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Fluorescence Resonance Energy Transfer Characterization of DNA Wrapping in Closed and Open *Escherichia coli* RNA Polymerase– λP_R Promoter Complexes

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Abstract

Initial recognition of promoter DNA by RNA polymerase (RNAP) is proposed to trigger a series of conformational changes beginning with bending and wrapping of the 40–50 bp of DNA immediately upstream of the –35 region. Kinetic studies demonstrated that the presence of upstream DNA facilitates bending and entry of the downstream duplex (to +20) into the active site cleft to form an advanced closed complex (CC), prior to melting of ~13 bp (–11 to +2), including the transcription start site (+1). Atomic force microscopy and footprinting revealed that the stable open complex (OC) is also highly wrapped (–60 to +20). To test the proposed bent-wrapped model of duplex DNA in an advanced RNAP– λP_R CC and compare wrapping in the CC and OC, we use fluorescence resonance energy transfer (FRET) between cyanine dyes at far-upstream (–100) and downstream (+14) positions of promoter DNA. Similarly large intrinsic FRET efficiencies are observed for the CC (0.30 ± 0.07) and the OC (0.32 ± 0.11) for both probe orientations. Fluorescence enhancements at +14 are observed in the single-dye-labeled CC and OC. These results demonstrate that upstream DNA is extensively wrapped and the start site region is bent into the cleft in the advanced CC, reducing the distance between positions –100 and +14 on promoter DNA from >300 to <100 Å. The proximity of upstream DNA to the downstream cleft in the

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00125. DNA sequence, methods of preparation of DNAs, and Figures S1–S9 (PDF)

Notes

The authors declare no competing financial interest.

advanced CC is consistent with the proposed mechanism for facilitation of OC formation by upstream DNA.

Graphical abstract

Efficient conversion of closed promoter complexes (generically called CC) to open complexes (generically OC) requires interactions of RNA polymerase (RNAP) with upstream and downstream DNA as well as with the central recognition region from the -35 element to the transcription start site ($+1$).^{1,2} Interactions of RNAP with the 20 bp region downstream of $+1$ are key players in the conversion of the initial unstable OC to the more stable OC.^{3–5} Interactions of RNAP with 40–50 bp of DNA upstream of the -35 element, including the region of the UP element and far-upstream DNA, are necessary for rapid isomerization of the CC to the OC. Truncations in this upstream region reduce isomerization rates of lacUV5 and λP_R promoters by up to 2 orders of magnitude.^{6–8}

How do upstream and downstream DNA exert these effects? Formation of the initial CC (often called RP_c or RP_{c1}) in which the promoter DNA is presumed to be linear and interactions with RNAP extend downstream only to the discriminator region (-7 to -2)^{9–11} sets in motion a series of conformational changes in RNAP and promoter DNA.^{1,2,7,8,12–14} To explain the large decrease in the isomerization rate during OC formation that results from the truncation of upstream DNA, as well as the features observed in real-time footprints of CC at full-length and truncated promoter DNA,⁸ we proposed that bending and wrapping of the UP element region and far-upstream DNA on RNAP are among the first of these large conformational changes.^{1,7}

Wrapping of upstream DNA around RNAP brings it near the α NTD (which act as hinges at the base of the active site cleft) and also near the downstream cleft, triggering conformational changes in the cleft and in downstream mobile elements (DMEs) of RNAP at the λP_R promoter.^{1,4,12} These conformational changes facilitate bending of the downstream duplex into the cleft to form the most advanced CC prior to opening, in which more than 100 bp of promoter DNA (-82 to $+20$) interacts with RNAP.^{1,2,4,7} The first kinetically significant intermediate (previously designated I_1) formed at the full-length λP_R

promoter is a population of advanced CC.^{7,8,14} Subsequent isomerization of I_1 was shown to involve DNA opening in the cleft by RNAP, forming the initial OC (a kinetically significant intermediate in the dissociation at λP_R promoter, designated I_2).¹⁵ At many promoters, after opening, rearrangements of the nontemplate strand in the cleft and of DME of RNAP that assemble on the downstream duplex rapidly convert the initial OC to a more stable OC.^{4,5} At λP_R , this rapid stabilization of I_2 occurs in several steps, with an additional, more advanced open intermediate (designated I_3 , which is kinetically significant during dissociation)¹⁶ on the pathway to formation of the most stable OC (designated RP_o at λP_R).^{4,5} In this work, the advanced λP_R closed complex investigated (designated CC) is that formed at equilibrium at temperatures below the opening transition (i.e., 0–5 °C).^{12,14,17} This is a RP_{c2} -like complex¹⁸ analogous to the kinetic intermediate I_1 .^{7,12} The λP_R open complex investigated (designated OC) is that formed at 19 °C (presumably largely RP_o).^{12,17}

While far-upstream (–65 to –82) interactions are eliminated in conversion of the advanced CC to the stable λP_R OC, footprinting reveals that at least 80 bp (–65 to +20) of λP_R promoter DNA remains in tight contact with RNAP,⁷ twice the length of the DNA contacted in the elongation complex (40 bp; –20 to +20).¹⁹ AFM DNA compaction studies reveal that the λP_R promoter DNA is wrapped around RNAP in stable OC,^{20,21} and also in abortively initiating complexes.²² Hence, evidence of extensive bending and wrapping of promoter DNA in both advanced λP_R CC and OC exists. Unwrapping must occur during or before promoter escape.

Bending and/or wrapping of DNA appear to be general properties of the CC and OC at other bacterial promoters and for eukaryotic RNAP. Extensive bending and wrapping of TyrT promoter DNA in a CC with RNAP were observed by AFM.²³ Transcription factors such as FIS and CAP bind to upstream DNA in the region of the UP element (–40 and –60), bending (CAP)²⁴ and wrapping (FIS)^{23,25} promoter DNA in the CC. Members of the metal-dependent MerR family of transcriptional regulators that recognize the spacer region of promoter DNA convert the early CC formed in the repressed (apo or metal-free) state, in which the –10 region is not contacted,^{26–28} to an activated state upon metal binding that unwinds and bends this region to contact σ region 2 in a more advanced CC.²⁷ Extensive wrapping of promoter DNA in CC formed by eukaryotic RNA Pol II was also observed by cross-linking and electron microscopy.^{29–31} Evidence of wrapping in the OC at other bacterial promoters has emerged from footprinting,^{9,32} supercoiling assays,³³ and gel shift assays.³⁴ To date, however, no direct spectroscopic assay for investigating structural, thermodynamic, and kinetic–mechanistic aspects of promoter bending and wrapping has been reported. Here we use ensemble Förster (fluorescence) resonance energy transfer (FRET) from Cy3 to Cy5 cyanine dyes attached at the upstream (–100) and downstream (+14) ends of λP_R promoter duplexes as a quantitative assay for the large reduction in the end-to-end distance of promoter DNA anticipated from bending and wrapping in both the CC and the OC. We also use RNAP-induced cyanine dye fluorescence enhancements often called PIFE effects^{35–41} at +14 to monitor the proximity of the +14 position of promoter DNA to RNAP in closed and open complexes. In ongoing research, we are applying these FRET and PIFE assays in fast kinetic studies of the roles of promoter bending and wrapping in the mechanism of formation and advancement of the initial CC, the isomerization to the

initial OC, and the subsequent stabilization of the OC by in-cleft and downstream conformational changes.

MATERIALS AND METHODS

Buffers

Storage buffer (SB) for the RNAP holoenzyme consists of 50% (v/v) glycerol, 10 mM Tris (pH 7.5 at 4 °C), 100 mM NaCl, 0.1 mM dithiothreitol (DTT), and 0.1 mM Na₂EDTA. Annealing buffer (AB) consists of 0.1 M potassium glutamate, 25 mM Tris-acetate (pH 7.6), and 10 mM magnesium acetate (“KGB buffer”).^{42,43} Buffer for FRET experiments (FB) consists of 40 mM Tris (pH 8), 10 mM MgCl₂, 120 mM KCl, 0.05 mg/mL BSA, 1 mM DTT, and 0.02% Tween. Buffer for transcription assays (TB) consists of 40 mM Tris (pH 8), 5 mM MgCl₂, 60 mM KCl, 0.1 mg/mL BSA, and 1 mM DTT. Quench dye buffer (QB) for transcription assays consists of TB with 8 M urea, 15 mM Na₂EDTA, 0.05% (w/v) xylene cyanol, and 0.05% (w/v) bromophenol blue. Stop solution for permanganate foot-printing consists of 1 M β -mercaptoethanol and 2.6 M ammonium acetate. Unless specified, all reagents were from Sigma-Aldrich (St. Louis, MO).

RNA Polymerase

Wild-type *Escherichia coli* RNAP core ($\alpha_2\beta\beta'\omega$) was overexpressed in *E. coli* BL21(λ DE3) cells from plasmid pVS10 and purified using standard protocols.⁴⁴ σ^{70} was overexpressed in *E. coli* strain M5219 containing plasmid pMRG8 and purified as previously described.⁴⁵ RNAP holoenzyme was reconstituted by incubating 5 μ M core RNAP with a 2-fold molar excess of purified σ^{70} for 1 h at 37 °C. Holoenzyme was stored at –20 °C for short-term use. Different preparations of reconstituted RNAP were 70–100% active in OC formation.

Preparation of Cy3- and/or Cy5-Labeled λ P_R Promoter and Cy3(+14)-Cy5(–100) Control DNA

Single-dye-labeled λ P_R [Cy3(+14) or Cy5(+14) λ P_R and Cy3(–100) or Cy5(–100) λ P_R], two-dye-labeled λ P_R [Cy3(–100)-Cy5(+14) or Cy3(+14)-Cy5(–100) λ P_R], and two-dye-labeled nonpromoter DNA [Cy3(+14)-Cy5(–100) nonpromoter DNA] as a control for FRET experiments were prepared from dye-labeled primers using the sequences and methods described in the Supporting Information.

Steady-State FRET and PIFE Fluorescence Methods

RNAP–promoter complexes were formed by incubating 100 nM Cy3-Cy5 λ P_R DNA and 20–120 nM RNAP for 20–90 min in FB at 2 °C (for CC) or 19 °C (for OC).^{12,17} No differences were observed between complexes incubated for these different times. FRET experiments with dye-labeled OC were performed at 19 °C because the fluorescence emission intensity of cyanine dyes decreases strongly with an increase in temperature (see also Figure S4).⁴⁶ Parallel series of experiments were performed with singly and doubly labeled 100 nM (Cy3, Cy5) promoter DNA. A Quantamaster C-60/2000 spectrofluorimeter (Photon Technology Instruments) equipped with a 75 W xenon compact arc lamp and single monochromators on the excitation and emission beam path was used for all experiments. Cuvettes were coated with Sigmacote before each experiment. Excitation and emission slit

widths were 3 and 5 nm, respectively. Samples were excited at 515 nm (for Cy3) or at 610 nm (for Cy5); these wavelengths were chosen to minimize direct excitation of Cy5 from excitation of Cy3 and to minimize overlap of Cy5 excitation and emission spectra. Emission spectra were collected between 550 and 720 nm for two-dye FRET experiments and for single-dye Cy3 experiments and between 620 and 720 nm for single-dye Cy5 experiments. Samples were removed from the instrument or shuttered when not being observed to minimize photodamage. For the temperature series experiment (Figure S4), the temperature was increased in steps and the cuvette equilibrated at each temperature before the emission scan.

Steady-State Fluorescence Anisotropy (FA) Methods

Anisotropy measurements were performed with a Quanta-master C-60/2000 spectrofluorimeter (Photon Technology Instruments) equipped with two polarizers in the paths for excitation and emission using the L-format method. Experimental settings were as in the FRET and PIFE experiments. Intensity values, I , were recorded over 120 s at 565 nm for Cy3 and 660 nm for Cy5 ($\lambda_{\text{max}}^{\text{em}}$ as determined from FRET and PIFE experiments). Anisotropy r was calculated as^{47,48}

$$r = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}} \quad (1)$$

The subscripts on I indicate the relative orientations (parallel and perpendicular) of the excitation and emission polarizers. G , the instrumental correction factor for different detection sensitivities for vertically and horizontally polarized light, was measured with Cy3- and Cy5-labeled primers separately. Individual values of $I_{\parallel}$ and $I_{\perp}$ used to calculate r from eq 1 were determined by averaging data from two scans in each determination. Values of r at 19 and 2 °C reported here are averages of three and two independent determinations, respectively. Uncertainties are reported as deviations from the average.

Predicted anisotropy r of the dye that is immobilized on DNA in the CC and OC and moves with only the entire dye–DNA–RNAP complex is estimated to be 0.4 (the maximal anisotropy) from the Perrin equation,⁴⁷ using Cy3 and Cy5 fluorescence lifetimes ($\tau \sim 2$ ns) and a characteristic time for rotational diffusion of the RNAP–DNA CC and OC modeled as spheres ($1/D_r \approx 700$ ns,⁴⁹ where D_r is the rotational diffusion coefficient). Because $\tau \ll 1/D_r$, the DNA–RNAP complex does not reorient significantly on the time scale of the fluorescence lifetime.

Calculation and Analysis of FRET Efficiency

FRET efficiency E was calculated from the emission of the acceptor (Cy5) by the “Ratio_A” method.^{47,50,51} For a CC or OC between Cy3–Cy5 labeled DNA and RNAP, Ratio_A at Cy5 $\lambda_{\text{max}}^{\text{em}}$ (660 nm)

$$\text{Ratio}_{A,660\text{nm}} = \frac{I_{660\text{nm}}^{\text{Cy3exc}}}{I_{660\text{nm}}^{\text{Cy5exc}}} \quad (2)$$

where $I_{660\text{nm}}^{\text{Cy3exc}}$ is the Cy5 emission intensity obtained by Cy3 excitation at 515 nm and $I_{660\text{nm}}^{\text{Cy5exc}}$ is the Cy5 emission intensity obtained by direct Cy5 excitation at 610 nm. For this calculation, the Cy5 portion of the emission spectrum for excitation of Cy3-Cy5 labeled DNA (at 515 nm) was determined by subtracting a reference spectrum obtained for Cy3 DNA (Figure S7). Prior to subtraction, this reference spectrum was normalized to match the fluorescence intensity of the Cy3-Cy5 labeled DNA-RNAP spectra over the wavelength range where only Cy3 emits (555–570 nm).

Analysis of Ratio_A yields FRET efficiency E from eq 3:^{50,51}

$$\text{Ratio}_{A,660\text{nm}} = E \frac{\epsilon_{\text{Cy3}}^{515\text{nm}}}{\epsilon_{\text{Cy5}}^{610\text{nm}}} + \frac{\epsilon_{\text{Cy5}}^{515\text{nm}}}{\epsilon_{\text{Cy5}}^{610\text{nm}}} \quad (3)$$

applicable when the same wavelength (here 660 nm) is used to measure acceptor emission from donor excitation (FRET) and from direct excitation of the acceptor.^{50,51} Extinction coefficients (ϵ) for Cy3 and Cy5 in the dye-labeled DNA at the excitation wavelengths used (515 nm for Cy3 and 515 and 610 nm for Cy5) were determined using variable-path-length technology (SoloVPE system from C Technologies). The absorbance of the Cy3- or Cy5-labeled DNA at a known concentration was measured for at least 10 different path lengths from 0.25 to 5 mm, from which $\epsilon_{\text{Cy5}}^{610\text{nm}}$, $\epsilon_{\text{Cy5}}^{515\text{nm}}$, and $\epsilon_{\text{Cy5}}^{515\text{nm}}$ were determined.⁴⁷ Values obtained ($\epsilon_{\text{Cy5}}^{610\text{nm}} = 9.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$; $\epsilon_{\text{Cy5}}^{515\text{nm}} = 5.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$; $\epsilon_{\text{Cy5}}^{515\text{nm}} = 9.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) are consistent with previously published values at other wavelengths when converted using the spectra of these dyes.⁵²

Values of E reported here have been corrected for the population fraction of free promoter DNA at each concentration of RNAP investigated. For the CC and OC formed by Cy3(–100)-Cy5(+14) λP_R DNA, Cy5(+14) PIFE is observed to make a 30% contribution (occupancy-corrected) to both the numerator and the denominator of eq 1 for Ratio_A and has no net effect on the calculation of the FRET E . For the CC and OC formed by Cy3(+14)-Cy5(–100) λP_R , Cy3(+14) PIFE is observed to make a 20% contribution (occupancy-corrected) to the numerator of Ratio_A and is accounted for in calculating Ratio_A .

Interdye distance R is calculated from efficiency E :

$$R = R_o \left(E^{-1} - 1 \right)^{1/6} \quad (4)$$

where the Förster distance, R_o , is the distance at which $E = 0.5$ for the Cy3-Cy5 pair and is given by⁴⁷

$$R_o = \left(9.78 \times 10^3 \right) \left[\Phi_D \kappa^2 \eta^{-4} J(\nu) \right]^{1/6} \text{ \AA} \quad (5)$$

In eq 4, refractive index $\eta = 1.33$, the spectral overlap integral for Cy3 and Cy5 is $J(\nu) = 7.2 \times 10^{-13} \text{ M}^{-1} \text{ cm}^3$,⁵³ Φ_D is the quantum yield of Cy3, and κ^2 is the orientation factor (see below).

The quantum yield of donor Cy3 (Φ_D) depends on temperature and on its environment, including attachment to single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA).⁴⁶ For cyanine dyes attached at position +14, RNAP (protein)-induced fluorescence enhancement (often called PIFE)^{35,36} contributes to the quantum yield and hence the emission intensity. In our dye-labeled DNA, Cy3 or Cy5 is inserted between position 5' of the end deoxyribose of dsDNA and position 3' of the first deoxyribose in a short ss region at the end of the duplex (see the Supporting Information). Correcting for PIFE effects by short extrapolation of a linear fit of Cy3-5' dsDNA Φ_D versus temperature⁴⁶ to 2 °C gives a Φ_D of 0.34 ± 0.03 and by interpolation a Φ_D of 0.21 ± 0.02 at 19 °C, when Cy3 is at +14. Similarly, $\Phi_D = 0.31 \pm 0.03$ at 2 °C and $\Phi_D = 0.19 \pm 0.02$ at 19 °C when Cy3 is at -100. These results are applicable at the salt concentrations used in our experiments.⁴⁶

Minimal and maximal values of orientation factor κ^2 for our DNA–RNAP complexes were estimated from anisotropy measurements of donor and acceptor dyes (r_a and r_d) using eqs 6 and 7:^{47,54}

$$\kappa_{\min}^2 \approx \frac{2}{3} \left(1 - \frac{\sqrt{\frac{5r_d}{2}} + \sqrt{\frac{5r_a}{2}}}{2} \right) \quad (6)$$

$$\kappa_{\max}^2 \approx \frac{2}{3} \left(1 + \sqrt{\frac{5r_d}{2}} + \sqrt{\frac{5r_a}{2}} + 3\sqrt{\frac{5r_d}{2}} \sqrt{\frac{5r_a}{2}} \right) \quad (7)$$

where r_d and r_a are the emission anisotropy of the donor and acceptor, respectively, measured at $\lambda_{\max}^{\text{em}}$ and the factor $5/2 = 1/r_{\max}$, where $r_{\max}$ is the maximal anisotropy obtained for an immobilized dye.⁴⁷ Together, eqs 6 and 7 determine a range of κ^2 values bracketing $\kappa^2 = 2/3$. Both $\kappa_{\min}^2$ and $\kappa_{\max}^2$ approach $2/3$ as r approaches zero (the random orientation situation) and diverge to define a wider range of possible κ^2 values, approaching 0 as r_a and r_d increase to 0.4 (the immobilized dye anisotropy).

Permanganate Footprinting Assay

Permanganate footprinting was performed to characterize the position and extent of opening of promoter DNA in a dye-labeled DNA⁵⁵ (see the Supporting Information). ³²P end labeling of dye-labeled primers was unsuccessful, and permanganate footprinting was therefore performed only for Cy5 (-100) or Cy3 (-100) λP_R where the upstream primer had an internal Cy5 or Cy3 while the downstream primer with no dye (Down primer) was end-labeled with [γ -³²P]ATP with T4 polynucleotide kinase (PNK) (NEB, Ipswich, MA). The MnO_4^- reference was ³²P-labeled λP_R without any fluorescent probe. DNA was purified as indicated above. DNA–RNAP complexes were formed in FB using ~1 nM promoter DNA and ~80 nM RNAP on ice (0 °C; CC) and at 19 °C (OC) for ~60 min. KMnO_4 was added to a final concentration of 10 mM and the reaction conducted for 10 s at 19 °C and for 60 s on ice (0 °C). Subsequent workup was performed as described previously.⁴ Footprinting gels were imaged using a Typhoon FLA-9000 instrument (GE Healthcare Biosciences, Pittsburgh, PA) and analyzed using Image Quant TL. Band intensity analyses were performed with ImageJ.⁵⁶

Transcription Assay

To form OC, solutions containing 100 nM λP_R promoter DNA and 80 nM RNAP were incubated as described above. Transcription was initiated with 50 μM ATP, CTP, and GTP and 170 nM/10 μCi [α - ^{32}P]UTP. Samples were incubated for 10 min at 19 °C after the addition of [α - ^{32}P]UTP. QB was then added to the samples that were heated to 90 °C for 2 min. Samples were centrifuged and loaded on a 8 M urea, 20% denaturing acrylamide gel along with a 10–100-nucleotide low-molecular weight RNA ladder from USB-Affymetrix (Santa Clara, CA) end-labeled with [γ - ^{32}P]ATP by T4 PNK. Transcription gels were imaged using a Typhoon FLA-9000 instrument, and line scans were analyzed using Image Quant TL or ImageJ.⁵⁶

Construction of Models of RNAP-Promoter Complexes

Models of the RNAP-promoter CC and OC were made and manipulated using PyMOL Molecular Graphics System, version 1.7.0.3 (Schrödinger LLC, New York, NY), and Coot.⁵⁷ For the CC, our starting point was the published model of a wrapped CC,⁷ which predated the *E. coli* RNAP structures and was therefore based on the *Thermus thermophilus* RNAP structure. The distance *R* between backbone (probe) positions at positions –100 to +14 in the original model is 85 Å. To update this CC model, we replaced *T. thermophilus* RNAP with *E. coli* RNAP [Protein Data Bank (PDB) entry 4LJZ],⁵⁸ which did not affect the distance between –100 and +14. We then used the previously described approach to refine this initial model to reduce the distance between –100 and +14 and to match the published footprinting data.⁷ We modeled the α CTD by analogy to the published wrapped CC model⁷ using PDB structure 1LB2 for the α CTD⁵⁹ and centering them at UP element positions –42 and –52. Key residue R265 is placed in the minor groove, and the acidic patch on the proximal α CTD (D259 and E261) is aligned with R603 on σ region 4.⁶⁰

No molecular model for bent, wrapped upstream DNA in an OC is available. To construct an OC model, we used the recent structure of the *E. coli* RNAP transcription initiation complex (PDB entry 4YLN).⁶¹ In this structure, the promoter DNA is open from –11 to +4 and surrounding duplex DNA extends from –37 to +13. We modeled the α CTD (1LB2) as in the CC model. Upstream DNA (–38 to approximately –112) was bent and wrapped on this structure as in the CC model described above, to be consistent with OC HO* footprinting data.⁷

RESULTS

Large Cy5 FRET Acceptor Signals from Exciting Cy3 Demonstrate DNA Wrapping in Advanced Closed and Open RNAP– λP_R Promoter Complexes

As a direct spectroscopic test of the proposed model of DNA bending and wrapping in the advanced CC⁷ and to compare extents of DNA wrapping in the advanced CC and OC, a far upstream (–100) position on the nontemplate strand backbone and a downstream (+14) position on the template strand backbone of a λP_R promoter DNA fragment were internally labeled with the Cy3/Cy5 donor/acceptor pair of cyanine dyes. Previous DNaseI footprinting¹⁴ revealed that the equilibrium 0 °C RNAP– λP_R complex, like the intermediate characterized in real time at a higher temperature,⁷ is an advanced CC with the downstream

duplex protected to +20 by interactions with RNAP. Far-upstream contacts in the 0 °C complex could not be characterized by footprinting because of the low DNaseI reactivity of this region of this promoter DNA. Here we investigate FRET between cyanine dyes at positions –100 and +14 in the advanced 2 °C λP_R CC and compare it with results for the stable 19 °C λP_R OC (RP₀) and with the fluorescence behavior of a nonpromoter Cy3–Cy5 labeled DNA fragment.

The signature of FRET is emission from the acceptor dye (Cy5) centered at its emission maximum, upon excitation of the donor (Cy3) in a two-dye construct. Figure 1 shows Cy5 emission spectra as a function of Cy3–Cy5 promoter DNA (100 nM) in complex with RNAP for both orientations of the FRET pair of Cy3 and Cy5 dyes –100 and +14 in the advanced λP_R CC. Figure 2 shows the analogous series of Cy5 emission spectra for the stable λP_R OC. A very significant FRET effect is demonstrated in both Figures 1 and 2 by the increases in Cy5 emission ($\lambda_{em}^{max}=660$ nm) with an increasing RNAP:promoter DNA concentration ratio up to approximately 0.6–0.8:1. (Additional series of titrations showing FRET for both the CC and the OC for both orientations of Cy3 and Cy5 are shown in Figure S2). In all cases, the excitation wavelength is 515 nm, near the Cy3 excitation maximum and a wavelength at which direct excitation of Cy5 is minimized.

Observation of FRET unambiguously demonstrates extensive bending and wrapping of promoter DNA on RNAP in both the CC and the OC, reducing the separation distance between –100 and +14 of the DNA backbone in both complexes from >300 Å on free DNA (a slightly flexible cylinder) to <100 Å (the upper bound distance⁴⁷ for detecting a FRET effect). These results provide the first spectroscopic demonstration of large-scale bending and wrapping in any closed or open RNAP–promoter DNA complex. No FRET is observed at either 2 or 19 °C for a 1:1 mixture of RNAP (100 nM) and an analogous Cy3–Cy5 labeled nonpromoter DNA (100 nM), lacking the –10, –35, and UP element sequences of the λP_R promoter (Figure S3; see the Supporting Information for sequence information). This demonstrates that the FRET effect, and DNA bending and wrapping by RNAP, are properties of the promoter CC and OC and not due to the dyes and DNA ends.

At RNAP:promoter DNA concentration ratios of 0.6–0.8:1, the FRET effect plateaus. In some cases, the FRET effect is reduced upon further titration with RNAP, perhaps indicating competition from weaker end binding modes of RNAP. Fluorescence intensities are significantly higher for both dyes at 2 °C than at 19 °C (Figures 1 and 2), as expected because of the reduction in quantum yield with increasing temperature for cyanine dyes (Figure S4).⁴⁶ Because of this, OC FRET measurements were made at 19 °C instead of 37 °C. Because 19 °C is well above the opening transition range (5–15 °C) for the λP_R promoter,^{12,17} all complexes are OCs at 19 °C.

For the two-dye-labeled promoter complexes, Figure 3 plots the acceptor:donor emission intensity ratio I_{Cy5}/I_{Cy3} as a function of RNAP:promoter DNA concentration ratio for both locations of the probes and both temperatures investigated. This analysis shows that I_{Cy5}/I_{Cy3} increases monotonically and linearly with an increasing RNAP:promoter DNA concentration ratio to a plateau value attained at a ratio of 0.6–0.8:1, completely consistent with the behavior of the Cy5 acceptor signal in Figures 1 and 2. The linearity of the plots in

Figure 3 demonstrates complete binding of the limiting reagent (RNAP) for all RNAP:promoter DNA concentration ratios of <1:1 for the concentrations of these experiments (100 nM promoter DNA). The data in these figures and Figures S1 and S2 provide the information needed to calculate FRET efficiencies and estimate distances between far-upstream (−100) and downstream (+14) positions of promoter DNA in the wrapped CC and OC.

Calculation of FRET Efficiencies and Implications for DNA Wrapping in RNAP– λ P_R Complexes

FRET efficiency E (a measure of the fraction of Cy3 excitation energy that is transferred to Cy5) of a Cy3-Cy5 labeled λ P_R promoter–RNAP complex is calculated from the ratio of the Cy5 emission intensity for excitation at the Cy3 wavelength (515 nm) to that for excitation at the Cy5 wavelength (610 nm), using the Ratio_A method⁵⁰ (see eqs 2 and 3 in Materials and Methods). Table 1 lists occupancy-corrected efficiencies E averaged from all experiments at RNAP:promoter DNA concentration ratios of <1:1 for the CC and OC.

For the CC and OC, the two different arrangements of donor and acceptor dyes at positions −100 and +14 yield similar FRET efficiencies (Table 1), averaging to an E_{CC} of 0.30 ± 0.07 and an E_{OC} of 0.32 ± 0.11 . FRET efficiencies of this magnitude indicate that the through-space distance between −100 and +14 is significantly less than 100 Å; model building (see Discussion) indicates that for this to occur both upstream and downstream regions of the promoter DNA must be highly bent and wrapped around RNAP in both the CC and the OC.

Interpretation of these FRET efficiencies using eq 4 shows that dye–dye distances in the CC and OC are approximately 15 and 10% larger, respectively, than the relevant Förster distance R_0 (eq 5) for these dyes. For the Cy3-Cy5 pair in either of the two-dye orientations, if the population-average orientation of one dye with respect to the other in CC and OC were relatively random so that κ^2 would equal 2/3, then R_0 would be 60 ± 1 Å for the CC and 56 ± 1 Å for the OC. From this R_0 and measured E values (Table 1), through-space distances R between −100 and +14 on promoter DNA would be 68 ± 8 Å for the CC and 62 ± 8 Å for the OC. These are also similar to the midrange values of κ^2 (between $\kappa_{\min}^2$ and $\kappa_{\max}^2$) and R_0 , from eqs 6 and 7, and so are relevant even if the random orientation limit is inapplicable. Determination of the anisotropies in donor dye and acceptor dye samples allows us to estimate $\kappa_{\min}^2$ and $\kappa_{\max}^2$.^{47,54}

Fluorescence Anisotropies of Cyanine Dyes on DNA and RNAP– λ P_R Complexes

We measured the steady-state fluorescence anisotropy, r , of RNAP–promoter complexes and free DNAs to obtain maximal and minimal estimates of κ^2 and to test the relevance of $\kappa^2 = 2/3$ for analysis of FRET data for the CC and OC. From the anisotropies, we determine relative mobilities of Cy3 and Cy5 dyes in CC and OC as compared to those of promoter DNA, primer DNA, and free dyes. Average r values obtained from multiple independent determinations with these different dye-labeled DNAs and RNAP–DNA complexes are listed in Table 2. Also listed are literature values of the anisotropies of the free dyes [$r \approx 0.24$ for Cy3,^{46,62,63} observed to be independent of temperature in the range examined (5–25 °C);⁴⁶ $r \approx 0.17$ for Cy5^{62–64}] and the upper limit for anisotropies ($r = 0.4$),⁴⁷ applicable

to situations in which the dye is immobile on the time scale of its fluorescence lifetime^{46,47} (~2 ns). If these dyes were completely rigidly attached to DNA, and rotated only on the time scale of the DNA or RNAP–DNA complex, their anisotropy is predicted to be 0.4.⁴⁷ Anisotropies between those of the free dyes and the immobilized limit indicate the extent to which the dye can rotate independently of the DNA to which it is attached.

Table 2 shows that, as expected, covalent attachment to the DNA backbone increases the anisotropy of both Cy3 and Cy5 as compared to that of the free dyes. Anisotropies of both Cy3 and Cy5 increase upon progressing from the free dye to the ss dye-labeled primer (26 or 40 bases) to the 114 bp promoter duplex with the dye at an end. Anisotropies of both dyes on both DNAs are somewhat larger at 2 °C than at 19 °C. Because the anisotropy of free Cy3 is observed to be independent of temperature,⁴⁶ these small differences may result from increased stacking of these dyes on neighboring nucleobases at 2 °C versus that at 19 °C. The larger anisotropies of Cy3 and Cy5 on ss primers and ds promoter DNA than for the free dyes result from restrictions on rotation of these dyes by incorporation into the backbone of a ss region of DNA and from relatively weak stacking interactions especially with the end of the duplex in promoter DNA.^{46,53}

For Cy3 located at +14, the anisotropy determined for the OC (19 °C; 0.36 ± 0.01) probably exceeds that of the CC (2 °C; 0.34 ± 0.02), and both definitely exceed the anisotropy of Cy3(+14) on unbound duplex promoter DNA (0.31 ± 0.01 at 19 °C and 0.32 ± 0.01 at 2 °C). These anisotropy increases are consistent with footprinting results that indicate that +14 of promoter DNA is contacted by RNAP in the CC and more strongly contacted in the OC. We conclude that the Cy3(+14) is less mobile in the OC and CC than in free promoter DNA but nonetheless is capable of rotational reorientation independent of the complex, because these anisotropies are significantly less than 0.4.

For Cy5 located at –100, the anisotropy determined for the OC (19 °C; 0.31 ± 0.03) is the same or slightly lower than that of the CC (2 °C; 0.33 ± 0.03), and neither is significantly different from the anisotropy of Cy5–100 on unbound duplex promoter DNA (0.31 ± 0.03 at 19 °C and 0.32 ± 0.02 at 2 °C). These similar anisotropies at –100 for DNA in the CC and OC are consistent with footprinting results that indicate that –100 of promoter DNA is not in contact with RNAP in either the CC or the OC.⁷ We conclude that Cy5–100 is as mobile in the OC and CC as in free promoter DNA.

Enhanced Fluorescence (PIFE) Is Observed at +14 in the Advanced CC and Stable OC

Equilibrium studies with a single dye (Cy3 or Cy5) at +14 of λP_R promoter DNA were performed to study the interactions of this region of the downstream duplex with RNAP in the advanced CC and OC. Cy3 samples were excited at 515 nm and Cy5 samples at 610 nm. Representative emission spectra for 100 nM Cy5(+14) λP_R in complex with RNAP are shown in Figure 4. With Cy5 at +14, the fluorescence emission intensity at $\lambda_{\text{max}}^{\text{em}}$ increases with an increasing RNAP:promoter DNA concentration ratio of up to 1:1 in both the CC [2 °C (Figure 4A)] and the OC [19 °C (Figure 4B)]. The fluorescence increase upon RNAP binding observed here [often called PIFE (protein-induced fluorescence enhancement)] is from the proximity of the dye to the protein^{35,37,38,40,41,65} and is consistent with DNA footprinting evidence⁷ that the +14 region of DNA interacts with the RNAP cleft in both the

advanced CC and OC.⁷ PIFE effects at +14 ($30 \pm 5\%$ increase in Cy5 fluorescence intensity and $20 \pm 5\%$ increase in Cy3 fluorescence intensity for complexes compared to free DNA) are similar in the CC and OC (Figure 4 and Figures S5 and S6).

The strong Cy3(+14) PIFE effect masks its expected FRET donor quenching effect in experiments with Cy3(+14)-Cy5(−100) λP_R , as shown in the Cy3 donor emission spectra centered between 555 and 570 nm in Figure S1. Enhanced fluorescence of the Cy3 donor is observed instead of quenching at all RNAP:promoter DNA concentration ratios, relative to that of a single-dye Cy3 control in the absence of RNAP. This indicates the dominance of the PIFE effect over the donor quenching FRET effect when Cy3 is at +14.

Dye-Labeled RNAP- λP_R Promoter OCs Are Transcriptionally Active and Permanganate Reactive in the Open Region

Transcription assays were performed to determine whether the OCs formed by RNAP on the cyanine dye-labeled promoter DNAs are capable of specific initiation. Figure 5 demonstrates that all dye-labeled λP_R promoter fragments initiate transcription and yield significant amounts of the expected-length runoff transcript at 19 °C. The observed differences in transcript length result from differences in the length of duplex DNA downstream of the transcription start site at +1. For promoter DNAs with a cyanine dye at +14, the major transcript is 13–14 bases in length, indicated by the black boxes in Figure 5. For promoters with no dye at +14 (unlabeled λP_R and λP_R with a cyanine dye only at −100), the downstream duplex extends to +70, and the longest transcript is observed to be 70 bases long (also indicated by black boxes in Figure 5).

We also compared MnO_4^- footprints of the dye-labeled and unlabeled (control) RNAP–promoter OC at 19 °C to test whether these are equally open and have the same pattern of reactive thymine bases in the open region. Nontemplate strands of these promoters were dye-labeled at the upstream end (−100), and template strands of both promoters were ³²P-labeled at the downstream end (see Materials and Methods). Transcription experiments (Figure 5) reveal no systematic differences between unlabeled and Cy3(−100)- or Cy5(−100)-labeled promoter DNA. Figure S8A compares template strand MnO_4^- footprints of RNAP complexes with unlabeled and Cy5(−100)-labeled λP_R promoter DNA. Thymine bases at the expected positions (−11, −9, −8, and +1) were detected in both the Cy5(−100) and control λP_R –RNAP complexes, demonstrating that the dye does not perturb the open region. No such bands are seen in the −RNAP control lanes for either DNA. Intensities of the thymine bands at −11, −9, −8, and +1 were quantified and normalized by the intensity of the full-length (unreacted) DNA band to correct for the loading difference between lanes. After correction, no significant difference in permanganate reactivity between the dye-labeled and control λP_R OC is observed for any of these four thymines. By contrast, MnO_4^- experiments performed at 0 °C on the CC formed with Cy3(−100) λP_R DNA and dye-free λP_R DNA reveal no reactivity above background (Figure S8B).

DISCUSSION

FRET between Far-Upstream and Downstream Dyes Indicates Extensive Bending and/or Wrapping of Promoter DNA on RNAP in Both Closed and Open Promoter Complexes

The observation of FRET between dyes at -100 and $+14$ demonstrates that the through-space distance between these dyes is <100 Å in both the CC and the OC. Because the dye–dye distance between -100 and $+14$ in free DNA is >300 Å, both upstream and downstream regions of promoter DNA must be highly bent and wrapped by *E. coli* RNAP in both complexes. For the λP_R OC, this conclusion is consistent with that derived from AFM compaction measurements, DNaseI and HO[•] footprinting, and other assays at both λP_R and other promoters, summarized below. For the advanced λP_R CC, this conclusion is consistent with HO[•] footprinting data, the model proposed by Davis et al.,⁷ and AFM compaction measurements at the TyrT promoter.²³

On the basis of the kinetic and footprinting results summarized in the introductory section, we^{1,7,12} proposed that upstream wrapping occurs as the RNAP molecular machine converts the earliest CC to an advanced CC, and that this upstream wrapping causes conformational changes in RNAP that make it possible to bend the downstream duplex at -11 into the active site cleft, nucleating promoter DNA melting. The significance of wrapping for the properties of OC, including its stability and ability to initiate efficiently upon addition of NTPs, is not yet known.

The +14 PIFE Effect at 2 °C Indicates the CC Is Advanced, with Downstream DNA Bent into the RNAP Cleft

The enhanced fluorescence of DNA-attached cyanine dyes was originally observed upon binding of SSB, BamHI, RecA, and RadH1 to neighboring sites.^{35,36,39} Myong et al. designated this effect PIFE and showed that it is detectable at a dye–protein distance of 34 Å and increases with a decreasing dye–protein distance.³⁶ PIFE between RNAP and a cyanine dye at $+2$ on promoter DNA has recently been used to study initiation and backtracking events upon addition of NTP to OC,^{37,38} OC dissociation kinetics,⁴¹ and effects of the CarD regulatory protein on OC formation.⁴⁰ PIFE (and also FRET) has also been used to investigate interactions of histone H1 with transcriptional activator Gal4.⁶⁵ Here the observation of $+14$ PIFE in the 2 °C CC corroborates information from footprinting¹⁴ and FRET that this CC is advanced, with the downstream duplex bent into the active site cleft as proposed previously.^{1,12} These $+14$ PIFE effects in CC may reflect interactions with DME of RNAP, which include the lineage-specific insert $\beta i4$ (also called Sequence Insertion 1 or SII), the β clamp, and the β jaw (see Figure 6A,B).^{4,5} In the early CC at other promoters (RP_c), where the promoter DNA is linear (not yet bent), the downstream boundary of the HO[•] or DNaseI footprint is at the discriminator region (-7 to -2).^{9,10} Hence, in RP_c, the distance between RNAP and $+14$ of promoter DNA greatly exceeds 34 Å and no PIFE would be expected. The $+14$ PIFE effect observed for the stable OC is expected on the basis of footprinting and RNAP deletion variant data that indicate that this position is contacted by the β jaw and possibly other DMEs.^{4,5}

Through-Space Distances between Far-Upstream and Downstream Regions of Promoter DNA Bent and Wrapped on RNAP in Closed and Open Complexes

Here we interpret FRET efficiencies and anisotropies for the CC and OC to estimate upper and lower bounds on interdyer distances in the bent/wrapped CC and OC and compare them with predictions from AFM compaction data and, in a subsequent section, from structural models built with input from DNA footprinting data.

OC and CC anisotropy data (Table 2) demonstrate that Cy3 at +14 is relatively immobile while Cy5 at –100 is more mobile. For the situation in which one dye is immobile and the other is free to rotate independently, a 4-fold range of values of κ^2 is possible, centered at $\kappa^2 = 2/3$ ($\kappa_{\min}^2 = 1/3$, and $\kappa_{\max}^2 = 4/3$). This 4-fold range of possible values of κ^2 translates into only an additional $\pm 26\%$ uncertainty in distance R between positions –100 and +14 because R varies with $(\kappa^2)^{1/6}$. Combined with the experimental uncertainty in FRET efficiency, this leads to an uncertainty in R of $\pm 36\%$ in dye–dye distances for both the CC and the OC. However, while the anisotropy of Cy5(–100) is approximately the same within uncertainty in the CC and OC as in free DNA, it exceeds that of the free DNA and the ss primer DNA (Table 2), presumably because cyanine dyes at the end of a duplex, like Cy5(–100), are partially stacked on the duplex and not completely free to reorient.^{46,53} Application of eqs 6 and 7 to obtain minimal and maximal values of κ^2 , R_o , and R from the anisotropy results increases the combined uncertainty in the determination of R for the CC and OC from the measured E and r values to $\pm 60\%$, in a range centered on the R values for these complexes calculated for $\kappa^2 = 2/3$.

Structural Models for the CC and OC Consistent with FRET and Footprinting Data

To interpret the strong FRET efficiencies of closed and open RNAP–promoter complexes and to obtain structural models for DNA wrapping tracks on RNAP, we model upstream (and, for CC, downstream) DNA onto recent crystal structures of *E. coli* RNAP holoenzyme (for CC)⁵⁸ and a ternary initiation complex with an open region from –11 to +4 and a downstream duplex extending to position +13 (for OC),⁶¹ using HO• footprinting data for promoter DNA in the advanced CC and the stable OC as a guide.

Advanced Closed Complex—Our modeling of DNA bending and wrapping in the advanced CC updates and extends the previously published model, which was based on the structure of *T. thermophilus* RNAP.⁷ In that CC model, the promoter DNA is wrapped in an approximate U-turn around RNAP, with an overall bend angle of $>200^\circ$, resulting in upstream contacts between RNAP and promoter DNA to position –81/–82 as evident in the HO• footprint. The distance R between upstream (–100) and downstream (+14) positions in this model is 85 Å, near the upper end of the range consistent with the FRET efficiency reported here (Table 1). Replacement of *T. thermophilus* RNAP by *E. coli* RNAP has little effect on upstream contacts or on the distance R between –100 and +14. Slight bending of the upstream DNA at –65 in this CC model creates a series of wrapped complexes with smaller distances R but similar upstream boundaries near –82 (see Figure S9). For example, an $\sim 8^\circ$ bend at –65 reduces R to ~ 50 Å, the minimal distance that is obtained by this approach without introducing steric clash. An $\sim 5^\circ$ bend at –65 yields the CC structure of Figure 6A with an R of 68 Å, the midrange distance deduced from the FRET efficiency and

isotropy of the CC. Models of the advanced CC built with an R of <50 Å introduce steric clash and are also incompatible with the known footprinting data.⁷ Any large conformational changes in RNAP in the conversion from the free protein⁵⁸ to the advanced CC modeled here could affect this conclusion.

Stable Open Complex—Though the FRET efficiencies for energy transfer between positions -100 and $+14$ are similar for the advanced CC and OC λP_R -RNAP complexes, the structures of these complexes must differ significantly in both upstream and downstream regions. Upstream DNA in both the advanced CC and the stable OC is predicted to be strongly bent between -35 and -55 ,^{7,13} indicating the trajectory of the upstream wrap begins similarly in both complexes, but they exhibit very different upstream boundaries of HO[•] protection ($-81/-82$ in the advanced CC and $-60/-65$ in the stable OC).⁷ Hence, the trajectory of the far-upstream DNA (between $-60/-65$ and $-81/-82$) and/or the positions of elements of RNAP, including β residues 808–912 (previously designated the UP clamp),^{1,4} that appear to make contact with this far-upstream DNA must shift in the conversion of the advanced CC to the stable OC.

Likewise, the trajectories of the downstream DNA in the advanced CC and stable OC appear to be different. Both complexes have an $\sim 90^\circ$ bend at the upstream end of the -10 region and the same downstream boundary of HO[•] protection ($+20$).⁷ However, while the DNA downstream of the bend at -11 is thought to be linear in the advanced CC, additional strong bending in the open region is observed in the recent structure of an initiation complex,⁶¹ altering the downstream duplex trajectory. The $+14$ region of downstream DNA is in the proximity of, and possibly interacting with, β SI1 (residues 227–308) in the advanced CC (Figure 6A) but is farther from it in the OC, indicating rearrangement of either this region of RNAP or the downstream DNA in the conversion of the CC to the OC. The β jaw (residues 1150–1208) is also closer to DNA downstream of position $+10$ in the OC (Figure 6B), supporting earlier evidence of stabilizing interactions between the β jaw and this region of downstream DNA in the OC.^{4,5}

Starting from the structure of the initiation complex,⁶¹ we modeled in the upstream DNA as described in Materials and Methods to create upstream-wrapped structures with distances R between -100 and $+14$ in the range of 40 – 80 Å as specified by the FRET efficiency, in which the upstream DNA contacts RNAP only to the upstream end of the UP element at $-60/-65$ to match the upstream HO[•] footprint boundary, and with the extreme wrapping deduced from DNA compaction by AFM.²⁰ If upstream DNA (from -37 to -99) from the CC model (Figure 6A) is modeled into the structure of the initiation complex without any additional bending, we obtain an OC model with an R of >100 Å and in which the tethers of the α CTD must be somewhat extended to make contacts with the UP element DNA.

This OC model does not have sufficient upstream bending and wrapping to explain the OC FRET efficiency. To reduce R , bends in upstream duplex DNA at positions of DNaseI or HO[•] enhancements^{7,14} were increased by small amounts ($\sim 3^\circ$) at -38 and -44 and substantially ($\sim 25^\circ$) at -54 , relative to the path of the DNA in the original CC model. This OC structure ($R = 62$ Å) is shown in Figure 6B. More extensive bending at these or other positions would force contacts between upstream DNA and RNAP beyond $-60/-65$, not

observed in the OC HO* footprint.⁷ Insertion of an ~25° bend at -54 changes the trajectory of upstream DNA from that of the CC model (in which it is near the α -NTD and the β groove), shifting the upstream DNA toward the ω subunit. Upstream DNA beyond -65 passes near the β subunit without a direct interaction, consistent with the end point of the HO* footprint at -65 on the nucleotide strand.⁷ The upstream (-100)– downstream (+14) distance R in this structure is 62 Å, which is also the midrange distance from FRET/ anisotropy data. Smaller values of R are not possible in this model because of steric interactions, unless there are large conformational differences in the region of RNAP contacting the upstream DNA between the stable OC and the initiation complex. While phasing and positioning of upstream DNA with respect to RNAP in this model are approximate and traced by eye, we think it provides a good estimate of the most probable distance in the OC between -100 and +14.

Possible Roles of Promoter DNA Wrapping and Unwrapping in OC Formation and Transcription Initiation

Truncations of far-upstream promoter DNA (between -65 and -100)⁶ and within the UP element (-38 to -65)^{6,8} both dramatically reduce the rate of isomerization of closed to open complexes at λP_R and lacUV5 promoters. While the real-time footprint of the ensemble of advanced closed complexes at λP_R extends from -82 to +20, truncation at -47 results in a less advanced closed complex, with a downstream boundary in real-time footprinting of only +2/+7 that indicates that the downstream duplex is only partially bent into the active site cleft.⁷ From this information, we proposed that upstream bending and wrapping, set in motion by initial binding to the -35 region and the UP element to form a linear complex (RP_C), are necessary to bend the downstream duplex fully into the cleft in the advanced CC prior to opening. Presumably, this is because upstream bending and wrapping play key roles in opening the cleft and moving obstacles like sigma 1.1 and DME of core RNAP to allow bending of the downstream duplex at -11 and its entry into the active site cleft before opening.^{4,5,12,66}

The significance of wrapping for the properties of OC is unknown. AFM studies with full-length λP_R indicate similar degrees of wrapping in OC and in an initiation complex paused at +3.²² Because elongation complexes (after escape from the promoter) exhibit a short (40 bp) footprint¹⁹ and are therefore unwrapped, unwrapping of the upstream DNA may therefore be a significant element of the transition from initiation to elongation that occurs after formation of a moderately long transcript (~10 bp RNA–DNA hybrid), which necessitates and may be driven by scrunching of promoter DNA in the cleft.^{67,68} Release of upstream wrapping presumably coincides with release of specific RNAP–promoter DNA interactions.

Comparison of Promoter DNA Wrapping in the CC Formed by Prokaryotic and Eukaryotic RNAP

Wrapping of upstream DNA and bending of downstream DNA appear to be general features of preinitiation complexes for eukaryotic as well as bacterial RNAP. After binding the TATA box, human and yeast TATA binding proteins (TBP) and transcription factor TFIIB together bend this sequence of duplex DNA by 80–100°⁶⁹ in a manner reminiscent of the bending of

the region upstream of the -35 element by *E. coli* RNAP to form an advanced CC.^{1,14} Assisted by the essential factor TFIIF (required for binding of RNA pol II to promoter DNA) and TFIIE, another $\sim 90^\circ$ bend is formed near the transcription initiation site^{30,70} (+1), which may be analogous to the downstream bending of DNA at -11 into the active site cleft in *E. coli* RNAP in an advanced CC^{1,12,66} that also has been implicated in nucleation of DNA opening.⁷¹ Extensive protein–DNA cross-linking is observed over a 90 bp region of promoter DNA (-56 to $+34$)³¹ in an advanced CC formed with TBP, TFIIB, RNA pol II, TFIIF, and TFIIE, indicating extensive DNA wrapping analogous to the ~ 100 bp of DNA wrapped by *E. coli* RNAP in the advanced CC (from $-80/-81$ to $+20$).⁷ We hypothesize that eukaryotic initiation factors such as TFIIE and TFIIF, and *E. coli* RNAP β lobe (including SII) and the β' UP clamp,⁴ may function similarly in wrapping promoter DNA in the advanced CC. Kinetic studies are underway in our laboratory to determine the progression of upstream DNA wrapping and downstream DNA bending as the initial closed complex (RP_C) advances to bend the downstream duplex into the active site cleft and open it.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

RNAP	RNA polymerase
CC	closed complex
OC	open complex
DME	downstream mobile element
PIFE	protein-induced fluorescence enhancement
AFM	atomic force microscopy
CTD	C-terminal domain
NTD	N-terminal domain
SB	storage buffer
FB	buffer for fluorescence experiments
AB	annealing buffer
TB	transcription buffer

QB	quench buffer
SI	sequence insertion
t	template
nt	nontemplate
wt	wild-type
HO[•]	hydroxyl radical

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Figure 1.

Donor (Cy3) to acceptor (Cy5) fluorescence energy transfer (FRET) demonstrating wrapping in CC. Cy5 fluorescence emission spectra (620–720 nm; counts per second) obtained by excitation of Cy3 at 515 nm as a function of RNAP:promoter DNA concentration ratio (0–1.2:1; 100 nM DNA) for advanced closed (CC; 2 °C) RNAP– λP_R promoter DNA complexes: (A) Cy3(–100)–Cy5(+14) λP_R and (B) Cy3(+14)–Cy5(–100) λP_R . DNA spectra (–RNAP) were obtained with 100 nM Cy3-labeled DNA and Cy3–Cy5 labeled DNA.

Figure 2.

Donor (Cy3) to acceptor (Cy5) fluorescence energy transfer (FRET) demonstrating wrapping in OC. Cy5 fluorescence emission spectra (620–720 nm; counts per second) obtained by excitation of Cy3 at 515 nm as a function of RNAP:promoter DNA concentration ratio (0–1.2:1; 100 nM DNA) for open (OC; 19 °C) RNAP– λP_R promoter DNA complexes: (A) Cy3(–100)-Cy5(+14) λP_R DNA and (B) Cy3(+14)-Cy5(–100) λP_R . DNA spectra (–RNAP) were obtained with 100 nM Cy3-labeled DNA and Cy3-Cy5 labeled DNA.

Figure 3.

Demonstration of FRET from intensity ratios (I_{Cy5}/I_{Cy3}) for Cy5 and Cy3 emission at λ_{max}^{em} upon Cy3 excitation vs RNAP:promoter DNA concentration ratio. Data for both the CC (2 °C) and the OC (19 °C) of 100 nM dye-labeled Cy3(−100)-Cy5(+14) λP_R and Cy3(+14)-Cy5(−100) λP_R DNA in complex with RNAP are shown. $\lambda_{max}^{em}=660$ nm (Cy5); $\lambda_{max}^{em}=565$ nm (Cy3). Cy3 excitation at 515 nm. Data are from Figures 1 and 2. Lines interpret the FRET effect as a linear increase up to RNAP:promoter DNA concentration ratios of 0.6–0.8:1 and a plateau at higher RNAP:promoter DNA concentration ratios.

Figure 4.

Observed Cy5(+14) fluorescence enhancement (PIFE) in CC and OC: 100 nM Cy5(+14) λP_R promoter DNA in complex with RNAP (50, 75, and 100 nM) for the CC (2 °C; panel A) and the OC (19 °C; panel B). Samples were excited at 610 nm. DNA spectra (–RNAP) were obtained with 100 nM Cy5(+14) λP_R DNA.

Figure 5. Gel analysis of transcription products for two-dye (left, center), single-dye (center, right), and unlabeled λP_R (right lane) promoter DNA. A RNA ladder is shown at the left. Boxed areas show full-length transcripts for each fragment.

Figure 6.

Models of bent and wrapped promoter DNA in (A) CC and (B) OC with *E. coli* RNAP. Models built for midrange values of the dye–dye distances, (A) $R = 68 \text{ \AA}$ for the CC and (B) $R = 62 \text{ \AA}$ for OC, as determined from FRET and anisotropy data (Table 1). Models based on structures of *E. coli* RNAP: PDB entry 4LJZ for the CC and PDB entry 4YLN for the OC. Cyanine dyes are shown at positions -100 (pink) and $+14$ (blue) as ellipsoids. RNAP subunits α NTD (cyan), α CTD (yellow; modeled from PDB structure 1LB2), β (pale pink), β' (green), ω (blue), and σ (orange) are shown. β SII (dark pink, residues 225–343), β' clamp

(dark green, residues 131–347 and 1260–1368), β' UP clamp (bright green, residues 808–912), and β' jaw (brown, residues 1150–1208) are highlighted. Promoter DNA is colored light gray (nt) and dark gray (t). A “backside” view of OC is also shown in panel B.

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Table 1

FRET Efficiencies and Distances R between Promoter Positions -100 and $+14$ in Advanced Closed and Stable Open Complexes

Property	Advanced Closed Complex (CC; 2 °C)	Stable Open Complex (OC; 19 °C)
FRET efficiency E^a		
Cy3(-100)-Cy5($+14$) λP_R	0.36 ± 0.10	0.36 ± 0.14
Cy3($+14$)-Cy5(-100) λP_R	0.23 ± 0.06	0.29 ± 0.09
Average	0.30 ± 0.07	0.32 ± 0.11
Estimates of interdyne distance R^b from FRET efficiency E		
Random orientation limit ($\kappa^2 = 2/3$)	$R_{2/3} = 68 \pm 8 \text{ \AA}$	$R_{2/3} = 62 \pm 8 \text{ \AA}$
Anisotropy maximum ($\kappa_{\max}^2 = 3.59$)	$R_{\max} = 91 \pm 9 \text{ \AA}$	$R_{\max} = 82 \pm 8 \text{ \AA}$
Anisotropy minimum ($\kappa_{\min}^2 = 0.052$)	$R_{\min} = 47 \pm 6 \text{ \AA}$	$R_{\min} = 42 \pm 4 \text{ \AA}$
Midrange value from anisotropy	$R_{\text{mid}} = 68 \pm 22 \text{ \AA}$	$R_{\text{mid}} = 62 \pm 20 \text{ \AA}$
Structural minimum value	$R_{\text{min-str}} \sim 50 \text{ \AA}$	$R_{\text{min-str}} \sim 60 \text{ \AA}$

^aFRET efficiencies E are averages of results shown in Figures 1 and 2 and Figures S1 and S2 for RNAP:promoter DNA concentration ratios of $< 1:1$, corrected for occupancy. Uncertainties in E are ± 1 standard deviation.

^bUncertainties in R are deviations from the average.

Table 2Fluorescence Anisotropies (*r*) of Cyanine Dyes, Dye-Labeled DNAs, and RNAP–Promoter Complexes

	2 °C	19 °C
Dye immobilized on DNA	0.40	0.40
Cy3(+14) λ P _R DNA–RNAP complex	(CC) 0.34 $\pm$ 0.02	(OC) 0.36 $\pm$ 0.01
Cy3(+14) duplex λ P _R DNA	0.32 $\pm$ 0.01	0.31 $\pm$ 0.01
Single-stranded Cy3(+14) primer	0.28 $\pm$ 0.01	0.27 $\pm$ 0.01
Cy3 free dye	0.24 $\pm$ 0.02 ^{a,b}	0.24 $\pm$ 0.02 ^{a,b}
Cy5(–100) λ P _R DNA–RNAP complex	(CC) 0.33 $\pm$ 0.03	(OC) 0.31 $\pm$ 0.03
Cy5(–100) duplex λ P _R DNA	0.32 $\pm$ 0.02	0.31 $\pm$ 0.03
Single-stranded Cy5(–100) primer	0.29 $\pm$ 0.02	0.25 $\pm$ 0.03
Cy5 free dye	0.17 $\pm$ 0.04 ^{a,b}	0.17 $\pm$ 0.04 ^{a,b}

^a Average of values reported at 20–25 °C for Cy3^{46,62,63} and Cy5.^{62–64}^b Anisotropy of free Cy3 observed to be independent of temperature in the range investigated (5–25 °C);⁴⁶ temperature-independent anisotropy assumed for Cy5.