Mapping ATP-dependent Activation at a $\sigma^{54}$ Promoter

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The $\sigma^{54}$ promoter specificity factor is distinct from other bacterial RNA polymerase (RNAP) $\sigma$ factors in that it forms a transcriptionally silent closed complex upon promoter binding. Transcriptional activation occurs through a nucleotide-dependent isomerization of $\sigma^{54}$, mediated via its interactions with an enhancer-binding activator protein that utilizes the energy released in ATP hydrolysis to effect structural changes in each endows the resulting holoenzyme with distinct properties (1–3). Core RNAP binding is known to induce conformational changes in the $\sigma^{70}$ subunit required for recognition of consen- sus promoter sequences (4–7). In contrast to $\sigma^{70}$, $\sigma^{54}$ can bind promoter sequences in the absence of core RNAP. Specific differences in the interactions with promoter DNA between $\sigma^{54}$ and $\sigma^{54}$-RNAP (E$\sigma^{54}$) suggest that interactions with core RNAP induce conformational changes in the $\sigma^{54}$ subunit (8, 9). These changes and the organization of $\sigma^{54}$ domains within the closed complexes have been probed previously using single cysteine $\sigma^{54}$ variants to map the proximity of specific $\sigma^{54}$ residues to regions within the promoter DNA in the presence of the tethered chemical nuclease reagent Fe-BABE (10–13). These analyses revealed that $\sigma^{54}$ adopts a C- to N-terminal orientation in the 5′ to 3′ direction with respect to the non-template strand and demonstrated that the regulatory N-terminal domain of $\sigma^{54}$ (Region 1; Fig. 1A) and promoter DNA both undergo conformational changes, induced by the binding of core RNAP to an initial $\sigma^{54}$-DNA complex leading to the formation of a conformationally stable and transcriptionally silent closed complex (11, 14). The $\sigma^{54}$ Region I and Region III domains are required for recognition of the start site proximal consensus promoter recognition sequence (the −12 region) where DNA opening nucleases. The interactions between Region I and Region III with the −12 site are of regulatory significance and are required for preventing spontaneous isomerization of the closed complex to the open promoter complex in the absence of activation (10, 15–20). Truncated versions of $\sigma^{54}$ lacking Region I ($\sigma^{54}$ΔRI) can bind to core RNAP, forming E$\sigma^{54}$ΔRI complexes capable of activator-independent transcription from supercoiled and so called late-melted promoters that are mismatched between positions −10 and −1, thus mimicking the confor- mation adopted by the promoter DNA in the open complex but not on DNA constructs mismatched between −12 and −11, which mimic the conformation adopted by the promoter in the closed complex. This implies that in closed complexes formed by E$\sigma^{54}$ΔRI there is a loss of interactions with regions of DNA inhibiting open complex formation and an increase in interactions with single-stranded DNA (16). The C-terminal RpoN box (Fig. 1A) is a $\sigma^{54}$ signature sequence implicated in the binding to consensus DNA sequences at −24 (12, 20).

Transcriptional activation in the $\sigma^{54}$ system is effected by a AAA$^+$ protein (ATPase associated with various cellular activities) bound to an upstream enhancer element being brought...
into contact through DNA looping with the closed complex (21, 22). The interaction between subunits of the Er\(^{\sigma_{54}}\) activator phage shock protein F (PspF) and Er\(^{\sigma_{54}}\) closed complex are conditioned by the binding and hydrolysis of ATP, which drives PspF oligomerization and leads to an isomerization of Er\(^{\sigma_{54}}\) closed complexes and subsequent formation of transcription-
ally active open complexes (23, 24). Substitution of ATP with analogues (ATPγS or AMP-PNP) or the ATP hydrolysis transition state analogue, ADP-AlF₄⁻, suggests that nucleotides drive the formation of different functional states of PspF and alter interactions between Eσ₅₄ and a fork junction DNA structure at the −12 promoter consensus region in the closed complex (23, 25–27). This is consistent with the idea that PspF acts via several discrete conformational states, each making functionally distinct interactions with Eσ₅₄-promoter DNA complexes before completing ATP hydrolysis for full transcriptional activation. The network of interactions governing these events at the point of ATP hydrolysis has recently been elucidated by combining the crystal structure of PspF with cryogenic electron microscopy images of σ₅₄ in complex with oligomeric PspF bound to ADP-AlF₄⁻ (27, 28). Using these structural data, site-directed PspF mutants were assayed for ATP binding and hydrolysis activity and the ability to trigger open complex formation. It appears that structural changes occurring in the nucleotide binding pockets formed by the PspF hexamer are communicated via relocation of a conserved loop motif to make stable contacts with σ₅₄ (27). Assaying the ability of various σ₅₄ mutants to form transcriptionally active open complexes indicated the nucleotide-bound form of PspF targets Region I of σ₅₄ (11, 29, 30). However, because of the transient nature of the initial contact, the events and associated processes whereby weak interactions between PspF and σ₅₄-closed complex in the absence of nucleotide lead to the formation of a stable complex capable of isomerization remain unclear.

Spectroscopic methods provide a powerful means of analyzing complex processes involving macromolecular interactions. Fluorescence resonance energy transfer (FRET) has become established as a method of choice in the dissection of protein-protein and protein-oligonucleotide interactions due to the sensitivity of non-radiative energy transfer over nanometer scales. The ease with which fluorophores with high quantum yields can be incorporated into both DNA-binding proteins and their substrates has given added impetus to the analysis of structure-function relationships between σ factors and multisubunit RNA polymerases and the formation of transcriptionally active complexes (7, 31).

Here we describe a FRET assay capable of assigning relative separations of dye labels incorporated into defined sites on the σ₅₄ subunit and the ends of DNA fragments encompassing the Sinorhizobium meliloti nifH DNA promoter sequence. Labeled positions in σ₅₄ were chosen based on Fe-BABE analyses (11, 12). The FRET assay has been used to probe the structural changes

![Image](237x26 to 265x38)

**TABLE 1**

| Promoter binding by wild-type and dye-labeled σ₅₄ and Eσ₅₄ in the presence or absence of nucleotide-dependent activator protein | Early-melted promoter | Late-melted promoter |
|---|---|---|
| | Unactivated | Activated³ | Activated⁴ | Unactivated | Activated⁴ |
| σ₅₄ | σ-DNA | σ-DNA | ss | Eσ | Eσ |
| Wild type | 58 ± 8 | 38 ± 7 | 31 ± 5 | 24 ± 2 | 28 ± 4 |
| C20 | 68 ± 6 | 39 ± 9 | 31 ± 5 | 23 ± 2 | 22 ± 4 |
| C463 | 62 ± 5 | 32 ± 3 | 38 ± 4 | 23 ± 3 | 19 ± 5 |
| C474 | 59 ± 8 | 34 ± 5 | 34 ± 6 | 22 ± 2 | 20 ± 5 |

³ Activation of σ-DNA and isomerized supershifted σ-DNA complexes (ss) in the presence of PspF₁₋₂₇₅ and ATP.
⁴ Activated Eσ represents complexes isomerized in the presence of PspF₁₋₂₇₅ and ATP that remain after heparin challenge.

![Graph](33719)

**FIGURE 2. FRET assays of σ₅₄ binding to nifH early-melted promoter.** Representative steady-state fluorescence emission spectra with excitation at 488 nm are shown. Spectra obtained from the Alexa 488-labeled early-melted promoter (50 nM) in the absence (blue line) or presence (green line) of unlabeled Eσ₅₄ (63 nM E) are shown, revealing the extent of non-FRET quenching. FRET is observed upon the addition of dye-labeled σ₅₄ cysteine mutants (250 nM) to the DNA in both the absence (black line) or presence (red line) of core RNAP, and these are shown after corrections for non-FRET quenching by σ₅₄ and core RNAP as appropriate (see supplemental data). Schematics indicate the expected positions of donor and acceptor labels, whereas the approximate position of the σ factor is shown in gray and core RNAP as a red dotted outline.
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The addition of PsPF\(_{1-275} \) to the \( \sigma^{54} \)-DNA complexes alters FRET signals in a nucleotide-dependent manner. \( \sigma^{54} \)-DNA complexes (black lines) were incubated with PsPF\(_{1-275} \), alone (6 \( \mu \)M; green lines) or in the presence of ATP (2 mM; blue lines) or ADP-AIF\(_{2} \) (200 \( \mu \)M; red lines). The dotted lines represent similar assays in the presence of ATP using the PsPF\(_{1-275} \) variant, T86A, that cannot couple ATP hydrolysis with isomerization. The insets show expanded views of the acceptor fluorescence emission region. \( \sigma^{54} \)-DNA complexes were formed after incubation of Alexa 488-labeled early-melted promoter (50 nM) with dye-labeled donor and acceptor labels, with the FRET curves have been corrected for non-FRET quenching of PsPF and/or nucleotides. Schematics indicate the expected positions of donor and acceptor labels, with the 54 complex because the latter is sensitive to the restrictive DNA conformation at -12 and -11 (16). In contrast, the late-melted promoter isomerizes with E\( \sigma^{54} \) (in response to activation), is competitor (heparin)-resistant, and supports transcription, i.e. it shows most of the features of the natural open complex. Structural alterations occurring upon nucleotide hydrolysis-dependent closed to open complex transition were inferred from assays utilizing truncated PsPF (PsPF\(_{1-275} \)) lacking a DNA binding motif. PsPF\(_{1-275} \) retains the ability to isomerize \( \sigma^{54} \) and E\( \sigma^{54} \) promoter complexes and activate transcription efficiently (11, 33). In addition, the use of PsPF\(_{1-275} \) facilitates experimental design since it does not rely on upstream activating sequences for transcription activation. FRET assays of the interactions of PsPF\(_{1-275} \) with \( \sigma^{54} \) and E\( \sigma^{54} \) promoter complexes (formed on the early- or late-melted fragments) in the presence or absence of hydrolysable and non-hydrolysable nucleotides provide novel insights into the structural alterations occurring during formation of the open complex.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification—**Klebsiella pneumoniae wild-type \( \sigma^{54} \) and single-cysteine variants (C20, C463, and C474) and the catalytic domain (residues 1–275) of the \( \sigma^{54} \)-dependent activator phage shock protein F (PsPF\(_{1-275} \)) were purified as N-terminal His\(_{6} \)-tagged fusion proteins. All proteins were overexpressed in BL21 Escherichia coli cells in 2YT medium in the presence of 50 \( \mu \)g/ml kanamycin. After growth to \( A_{600} \) of ~0.6 at 37 °C, cells were induced by the addition of 0.8 mM isopropyl \( \beta \)-D-galactopyranoside, incubated at 30 °C for 3 h, then harvested by centrifugation at 11,000 \( \times g \) for 30 min at 4 °C. All subsequent steps were performed at 4 °C in the presence of protease inhibitor mixture (Complete-EDTA; Roche Diagnostics) unless stated otherwise. Cells pellets were resuspended in 50 mM phosphate buffer, pH 8.0, 150 mM NaCl, 10 mM imidazole, 5 mM \( \beta \)-mercaptoethanol, 10% (v/v) glycerol, 1% (v/v) Tween 20. Resuspended cells were then lysed with a French press, and insoluble material removed by centrifugation at 11,000 \( \times g \) for 30 min.

Cell lysates were loaded onto a 5-ml immobilized metal affinity column (HisTrap; Amersham Biosciences) and washed with 50 ml of 50 mM phosphate buffer, pH 8.0, 300 mM NaCl, 5 mM \( \beta \)-mercaptoethanol, 5% (v/v) glycerol, then 50 ml of the above wash buffer containing 20 mM imidazole. Specifically bound protein was eluted by a step elution of 15 ml of wash buffer containing 600 mM imidazole, and the eluted fraction was exchanged into 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 5% (v/v) glycerol by passage through a HiPrep 26/10 desalting column. Fractions containing the peak of protein were loaded onto a 1-ml Sepharose Q col-
TABLE 2
Apparent dye separations based on FRET efficiency data

| Donor                  | Acceptor | Energy transfer (Å) | Inferred fluorophore separation (Å) |
|------------------------|----------|---------------------|-------------------------------------|
| Early-melted DNA       | C20      | 0.20                | 80                                  |
| template               | C463     | 0.24                | 75                                  |
|                        | C474     | 0.15                | 95                                  |
|                        | C20-RNAP | 0.28                | 70                                  |
|                        | C463-RNAP| 0.35                | 65                                  |
|                        | C474-RNAP| 0.29                | 70                                  |
| Late-melted DNA        | C20      | 0.20                | 80                                  |
| template               | C463     | 0.4                 | 60                                  |
|                        | C474     | 0.4                 | 60                                  |
|                        | C20-RNAP | 0.30                | 70                                  |
|                        | C463-RNAP| 0.57                | 50                                  |
|                        | C474-RNAP| 0.85                | 30                                  |
| Late-melted DNA        | C20-RNAP | 0.55                | 50                                  |
| non-template           | C463-RNAP| 0.5                 | 55                                  |
|                        | C474-RNAP| 0.3                 | 70                                  |
|                        | C20-RNAP | 0.43                | 60                                  |
|                        | C463-RNAP| 0.63                | 40                                  |
|                        | C474-RNAP| 0.92                | 15                                  |

* The $R_0$ of Alexa Fluor 488 and Alexa Fluor 594 is 54 Å (35).

umn, washed with 5 ml of 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 5% (v/v) glycerol, 0.1 mM EDTA, then eluted with a linear gradient of NaCl (50–1000 mM) in the same buffer over 20 ml. Purified protein was dialyzed overnight against 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 5% (v/v) glycerol, 0.1 mM EDTA for storage at −80 °C. Cysteine mutant α54 subunit proteins were purified in an identical fashion before fluorescent labeling except DTT was omitted from chromatography and dialysis buffers.

Fluorescent Labeling of Cysteine Mutant α54 Subunit Protein—Purified cysteine mutant α54 subunit protein (1 ml) was incubated on a Reduce-Inm column (Pierce) for 1 h at room temperature in 10 mM Tris, pH 7.5, 50 mM NaCl, 5% (v/v) glycerol, 0.1 mM EDTA. The column was eluted with the same buffer, and the fraction containing the peak of protein was incubated with a 10–20 M excess of Alexa Fluor 594 maleimide for 2 h at room temperature after which the reaction was quenched with 10 mM β-mercaptoethanol. The quenched reaction mixture was adjusted to 30% saturated (NH₄)₂SO₄ and loaded onto a 1-ml phenyl-Sepharose column. The column was washed with 15 ml of 50 mM Tris, pH 7.5, 1 mM DTT, 5% (v/v) glycerol, 0.1 mM EDTA, 30% saturated (NH₄)₂SO₄ then eluted with a linear gradient of 30 to 0% saturated (NH₄)₂SO₄ in 50 mM Tris, pH 7.5, 1 mM DTT, 5% (v/v) glycerol, 0.1 mM EDTA over 15 ml. Purified labeled fractions were dialyzed as above for long term storage at −80 °C. Comparison of elution profiles of labeled and unlabeled α54 protein preparations from phenyl-Sepharose columns and SDS-PAGE analysis of eluted fractions indicates that hydrophobic interaction chromatography separates protein from free dye and resolves labeled and unlabeled protein (data not shown). Surface-enhanced laser desorption ionization (SELDI) mass spectrometry of labeled cysteine mutant α54 subunit protein samples immobilized on nickel-charged supports confirmed incorporation of single dye molecules (data not shown). Fluorophore concentrations in eluted fractions were quantified by amino acid analysis. The efficiency of labeling of cysteine mutant α54 subunit protein preparations, as determined by the molar ratio of fluorophore to protein, was typically ≥70%.

Promoter DNA Fragments—Synthetic DNA oligonucleotides corresponding to −38 to +6 bp of the S. meliloti nifH promoter sequence were used in the formation of fragments to assay wild-type and fluorescently labeled cysteine mutant α54 function. Incorporation of mismatches at positions −12 and −11 or from −10 to −1 generates mismatched DNA constructs that mimic the early- and late-melted promoter conformations, respectively (2, 11, 16). Mismatched DNA constructs used in native gel mobility shift assays were formed after the method of Wigneshweraraj et al. (34) by annealing a radiolabeled promoter strand with a 2-fold molar excess of the unlabeled complementary strand. FRET measurements were performed using...
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identical DNA sequences terminating in a 5'-amino group labeled with Alexa Fluor 488 succinimidyl ester according to the manufacturer’s instructions and purified by high-performance liquid chromatography. Mismatched DNA constructs were then formed by annealing either the labeled template strand (bottom) or labeled non-template strand (top) with the unlabeled complementary strand.

Native Gel Mobility Shift Assays—Binding reactions were conducted in 25 mM Tris acetate, pH 8.0, 8 mM magnesium acetate, 100 mM KCl, 1 mM dithiothreitol, and 3.5% (w/v) polyethylene glycol 6000 in a final volume of 20 $\mu$L at 30 °C using 100 $\mu$L radiolabeled promoter probe and 500 $\mu$L unlabeled complementary strand. The ability of the preformed $\sigma^{54}$-DNA complexes to undergo isomerization was then assayed by the addition of 15 $\mu$L PsP$_{1-275}$ and 2 mM ATP, and the reaction was incubated for a further 5 min. In experiments utilizing late-melted promoter, isomerized heparin-stable $\sigma^{54}$ promoter complexes were isolated by the addition of 10 $\mu$g/ml heparin after completion of the isomerization reaction. Reactions were analyzed on a 4.5% polyacrylamide gel under non-denaturing conditions in 25 mM Tris, 200 mM glycine buffer, pH 8.6, at room temperature. The dried gel was visualized, and bound DNA was quantified using phosphorimaging.

FRET Assays and Relative Separation Calculations—Fluorescence measurements were conducted using a Shimadzu RF-5301 spectrophotometer with excitation at 488 nm and excitation and emission slit band-pass settings at 5 nm. Assays were performed in a 40-$\mu$L, 3-mm path length microcuvette inside a temperature-controlled sample chamber at 30 °C utilizing buffer and incubation conditions identical to gel shift assays. Formation of $\sigma^{54}$-RNAP was achieved by the addition of E. coli core RNA polymerase (Epicenter Technologies) to give a 1:4 molar ratio of core to $\sigma^{54}$. Emission spectra were measured after 5 min and corrected for dilution. In experiments analyzing the effect of activator protein in the presence or absence of nucleotides, spectra were measured before and after the addition of PsP$_{1-275}$ and corrected for dilution and the presence of activator. Nucleotide was then added, and spectra were recorded after 10 min and corrected for dilution and the presence of nucleotide. The transition state analogue, ADP-AlF$_3$, was formed in situ by the addition of 0.2 mM AlCl$_3$ to an assay mixture containing 0.2 mM ADP and 5.0 mM NaF.

The efficiency of fluorescence resonance energy transfer was calculated using the equation $E_{\text{FRET}} = 1 - (I_{DA}/I_D)$, where $I_{DA}$ is the integrated donor fluorescence intensity in the presence of labeled acceptor proteins, and $I_D$ is the integrated donor fluorescence intensity in the presence of unlabeled acceptor proteins. The relative change in FRET was then corrected for the percentage of each bound species estimated using the population distribution in an identical gel shift assay (see the supplemental data).

The relative separation ($R$) between donor and acceptor was calculated from FRET efficiencies using $R = R_0((1/E_{\text{FRET}}) - 1)^{1/6}$, where $R_0$, the Förster distance, is the distance at which energy transfer is 50% of the maximum value. A value for $R_0$ of

![FRET assays of holoenzyme binding to late-melted DNA.](image)
54 Å for the Alexa Fluor 488/Alexa Fluor 594 dye pair calculated from ensemble FRET measurements of donor and acceptor fluorophores, separated by known distances in a DNA ladder, agreed with previously published data (35).

RESULTS AND DISCUSSION

Fluorescent Labeling of $\sigma^{54}$ and DNA Promoters Does Not Affect Recognition and Isomerization—Previously single cysteine variants of $\sigma^{54}$ have been created to provide proximity-based footprinting tools. These have been used here as the points of attachment of the acceptor for FRET. Three mutants were chosen on the basis of their cysteine locations in the activator binding (C20 of Region I) or DNA binding (C463 and C474 in Region III) domains (Ref. 11; Fig. 1). These were labeled (efficiency $\geq 70\%$) with Alexa Fluor 594 maleimide and purified away from unlabeled protein and free dye by hydrophobic interaction chromatography on phenyl-Sepharose (Fig. 1B).

The labeled proteins were then assayed for their abilities to bind radio- or fluorescently labeled (data not shown) promoter fragments. Two types of promoter fragment, early- and late-melted (see “Experimental Procedures”) were used in electrophoretic mobility shift assay and isomerization assays (Fig. 1, C and D). Gel-shift assays of $\sigma^{54}$ and E$\sigma^{54}$ complexes on either DNA fragment that have undergone nucleotide-dependent isomerization by PspF$_{1-275}$ reveal the presence of differently migrating species in the presence of ADP-AlF$_{4}$ or ATP. Complexes formed by the action of PspF$_{1-275}$ in the presence of transition state analogue are described as being “trapped” in a state conformationally distinct from that found in the fully isomerized $\sigma^{54}$ early-melted promoter construct (supershifted) species generated in response to ATP hydrolysis by PspF$_{1-275}$. Such fully isomerized complexes on the late-melted promoter are defined by their ability to withstand a challenge with heparin. The percentages of total radiolabeled DNA bound in the non-isomerized, fully isomerized, and trapped species for both DNA fragments interacting with wild-type and dye-labeled $\sigma$ factors are listed in Table 1. The results suggest that the labeling does not interfere with DNA binding, activation, and subsequent isomerization.

FRET Analysis of the Activation Pathway on an Early-melted Promoter—FRET assays were then carried out using donor-labeled (Alexa 488) DNA with the dye on either the non-template or template strand. Spectra were recorded for the unbound early-melted DNA, the DNA with saturating levels of $\sigma^{54}$ or core RNAP, and finally for the addition of core RNAP to the $\sigma^{54}$ early-melted DNA complex. Data were corrected by subtraction of appropriate control spectra (see details in the supplemental data). The results are shown in Fig. 2. The addition of core RNAP to early-melted DNA results in reductions in donor emission intensity compared with the protein-free promoter, suggesting that significant fluorescence quenching occurs. Adding the $\sigma$ factor to the free DNA also causes a decrease in donor intensity and an increase in acceptor fluorescence with an emission peak at 610 nm (Fig. 2, A–D). These results confirm that FRET occurs between $\sigma^{54}$ and the early-melted DNA. Assembling E$\sigma^{54}$ with the labeled $\sigma$ factor results in further decreases in donor emission that are larger than the effect(s) caused by core RNAP quenching, presumably due to alterations in the conformations in the E$\sigma^{54}$ complex. These FRET effects occur with differing efficiencies, as expected, for the different sigma variants and the different positions of the donor. Higher FRET efficiencies are apparent in the assays of $\sigma^{54}$C20, $\sigma^{54}$C463, or $\sigma^{54}$C474 binding to the DNA with the dye on the non-template strand (Fig. 2, B, D, and F) compared with the template strand (Fig. 2, A, C, and E). This is consistent with proximity expectations based on the Fe-BABE footprinting and other structural studies of these complexes (11).

Isomerization of the $\sigma^{54}$ early-melted complex (in the absence of core RNAP) via the ATP-dependent action of PspF$_{1-275}$ results in significant conformational changes, as shown by altered migration in gel shift assays (Fig. 1C, ss). In the FRET assay, the addition of PspF$_{1-275}$ to the complex in the absence of nucleotide induces slight decreases in FRET efficiency that are most easily seen when the donor is on the template strand (Fig. 3). The addition of ATP to this mixture, which would be expected to produce the isomerization seen in Fig. 1C,

FIGURE 6. Mapping changes in the relative separations of $\sigma^{54}$ domains and DNA during isomerization on the late-melted promoter. The apparent relative separations ($\Delta R_{app}$ Å) of the fluorophores are shown in the legend for Fig. 4 and are relative to the initial separations, which were as follows. A, E(σ)20 to position $-38 = 70$ Å; E(σ)20 to position $+6 = 70$ Å; B, E(σ)463 to position $-38 = 50$ Å; E(σ)463 to position $+6 = 65$ Å; C, E(σ)474 to position $-38 = 30$ Å; E(σ)474 to position $+6 = 70$ Å.
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TABLE 3
Effect of wild-type and mutant PspF1-275 on FRET between donor-labeled nirH promoters and acceptor-labeled α54 and Erα54

| Donor | Acceptor | Changes in apparent dye separation |
|-------|----------|-----------------------------------|
|       |          | No NTP | 0.2 mM ADP·AlF4 | 2 mM ATP |
| Early-melted DNA template | αC20-PspF1-275 | +20 | +25 | +30 |
| Early-melted DNA template | αC463-PspF1-275 | +10 | +25 | +30 |
| Early-melted DNA template | αC474-PspF1-275 | +15 | +25 | +30 |
| Early-melted DNA template | αC20-PspF1-275 T86A | - | ND | +10 |
| Early-melted DNA template | αC463-PspF1-275 T86A | - | ND | +20 |
| Early-melted DNA template | αC474-PspF1-275 T86A | - | ND | +20 |
| Early-melted DNA template | αC20-PspF1-275 | - | +20 | +25 |
| Early-melted DNA template | αC463-PspF1-275 | +15 | +25 | +30 |
| Early-melted DNA template | αC474-PspF1-275 | - | +35 | +40 |
| Early-melted DNA template | αC20-PspF1-275 T86A | - | ND | - |
| Early-melted DNA template | αC463-PspF1-275 T86A | - | ND | +15 |
| Late-melted DNA template | αC20-RNAP-PspF1-275 | - | - | - |
| Late-melted DNA template | αC463-RNAP-PspF1-275 | - | - | - |
| Late-melted DNA template | αC474-RNAP-PspF1-275 | - | - | - |
| Late-melted DNA template | αC20-RNAP-PspF1-275 T86A | - | ND | - |
| Late-melted DNA template | αC463-RNAP-PspF1-275 T86A | - | ND | - |
| Late-melted DNA template | αC474-RNAP-PspF1-275 T86A | - | ND | - |

Results in a more significant effect. Use of the non-hydrolysable ADP·AlF4 results in only a slight reduction in this effect compared with ATP, implying that the conformation of the complex during the transition state for nucleotide hydrolysis shares features found in the final (ATP hydrolysis-dependent) state. When the assays are repeated with a PspF1-275 variant (T86A) that can hydrolyze the ATP but is unable to couple this to isomerization of the σ factor, the FRET curves are essentially identical to those with PspF1-275 in the absence of nucleotide, establishing FRET changes as reflecting outcomes of energy coupling.

In FRET assays utilizing PspF1-275 in the absence of nucleotides, the slight difference observed for the C20 mutant on the early-melted template strand was no longer apparent. Clearly, upon binding of hydrolyzable or non-hydrolyzable nucleotide, PspF1-275 interacts with Region I of α54 bound to distort DNA, thereby altering FRET efficiencies without full isomerization. The importance of Region I in formation of the regulatory center at the −12 fork junction DNA was established in experiments using labeled homoduplexes (data not shown). The absence of FRET between C20 and donor label on the homoduplex confirms the importance of DNA distortion at this position for interaction with Region I, forming a nucleo-protein interface that is the target for interaction with the activator. These observations confirm that FRET can be used to identify and discriminate between the various forms of the activation complex and also that the mutant T86A PspF1-275 is unable to influence the conformation of the promoter bound σ factor.

The precise extent of conformational change occurring during the assays shown in Fig. 3 is not readily apparent from the spectra because only a fraction of the free DNA binds α54 and only a fraction of the bound α54 complex undergoes PspF1-275-mediated isomerization. The gel shift assays shown in Fig. 1C were carried out under essentially identical conditions to the FRET assays. Thus, it is possible to estimate the amount of each of these complexes present in the FRET assays by densitometry of the bands in Fig. 1C (Table 1). Very little affinity or functional difference between the wild-type and variant σ factors was evident.

Correcting the FRET curves for both the effects of quenching and the amounts of each species present allows us to estimate the relative dye separations when isomerization occurs (Table 2; Fig. 4; see the supplemental data). ATP hydrolysis-dependent isomerization or formation of the trapped transition state results in increases in separation of ~15–40 Å between the 5′ end of the early-melted non-template strand and the σ factor for all three single cysteine α54 mutants. This suggests something like a rigid body movement between the protein and the promoter in the direction of the transcript start (see Fig. 7A). However, there is also an apparent increase in separation (~10–25 Å) from the 5′ end of the template strand that is not nucleotide-dependent. This distance would be expected to decrease if the proteins moved toward the transcript start. Therefore, under these conditions either the σ factor is not properly engaged with the DNA or there is a “bending/scrunching” of the DNA and protein complex, probably due to the conformation of the early-melted promoter (see Fig. 7A).

**FRET Analysis of the Activation Pathway on a Late-melted Promoter**—To test the idea that the conformational changes described above were the result of the choice of promoter fragment, we then carried out equivalent assays with the late-melted promoter DNA. Acceptor fluorophore-labeled Erα54 binds to this DNA leading to decreases in donor fluorescence and increases in acceptor emission. The relative FRET efficiencies are different from those recorded for Erα54 on the early-melted promoter, suggesting that the holoenzyme is in a slightly different conformation on the two DNAs (Fig. 5, cf. Fig. 2). Similarly, in contrast to the results with the early-melted...
DNA, the addition of PspF$_{1-275}$ in the absence of nucleotide and heparin results in increased FRET efficiencies when the donor is on the non-template strand, similar to the results with the early-melted DNA. However, they increase when the donor is on the template strand, consistent with the FRET efficiencies when the donor is present on the template strand (Figs. 5 and 6, B, D, and F; Table 3). However, no significant changes to FRET occur with the donor on the non-template strand.

Adding ATP to these complexes results in decreased FRET efficiencies when the donor is on the non-template strand, similar to the results with the early-melted DNA. However, they increase when the donor is on the template strand, consistent with movement of $\sigma^{54}$ away from the upstream sequences and toward the transcription start site (Fig. 7B). Heparin was used to challenge the complexes being formed. Only ATP hydrolysis-dependent isomerized species are stable to heparin challenge, whereas intermediates are not (25, 31). The use of the transition state analogue, ADP-AlF$_4^-$, results in changes that are as great and in the same direction as those seen with ATP, consistent with the idea that this conformation is closer to the fully isomerized state than the starting state in the absence of nucleotides. As with the early-melted promoter experiments, use of the T86A PspF$_{1-275}$ variant mostly ablates these changes, confirming that they reflect specific PspF-mediated conformational rearrangements in the activation complex.

Correcting the FRET curves as described above, including following heparin challenge where appropriate, yields the FRET efficiency values listed in Table 3. Fig. 6 shows the inferred alterations in relative separation of the dyes in each case. A number of structural studies have been carried out on this system, allowing us to estimate the relative separations of protein domains from the ends of the promoter. Inspection of such structures suggests that the distance estimates for the non-isomerized $\sigma^{54}$ complexes are consistent with current cryo-electron microscopy models (36), confirming that the FRET corrections are sensible. Increases in separation from the upstream dye are closely matched by decreases in separation toward the dye located downstream. These results are consistent with an isomerization event in which the $\sigma$ factor alters its spatial arrangement on the DNA and moves toward the transcript start (Fig. 7B). This is entirely consistent with other biochemical assays of open complex formation (7, 31), although it cannot exclude a conformational rearrangement entirely within the DNA. Interestingly, the increases and decreases are different for the different mutant proteins and for the different functional states within each mutant and are not consistent with a simple rigid movement of the $\sigma^{54}$ protein domains with respect to the DNA, implying that core RNAP mediates some of the changes, which is not easily reconciled with an entirely DNA-mediated change.

Transcriptional activation is a complex, multistep process in which multiple protein complexes and promoter DNA undergo a series of defined conformational transitions. Biochemical and structural studies can only characterize and probe a limited number of these states, making it difficult to extract a temporal sequence of events defining the transcriptional activation pathway. In principle, single molecule fluorescence studies can be used to extract such information. The results presented here show that suitably labeled substrates will undergo the expected conformational rearrangements that are the prerequisite to transcription initiation. The fluorescent properties of the species generated have unique signals allowing their presence to be detected, making practical such experiments, and these are in hand.

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