fundamental insights that are required understand the mechanism of transcription.

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Abbreviations

2-AP	2-Aminopurine fluorescence
bp	Base pair
DNA	Deoxyribonucleic acid
ds	Double stranded
EC	Elongation complex
FRET	Förster resonance energy transfer
IC	Initiation complex
K_d	Equilibrium dissociation constant
mt	Mitochondrial
NTP	Nucleoside triphosphate
RNA	Ribonucleic acid
RNAP	RNA polymerase
ss	Single stranded
TAMRA	Tetramethylrhodamine

Fig. 6.1.

Fluorescence methods probe the specific steps of transcription initiation. RNA polymerase (E) binds to the promoter DNA (D) to form a closed complex (EDc), which is converted to an open complex (EDo), where the DNA is sharply bent and locally melted. The initiating NTPs bind at the active site of the RNAP and base pair with +1 and +2 template bases to form the initially transcribing complex (EDoNN) that undergoes reaction to form the 2-mer RNA bound to the initiation complex (IC₂). The 2-mer RNA is elongated to 8-mer and 12-mer in the initiation phase (IC₈₋₁₂), and then the RNAP makes the transition into the elongation complex (EC₁₂). Short 2–12 mer RNAs readily dissociate in solution to accumulate as abortive products

Fig. 6.2.

Fluorescence anisotropy methods measure the K_d and kinetic *on*- and *off*-rate constants. (a) Fluorescence anisotropy is lower when the fluorophore-labeled DNA is not bound to RNAP and increases when the DNA binds to RNAP, because the larger complex does not readily depolarize the light. (b) The yeast mt promoter (15S rRNA U12D8) labeled with fluorophore TAMRA (5 nM) is titrated with increasing Rpo41 (empty triangles) or 1:1.2 ratio mixture of Rpo41 and Mtf1 (red circles), and the increase in fluorescence anisotropy (excitation at 555 nm and emission at 580 nm) is fitted to Eqs. (6.1) and (6.3) to obtain the K_d values of the complexes. (c–e) TAMRA-labeled mt promoter DNA (40 nM) from one syringe of the stopped-flow instrument is rapidly mixed with Rpo41 or Rpo41–Mtf1 (120 nM) from a second syringe. The kinetics of the anisotropy increase (d) are fitted to a single exponential function (Eq. 6.4). The observed rates increase linearly with increasing protein concentration (e) and hence the slope estimates the *on*-rate constant. (f–h) Preformed

complex of Rpo41 or Rpo41–Mtf1 with TAMRA-labeled mt promoter (40 nM) from one syringe of the stopped-flow instrument is mixed with unlabeled mt promoter (1 μ M) from the other syringe. The decrease in fluorescence anisotropy with time at 100 nM Rpo41 (**g**) and 100 nM Rpo41–Mtf1 (**h**) is fit to a single exponential function (Eq. 6.4) to obtain the *off-rate* [19]

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Fig. 6.3.

FRET methods to study promoter bending and unbending. (a) The spectral overlap (yellow-striped region) between the donor (green) and acceptor (red) fluorophores attached to the DNA enable FRET measurements. When the RNAP binds to the promoter DNA and induces a bend, the distance (R_{DA}) between the two fluorophores decreases, leading to an increased E_{FRET} monitored as a decrease in donor's emission or increase in acceptor's emission intensity. The bending angle α is calculated from the R_{DA} value and the assumed upstream L_1 and downstream L_2 DNA lengths based on a single-kink model of DNA. Yeast mt promoter (b) and non-promoter DNAs (c), doubly labeled with TAMRA and Alexa Fluor 647, are titrated with increasing concentration of Rpo41 (empty triangles) or Rpo41–Mtf1 (red circles). The E_{FRET} provides the DNA bending angles and through titrations the K_d values of complexes [19]

Fig. 6.4.

Promoter opening and initial bubble collapse using 2-AP modified promoters. **(a)** The adenine analogue 2-AP base pairs with thymine in DNA. **(b)** The 2-AP fluorescence is quenched when 2-AP base is stacked in the duplex DNA and increases when the DNA is melted by RNAP. **(c)** Positions of the 2-AP (*red underlined*) in the template and non-template strands relative to the -8 to +1 consensus region (*dotted black box*) of the yeast mt 15S rRNA promoter. **(d)** Fluorescence mapping indicates that the -4 to +2 region is melted in the open complex [*dotted red box* in (c)]. **(e)** 2-AP(-4NT) mt promoter (200 nM) was titrated with increasing concentrations of a 1:1 mixture of Rpo41 and Mtf1. The 2-AP fluorescence increases and saturates at ~270 nM Rpo41-Mtf1 demonstrating the 1:1:1 stoichiometry of Rpo41-Mtf1-DNA. **(f)** Stopped-flow assay to measure the kinetics of promoter opening. The 2-AP modified T7 promoter from one syringe is mixed with T7 RNAP (at various concentrations) with and without the initiating NTPs. **(g)** The observed

rates in the absence (*red*) or the presence (*blue*) of 3'dGTP plotted as a function of T7 RNAP concentration provide estimates of promoter opening and closing rates [43] [71]

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Fig. 6.5.

FRET and 2-AP fluorescence assays to study conformational changes in the DNA during transition from initiation to elongation. **(a)** FRET between fluorophores attached to -4 and +18 bases of the T7 promoter measure DNA bending during transcription initiation. Complex RP (RNAP promoter) was formed by incubating T7 RNAP (120 nM) with dual labeled T7 promoter (100 nM). The IC2-7 and IC/EC10 stalled complexes were formed by addition of limited NTPs. The distances (R_{DA}) were calculated from E_{FRET} and R_0 . **(b)** Stopped-flow assay to measure the kinetics of DNA unbending and initial bubble collapse. T7 RNAP was walked to +5, +9, and +12 positions on the template using limited NTPs, and acceptor fluorescence and 2-AP fluorescence were measured as a function of time after mixing. **(c)** Representative kinetic traces of FRET and 2-AP fluorescence changes for the IC5-12 complexes showing temporal coupling between DNA unbending and initial bubble collapse [47, 50, 72]

Scheme 6.1. One-step binding of RNAP to DNA

Scheme 6.2. Two-step mechanism of RNAP binding to DNA