Sensing DNA Opening in Transcription Using Quenchable Förster Resonance Energy Transfer†

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ABSTRACT: Many biological processes, such as gene transcription and replication, involve opening and closing of short regions of double-stranded DNA (dsDNA). Few techniques, however, can study these processes in real time or at the single-molecule level. Here, we present a Förster resonance energy transfer (FRET) assay that monitors the state of DNA (double- vs single-stranded) at a specific region within a DNA fragment, at both the ensemble level and the single-molecule level. The assay utilizes two closely spaced fluorophores: a FRET donor fluorophore (Cy3B) on the first DNA strand and a FRET acceptor fluorophore (ATTO647N) on the complementary strand. Because our assay is based on quenching and dequenching FRET processes, i.e., the presence or absence of contact-induced fluorescence quenching, we have named it a “quenchable FRET” assay or “quFRET”. Using lac promoter DNA fragments, quFRET allowed us to sense transcription bubble expansion and compaction during abortive initiation by bacterial RNA polymerase. We also used quFRET to confirm the mode of action of gp2 (a phage-encoded protein that acts as a potent inhibitor of Escherichia coli transcription) and rifampicin (an antibiotic that blocks transcription initiation). Our results demonstrate that quFRET should find numerous applications in many processes involving DNA opening and closing, as well as in the development of new antibacterial therapies involving transcription.

Processes that open and close duplex DNA regions are essential in many fundamental biological processes such as gene transcription and replication (1). For example, promoter opening is an obligatory step for transcription and serves as the first step in gene expression. During transcription initiation in Escherichia coli, RNA polymerase (RNAP) binds to a double-stranded promoter DNA region to form a closed complex (RPo). At most promoters, this reaction is followed by isomerization in which double-stranded DNA (dsDNA) is partially opened (“melted”) to form a “transcription bubble” that comprises two single-stranded DNA (ssDNA) regions; this RNAP–promoter DNA complex is known as the “open complex” (RPo) and is transcriptionally active (2, 3). Upon formation of RPo, RNAP can access the information encoded in the specific template sequence and perform template-directed gene transcription (3, 4).

Opening and closing of promoter DNA have been studied using a variety of biochemical and biophysical assays (5). Standard methods use chemical probes that exploit the different reactivity of specific nucleotides (e.g., thymines and cytosines) in the dsDNA and ssDNA forms; these assays include permanganate footprinting (6), cytosine methylation (7), and hydroxyl radical footprinting (8, 9). Other assays look at the pre-steady-state kinetics of the formation of the first phosphodiester bond, which occurs at the start of transcription; typically, this assay monitors the formation of a bond between an initiating dinucleotide and the nucleotide complementary to the third base on the template strand. These assays were initially performed using radioactive nucleotides (10) and subsequently converted into fluorescence-based assays (11, 12). These assays, however, are indirect and based on the assumption that the rate-limiting step is RPo formation and not the initiation of RNA synthesis; moreover, these assays cannot report on intermediates populated during promoter opening.

Assays using ensemble fluorescence spectroscopy relied on quantum yield changes of either aminopurine (13–15) or coumarin (12) derivatives incorporated into the DNA region that melts upon RPo formation. Though useful for determining important parameters such as binding constants, these methods use fluorophores characterized by low signal intensities and thus cannot be used for single-molecule studies, which are highly desirable for capturing detailed views of the real-time kinetics, molecular dynamics, and heterogeneity in biological mechanisms (16). Other attempts such as protein-induced fluorescence enhancement (PIFE) monitor changes in the fluorescence intensity of a single fluorophore; such changes correlate with the proximity of the unlabeled protein (17).
Recently, a single-molecule assay based on magnetic tweezers (5) was introduced to study RP formation as well as transcription initiation, promoter escape, and elongation (18). This impressive assay showed that conformational changes in DNA (“DNA scrunching”), i.e., formation and dissolution of transient DNA loops and bulges within the transcription bubble) are needed for RNAP to break its strong interactions with the promoter and escape into elongation. The temporal resolution of this technique, however, was limited to ≈1 s, complicating the observation of individual steps during initial transcription or of individual events of RNA synthesis. Moreover, monitoring multiple coordinates within transcription complexes using combinations of magnetic tweezers with other methods presents difficult experimental challenges (19). Consequently, observing the coupling of conformational changes in DNA with RNAP structural rearrangements has not been possible. Access to such information would illuminate the mechanisms of promoter opening and initial transcription in many interesting transcription systems, such as α5-dependent transcription (20) or eukaryotic transcription initiation (21).

Here, we introduce a versatile promoter opening assay based on Förster resonance energy transfer (FRET); the assay monitors DNA opening within a specific region of any DNA fragment, at the ensemble and single-molecule levels, by employing a FRET donor fluorophore on one DNA strand and a FRET acceptor fluorophore in the proximity of the donor (≤2 nm) on the complementary strand. To study RP formation and initial transcription, the fluorophores are placed within the region of transcription bubble formation. Fluorophore proximity causes contact-induced quenching, suppressing fluorescence and hence FRET in the dsDNA form. Upon RP formation (i.e., when the DNA strands within the transcription bubble are separated), contact-induced quenching is removed and a strong increase in fluorophore brightness and a high FRET efficiency is observed. Because our assay is based on quenching and dequenching FRET processes, i.e., the presence or absence of contact-induced quenching, respectively, we have named it a “quenchable FRET” (quFRET). Using lac promoter DNA fragments, we demonstrate that quFRET can probe the compaction and expansion of the transcription bubble. Finally, we use quFRET to study the mode of action of specific inhibitors of bacterial RNAP in transcription initiation. Our results clearly demonstrate that quFRET can serve as a useful tool for studying DNA opening and closing, as well as biomolecules that modulate these two processes.

MATERIALS AND METHODS

DNA and Reagents. Unless otherwise stated, reagents of luminescent grade were used as received. Amino-modified oligonucleotides (IBA) were internally labeled with NHS-conjugated fluorophores Cy3B and ATTO647N (Invitrogen and ATTO-TEC) according to published protocols and purified using a liquid chromatography system (AKTA, GE Healthcare). Labeled and purified DNA single strands were annealed in hybridization buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 500 mM NaCl]. The two different DNA fragments will be called Cy3B and ATTO647N (donor fluorophore at position X and acceptor fluorophore at position Y with respect to transcription start site named +1) throughout this work. The DNA sequence (Figure 1a) is derived from a lac consensus promoter DNA to which bacterial RNA polymerase binds. For a photophysical characterization and comparison experiments, we used a DNA sample with an 18 bp separation between Cy3B and ATTO647N (22). The sequences of these oligonucleotides (IBA) are shown in Figure S1 of the Supporting Information.

Formation of the RNA Polymerase Open Complex and Initial Transcribing Complexes. The open complex (RP) of E. coli RNA polymerase was formed according to published procedures (16, 23, 24). Briefly, dsDNA (10 nM, rifampicin [0 or 250 nM (Sigma Aldrich)], and RNAP holoenzyme [50 nM (Epitcentre or USB)] were mixed in a total volume of 20 μL of KG7 buffer [40 mM HEPES-NaOH (pH 7), 100 mM potassium glutamate, 10 mM MgCl2, 1 mM DTT, 100 μg/mL BSA, 5% glycerol, and 1 mM mercaptoethanol] and incubated at 37°C for 15 min. Subsequently, heparin Sepharose [1 mg/mL (GE Healthcare)] was added to disrupt nonspecific RNAP–DNA complexes and remove free RNAP. After 30 s at 37°C, the samples were centrifuged, and 13 μL of supernatant was transferred to a different tube, supplemented with either 500 μM ApA (for RP<sub>sc</sub>), 500 μM ApA and 50 μM UTP (for RP<sub>sc3</sub>), 500 μM ApA, 50 μM UTP, and 50 μM GTP (for RP<sub>scd</sub>), or 500 μM ApA, 50 μM UTP, 50 μM GTP, and 50 μM ATP (a mix capable of forming RD<sub>on</sub>) for a total reaction volume of 10 μL. Before each single-molecule experiment, transcription complex samples were incubated for a further 20 min at 37°C.

RNAP Functional Assay Using in Vitro Transcription and Radioactive Nucleotides. RP was formed in T8 buffer [50 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM MgCl₂, 100 μg/mL BSA, 1 mM DTT, and 5% glycerol] as described in the previous section. Heparin Sepharose (1 mg/mL) was added to the reaction mixtures; they were incubated for 30 s at 37°C and centrifuged for 10 s, after which 5 μL of the supernatant was transferred to a prewarmed tube. The in vitro transcription reaction mixtures were set up by adding 1 μL of supernatant to 4 μL of KG7 buffer [40 mM HEPES-NaOH (pH 7), 100 mM potassium glutamate, 10 mM MgCl₂, 100 μg/mL BSA, 1 mM DTT, 1 mM MEA, and 5% glycerol] supplemented with 4 units of SUPERase-In [20 units/μL (Ambion, Inc.)], 500 μM ApA, 50 μM UTP (RP<sub>sc</sub>), or 50 μM UTP, 50 μM CTP, and 50 μM ATP (full transcript) in the presence of 0.3 μCi/μL [α-<sup>32</sup>P]GTP [10 μCi/μL (Perkin Elmer)] and incubated for 5 min at 37°C. Reactions were stopped by addition of 1 reaction volume of loading dye [80% (v/v) formamide, 10 mM EDTA, 0.04% (w/v) bromophenol blue, and 0.04% (w/v) xylene cyanol FF], and mixtures were incubated for 5 min at 95°C, electrophoresed on a 6 M urea–25% polyacrylamide sequencing gel, and visualized by autoradiography. To ensure an RNase-free environment, the labware was cleaned with RNaseZap wipes or solution (Ambion, Inc.); RNase-free solutions were prepared in the laboratory, filtered, and autoclaved, using only sterile (RNase-free) tubes and glassware.

gp2–RNA Polymerase Interaction Assay Using Gel Electrophoresis. Inhibition of transcription complexes by gp2 was studied by incubation of 50 nM RNAP with 1 μM gp2 for 20 min at 37°C, after which 10 nM promoter DNA was added, followed by incubation for 15 min at 37°C (route 1). Alternatively, 1 μM gp2 was incubated for 15 min at 37°C with preformed RP<sub>p</sub> (route 2). A fraction of each reaction mixture was challenged with 1 mg/mL heparin Sepharose. The resulting samples were used in single-molecule experiments or gel electrophoresis. For gel electrophoresis, 5 μL of each sample (reaction mixture or supernatant with 0.5 μL of 50% glycerol added) was run on a 4.5% native polyacrylamide gel.
Ensemble Absorption and Fluorescence Spectroscopy.

Absorption spectra were recorded on a standard absorption spectrometer (Cary 50 Bio, Varian). Fluorescence spectra were recorded using a scanning spectrofluorometer (PTI). The average configuration for recording spectra was a 1 s integration time per 1 nm wavelength interval over a total range of several hundred nanometers. Excitation was performed at 533 nm (Cy3B) or 640 nm (ATTO647N). Studies were performed on lacCy3B, −5/ATTO647N, −3 (Figure 1a) or singly labeled versions of the promoter bearing either Cy3B or ATTO647N. Fluorescence time traces collected to monitor transcription complex formation were recorded at 37 °C at a DNA concentration of ≈50–100 pM. The measurement time was ≈20 min for panels a and b.

Single-Molecule Fluorescence Spectroscopy.

For single-molecule fluorescence and FRET, a custom-built confocal microscope was used as described previously (26, 27). The setup allowed alternating-laser excitation of donor and acceptor fluorophores (28, 29). The fiber-coupled output of a green [532 nm (Samba, Cobolt)] and red laser [638 nm (Cube Coherent)] was alternated with a modulation frequency of 10 kHz. The spatially filtered beams were coupled into an inverted confocal microscope [IX71 (Olympus)] equipped with an oil-immersion objective [60×, 1.35 NA, UPLSAPO 60XO (Olympus)]. The average excitation intensities for measurements were 250 μW at 532 nm and 50 μW at 640 nm. The resulting fluorescence was collected using the same objective, separated from excitation light by a dichroic mirror, focused onto a 200 μm pinhole, and split spectrally on two avalanche photodiodes [SPCM-AQR-14 (PerkinElmer)] detecting the donor and acceptor fluorescence with appropriate spectral filtering (green for 585DF70 and red for 650LP). The detector signal was registered and evaluated using custom LabVIEW software. The temperature of the sample was controlled within ±1 °C.

Data Analysis. Fluorescence photons arriving at the two detection channels (donor detection channel, Dexc; acceptor detection channel, Aexc) were assigned to either donor (Dem) or acceptor (Aem) excitation on the basis of their photon arrival time as described previously (28, 29). Fluorophore stoichiometries S and apparent FRET efficiencies E* were calculated for each
fluorescent burst above a certain threshold, yielding a two-dimensional (2D) histogram. Here, S is defined as the ratio of the overall green fluorescence intensity to the total green and red fluorescence intensity and describes the ratio of donor-to-acceptor fluorophores in the sample (28, 29). Uncorrected FRET efficiency \( E^* \) [defined as \( D_{\text{exc}} A_{\text{em}} / (D_{\text{exc}} A_{\text{em}} + D_{\text{em}}) \)] monitors the proximity between the two fluorophores. Using published procedures to identify bursts corresponding to single molecules (30), we obtained bursts characterized by three parameters (M, T, and L). A fluorescent signal is considered a burst provided it meets the following criteria: a total of \( L \) photons having \( M \) neighboring photons within a time interval of \( T \) microseconds. Unless stated otherwise, we identified acceptor-containing molecules by applying a burst search on \( A_{\text{exc}} A_{\text{em}} \) using an \( M \) of 7, a \( T \) of 500 \( \mu \)s, and an \( L \) of 12; additional per-bin thresholds removed spurious changes in fluorescence intensity and selected for bright donor—acceptor molecules (\( A_{\text{exc}} A_{\text{em}} > 30–50 \) photons). Binning the detected bursts into a 2D \( E^* - S \) histogram allowed us to separate molecules labeled with only an acceptor fluorophore (\( S \approx 0.2 \)) from molecules with both fluorophores present (\( S \approx 0.6 \); see the dashed rectangle in Figure 1a). The one-dimensional (1D) \( E^* \) distribution for donor—acceptor species was obtained by using a 0.45 < \( S < 0.8 \) threshold; the \( E^* \) distributions were then fitted using a Gaussian function, yielding the mean \( E^* \) value for the distribution and an associated standard deviation \( \sigma \).

RESULTS AND DISCUSSION

Sensing RP\(_o\) Formation Using quFRET. Our assay relies on the proximity (<2 nm) of two fluorophores; because this proximity is disrupted upon strand separation and transcription bubble formation, we reasoned that the assay can be used to study RP\(_o\) formation and related processes. Our fluorophore pair comprises a “green” and a “red” fluorophore (i.e., excited by green and red excitation wavelengths, respectively) that can participate in FRET; the FRET pair in our experiments consists of Cy3B (donor) and ATTO647N (acceptor). When the fluorophores are on fully double-stranded DNA, their fluorescence is quenched because of transient or static contacts between the fluorophores, whereas upon DNA separation in the region of the fluorophores (i.e., DNA becomes partially single-stranded), contact-induced quenching is replaced by the occurrence of FRET; the FRET efficiency can then report on the extent of local DNA opening.

To demonstrate the principle of the quFRET assay, we monitored RP\(_o\) formation on lac promoter DNA fragments. We prepared a DNA fragment carrying the donor on the non-template strand at position \(-5 \) and the acceptor on the template strand at position \(-3 \), both positions being relative to the transcription start site; this DNA was named lac\(^{\gamma 3B-5/ATTO647N\sim} \). The chosen labeling sites place the fluorophores in close proximity (<2 nm (Figure 1a)] and within the DNA segment that gives rise to the transcription bubble (Figure 1a,b).

The considerable size of the organic fluorophores used for quFRET has the potential to perturb the system under study; to establish whether fluorophore labeling interfered with important RNAP functions (RP\(_o\) formation, initial transcription, and promoter escape), we performed in vitro transcription assays in the presence of radioactive nucleotides. The results showed that RNAP synthesizes similar amounts of abortive products regardless of the presence or absence of labels on the template DNA; however, the labeled construct led to an increased level of promoter escape (Figure S2 of the Supporting Information).

This tendency is probably attributable to slight destabilization of the transcription complexes when fluorophores are present. Such perturbation may be a concern, but in determining kinetic rate constants for promoter opening between our lac fragment and lacUV5 DNA studied in earlier work (see ref 12 and Monitoring the Kinetics of Promoter Opening Using quFRET), we found that promoter opening is not significantly affected. Because (i) a similar ladder of abortive products was obtained from unlabeled fragments, (ii) rates of promoter opening were in agreement with previous studies, and (iii) we see only apparent enhanced promoter escape, we reasoned that introduction of fluorophores within the transcription bubble did not significantly interfere with the biological activity of our studied system. Initial transcribing complexes were fully active, and any insights and conclusions obtained from our experiments are not affected by an increased level of promoter escape.

To detect RP\(_o\) formation, we performed single-molecule FRET spectroscopy with alternating-laser excitation [ALEX (28, 29)] on diffusing molecules of dsDNA and RP\(_o\) (Figure 1b). After identifying single fluorophore molecules, we used their fluorescence intensities to construct a 2D histogram of apparent FRET efficiency (\( E^* \)) and relative probe stoichiometry (\( S \)) (28, 29). For free double-stranded DNA molecules that carry a fluorescent acceptor, we observe two main species: a major subpopulation (≈85% of all molecules) with a low \( S \) value of < 0.4, corresponding to acceptor-only species, and a minor subpopulation with intermediate \( S \) value (0.45 < \( S < 0.8 \); ≈15% of all molecules), corresponding to donor—acceptor species. The 1D \( E^* \) histogram for the donor—acceptor species (corresponding to the dotted rectangle in Figure 1b) shows a broad and unstructured FRET distribution characterized by the absence of high-FRET species (\( E^* > 0.9 \)), which were expected to appear if contact-induced quenching was absent (e.g., see Figure 3). The number of detected molecules is only ≈120 (Figure 1b, left panel), a low number upon comparison of lac\(^{\gamma 3B-5/ATTO647N\sim} \) to a dsDNA standard carrying the same fluorophores with an 18 bp separation; this probe showed ≈1500 detected molecules with intermediate \( S \) values (Figure S3a of the Supporting Information).

Upon RP\(_o\) formation, we observed three major changes in the \( E^* - S \) histogram. First, the relevant fraction with intermediate \( S \) values increases substantially (from ≈15 to ≈60% of all molecules). Second, a population with a high \( E^* \) is observed [\( E^* \approx 0.7 \) (Figure 1b, right panel)]. Third, the number of detected molecules with intermediate \( S \) values increases substantially. These changes are consistent with removal of the contact-induced quenching between the fluorophores upon RP\(_o\) formation, presumably due to the separation of the two DNA strands. These observations and the quenching hypothesis are further supported by comparing the photon counting histograms [PCH (Figure S4 of the Supporting Information)] of dsDNA and RP\(_o\) from Figure 1, which were recorded for comparable concentrations (50–100 pM) and measurement times of 20 min. Upon RP\(_o\) formation, the PCHs show a significant increase in the total number of events for all three channels (Figure S4a–c of the Supporting Information, \( D_0 D_{\text{em}}, A_0 A_{\text{em}}, D_0 A_{\text{em}} \)). The mean number of detected photons in a burst also increases significantly for FRET [\( D_0 A_{\text{em}} \), mean of ≈60 photons (Figure S4c of the Supporting Information)] and acceptor-direct excitation photons [\( A_0 A_{\text{em}} \), mean of ≈30 photons (Figure S4b of the Supporting Information)] pointing to removal of contact-induced quenching upon RP\(_o\) formation.

Analyzing the data to retain all fluorescent bursts (donor-only, acceptor-only, and donor—acceptor species) reveals that in the
quenched form of lac\(^{Cy3B,−5\text{ATTO647N},−3}\), a substantial apparent fraction of the molecules (80%) is due to donor-only species, followed by acceptor-only (15%) and donor−acceptor (5%) species (Figure S5 of the Supporting Information, left panel; note that the fractions are not corrected for the different brightnesses of the two fluorophores). Upon formation of the open complex, we observe a significant increase in both the number of FRET events and their signal intensity, with ≈50% of all molecules being donor−acceptor species, ≈40% donor-only species, and ≈10% acceptor-only species. These results suggest that the quenching process mainly affects the acceptor fluorophore. We additionally observe a “smear” from the FRET to the donor-only population; this smear is likely caused by blinking and photobleaching of the acceptor fluorophore (25, 31).

To show that quFRET does not depend on the exact positions of the fluorophores (provided that they are within the range for contact-mediated quenching, <2 nm), we examined a variant of our DNA carrying the two fluorophores at different positions within the transcription bubble (lac\(^{Cy3B,−3\text{ATTO647N},−3}\), with Cy3B and ATTO647N at position −3 on the nontemplate and template strands, respectively). The results obtained for this dsDNA and its corresponding RP\(_0\) were essentially identical to the results obtained for lac\(^{Cy3B,−5\text{ATTO647N},−3}\): quenching for dsDNA and high \(E^*\) for RP\(_0\) (Figure S6a,b of the Supporting Information).

Characterization of Fluorophore−Fluorophore Interactions in quFRET. To characterize the process responsible for our single-molecule fluorescence observables, we used ensemble fluorescence spectroscopy to examine fluorophore emission spectra under nonquenching and quenching conditions (Figure 2).

The emission spectrum of Cy3B on dsDNA in the absence of ATTO647N (Figure 2a, lac\(^{Cy3B,−3}\)) shows a maximum at 570 nm and a shoulder at ≈615 nm. On the other hand, lac\(^{Cy3B,−5\text{ATTO647N},−3}\) with both fluorophores on DNA shows a similar spectral profile with an additional shoulder at ≈660 nm, but with ≈35% decreased emission intensity (Figure 2a, dotted line). Adding 1.5 mM anionic surfactant [sodium dodecyl sulfate (SDS)] leaves the intensity of Cy3B unaffected but strongly increases the long-wavelength emission centered at ≈660 nm (Figure 2a, dashed line). This long-wavelength emission is due to FRET processes in ATTO647N (Figure 2b).

On the other hand, the emission spectrum of ATTO647N in the absence of Cy3B (Figure 2b) shows a maximum at ≈660 nm and a broad emission tail extending beyond 700 nm. As for Cy3B, the ATTO647N emission is reduced by 70% for lac\(^{Cy3B,−5\text{ATTO647N},−3}\) (Figure 2b, dotted line); moreover, dequenching by 60% is observed upon addition of 1.5 mM SDS (Figure 2b, dashed line). Interestingly, the signal intensity of Cy3B in lac\(^{Cy3B,−5\text{ATTO647N},−3}\) is constant upon addition of SDS (Figure 2a, dotted line vs dashed line), likely because of the combined effects of dequenching of Cy3B (that increases Cy3B fluorescence intensity) and an increase in the level of FRET (that decreases Cy3B fluorescence intensity).

Interactions of the fluorophores and the protein−DNA complex were additionally investigated using anisotropy measurements. As expected, we find low anisotropies for the free dyes Cy3B (\(r = 0.05 \pm 0.02\)) and ATTO647N (\(r = 0.08 \pm 0.02\)) in aqueous solution. An increase to \(r\) values of 0.22 ± 0.02 (Cy3B) and 0.18 ± 0.02 (ATTO647N) was found for lac\(^{Cy3B,−5\text{ATTO647N},−3}\), these values for dsDNA were identical for Cy3B-only and ATTO647N-only forms on the lac promoter. A large anisotropy increase is observed for both dyes upon RP\(_0\) formation (for Cy3B, \(r = 0.31 \pm 0.03\); for ATTO647N, \(r = 0.30 \pm 0.04\)). Our results indicate the strong influence of the protein environment on the rotational freedom of the fluorophores. Thus, assigning FRET changes during and after
RP o formation purely to changes in intraprobe distances is not straightforward; however, one can obtain FRET signatures for specific transcription complexes (that can be preferentially formed upon addition of DNA and nucleotide subsets) and then monitor the transitions between the calibrated FRET states.

To check whether static quenching is a contributing mechanism for the observed quenching, we recorded the absorbance spectra of free Cy3B and DNA (Figure S7 of the Supporting Information). The results show that the absorption maximum of Cy3B (≈560 nm) is blue-shifted to ≈548 nm when ATTO647N is present (Figure S7 of the Supporting Information). This blue shift is an indication of the presence of static quenching based on the formation of H-dimers, as discussed previously (32). The observed quenching can, however, also be in part due to (i) contact-induced quenching of both fluorophores with a dynamic origin (32) or (ii) DNA-mediated photoinduced electron transfer (33–35).

We also performed experiments at the single-molecule level using ALEX spectroscopy. Upon addition of either 50% (v/v) ethanol (Figure 3b) or 1.5 mM SDS (Figure 3c), contact-induced quenching seen in dsDNA (Figure 3a) is eliminated and distributions with very high FRET values emerge (maximal E° > 0.9).

We observe that dequenching appears to have distinct threshold concentrations of ≈30% (v/v) ethanol and ≈0.75 mM SDS (data not shown). To rule out specific interactions between SDS and the two fluorophores, similar experiments were performed using the same FRET pair with an 18 bp separation; no FRET change was observed upon addition of SDS (Figure S3 of the Supporting Information). Our findings provide strong support for the presence of contact-induced quenching in dsDNA and the dequenching caused by either surfactants or partial opening of DNA, e.g., due to RP o formation. The observed dequenching is possibly due to the attenuation of hydrophobic interactions between the two fluorophores and the formation of a solvation shell around the fluorophores caused by favorable interactions between ethanol or SDS and hydrophobic parts of the fluorophore molecules (32). While revealing the exact underlying mechanism causing our observations is worthy of a study in its own right, we chose to focus our efforts in applying the assay to provide insight into biological systems. A more detailed description of the underlying dye photophysics is found in refs (32–35) and could be the principal subject of a future publication.

**Monitoring the Kinetics of Promoter Opening Using quFRET.** Kinetic analysis of transcription on different promoters and promoter-proximal regions can establish rate-limiting steps that modulate transcription either by endogenous factors or by small molecules that can serve as antibacterial agents. In initial transcription, this step is often the isomerization from a closed RNAP-DNA complex to RPo (12–15). To follow the kinetics of RP o formation, we monitored the time dependence of the ensemble FRET signal increase due to fluorophore dequenching in lacC3B–5/ATTO647N–3 (Figure 4).

Upon mixing lacC3B–5/ATTO647N–3 (20 nM) with the RNAP holoenzyme (100 nM) at 37 °C, we observe a biexponential increase in fluorescence intensity due to RP o formation. The measured rate constants of four individual experiments show the following mean values: \( k_1 = (63 \pm 56) \times 10^{-3} \, \text{s}^{-1} \) (amplitude of 45%), and \( k_2 = (4.9 \pm 2.8) \times 10^{-3} \, \text{s}^{-1} \) (amplitude of 55%). The rates are in good agreement with published values for a related promoter (lacUV5) determined using a gel shift assay where biexponential kinetics were also observed (32–35) with an amplitude of 60%, interpreted as formation of closed complexes, and \( k_2 = 12 \times 10^{-3} \, \text{s}^{-1} \) with an amplitude of 40%, interpreted as isomerization (12). The 2-fold difference between the studies may reflect measurement errors (partly due to manual mixing) and the differences between the lac sequences used in the two studies (36).

Our results establish the ability of quFRET to monitor the kinetics of promoter opening in a simple yet quantitative fashion. The full compatibility of this assay with single-molecule fluorescence detection should allow monitoring of the kinetics of promoter opening at the level of diffusing single molecules [with a temporal resolution of minutes (29)] or at the level of immobilized single molecules with millisecond time resolution (37).

**Monitoring Abortive Transcription and Promoter Escape Using quFRET.** Mechanistic steps occurring after RP o formation, such as abortive initiation (i.e., the reiterative synthesis and release of RNA transcripts two to nine nucleotides in length by initial transcribing complexes, RP o), also change the size and shape of the transcription bubble. Prior work in our laboratory (23) and by others (17) showed that abortive initiation by bacterial RNAP proceeds by a DNA scrunching mechanism, involving formation and dissolution of transient bulged or looped DNA structures within the transcription bubble. Neither of the methods, however, observed DNA scrunching directly; also unclear is which parts of the bubble move (or become scrunch) during abortive initiation. We thus tested whether quFRET can provide a means of addressing these questions.

To probe DNA conformational changes using quFRET, we used the DNA fragment lacC3B–5/ATTO647N–3, which carries both fluorophores within the transcription bubble of RP o. We prepared initial transcribing complexes capable of synthesizing abortive RNA of different maximum lengths by using nucleotide subsets dictated by the initial transcribed sequence of our lac fragment (Materials and Methods) and observing their smFRET signatures (Figure 5).

Upon addition of 500 μM initiating dinucleotide ApA to RP o, we formed complex RP o,c (equivalent to a transcription complex in which RNAP has synthesized the first dinucleotide, ppApA); FRET analysis of the donor–acceptor species showed a distinct FRET peak with a mean E° of ≈0.68 (a value essentially identical to that of RP o,c compare to Figure 1b) and a standard deviation of
in the RNAP initiation complexes, where the scrunched DNA is
results from quFRET in combination with additional systematic
Hwang and A. N. Kapanidis, unpublished observations). These
signatures of RP\textsubscript{o}, RP\textsubscript{itc,2}, and RP\textsubscript{itc,4} and the signature of RP\textsubscript{itc,7}.

Similar results [i.e., decrease in apparent FRET and distribution
broadening upon movement from RP\textsubscript{itc,2} to RP\textsubscript{itc,7} (see Figure S6
of the Supporting Information)] were obtained for a different
DNA, in which the donor is incorporated at position
3\textsuperscript{th} on the nontemplate strand (L. C.
Hwang and A. N. Kapanidis, unpublished observations). These
results from quFRET in combination with additional systematic
smFRET experiments within several RP\textsubscript{itc} complexes of the bacterial RNAP should allow identification of the position and
dynamics of scrunched DNA in the initial transcription for
multisubunit RNAP. In a future publication, we intend to
compare the predictions and validity of different models, e.g.,
the “steric exclusion model” \cite{23, 38}, which describes scrunching
in the RNAP initiation complexes, where the scrunched DNA is
accommodated within or close to the active site pocket of RNAP,
or other existing models \cite{39, 40}.

We also considered whether our FRET pair can sense the
downstream movement of the transcription bubble upon
promoter escape and formation of RNAP–DNA elongation complexes (RD\textsubscript{e}) \cite{2, 3}. To form the first stable elongation complex
(RD\textsubscript{e,11}, a stable elongation complex in which RNAP has
synthesized an 11-mer RNA transcript still bound within the
complex), we added UTP, GTP, and ATP to RP\textsubscript{o}. In this case,
although the DNA between the two fluorophores is expected to
reclose, it was unclear whether the quenched state would be
reformed, since the local presence of protein residues may affect
quenching in a manner similar to that of ethanol or SDS. Our
results for RD\textsubscript{e,11} show a broad E\textsuperscript{*} distribution, but with many
more donor–acceptor molecules than in free DNA (Figure 5e,
with a measurement duration 3-fold shorter than that for
Figure 5a). Our result most likely represents a mixture of species:
elongation complexes that escape to elongation and assume a
fully or partially quenched state, transcription complexes unable
to escape to elongation [with this species ranging from \(\approx\)15 to
40\% \cite{21, 41, 42}], and free DNA molecules due to heparin
challenge. Further experiments with immobilized molecules will
help distinguish between such subpopulations.

To confirm that the observed FRET changes in initial
transcription and elongation are due to transcription reactions,
we performed control experiments using rifampicin, an antibiotic
that prevents synthesis of RNA transcripts longer than two or
three nucleotides (Figure 6), even in the presence of all four
nucleotides \cite{43}.

Indeed, preincubation of RNAP with rifampicin before RP\textsubscript{o}
and RP\textsubscript{itc,7} formation (Figure 6c) abolishes the 8\% decrease in E\textsuperscript{*}
seen upon the addition of UTP and GTP to RP\textsubscript{o} (to form RP\textsubscript{itc,7})
in the absence of rifampicin (Figure 6a,b). These results establish
that the FRET changes shown in Figures 5 and 6 are due to RNA
synthesis and conformational changes in DNA. These
experiments also show that quFRET can be used to study the mode
of action of antibiotics that block initial transcription and promoter
escape and to screen novel antimicrobial compounds that target
transcription.
FIGURE 7: gp2 inhibits transcription by preventing DNA opening. (a) Results of gp2 inhibition of RP₀ formation at lacCy3B−5/ATTO647N−3 using gel electrophoresis; the images shows acceptor emission upon acceptor excitation (a signal that is not affected by FRET but is affected by contact-induced quenching). Different lanes correspond to different sample mixtures or orders of addition: (1) dsDNA only, (2) dsDNA and RNAP, with heparin Sepharose challenge, (3) dsDNA, RNAP, and gp2, with heparin Sepharose challenge, and (4) RNAP, gp2, and dsDNA, with heparin Sepharose challenge. (b) ALEX-based Eᵣ histograms of single diffusing molecules of transcription complexes formed using lacCy3B−5/ATTO647N−3 DNA at 37 °C and a DNA concentration of ≈50–100 μM. The panels show apparent FRET of samples found in gel lanes 1–4 (panel a) as indicated in the figure. The measurement time was ≈30–40 min for each panel.

Using quFRET To Study the Mechanism of a Specific Inhibitor of Promoter Opening. To demonstrate the ability of quFRET to study protein–protein interactions that modulate transcription, we studied the mechanism by which gp2, a small T7 phage-encoded protein and potent inhibitor of E. coli RNAP, blocks transcription (44). Recent experiments have suggested that gp2-based inhibition involves prevention of RNAP–promoter DNA interactions required for strand separation and formation of the transcription bubble (44).

To test the proposed mechanism of gp2 action, we used quFRET on lacCy3B−5/ATTO647N−3. To establish whether the fluorophores perturb the inhibitory activity of gp2 on formation of RNAP–promoter DNA complexes, we performed a gel mobility shift assay (Figure 7a, native gel electrophoresis).

As expected, gp2 cannot disrupt preformed RNAP–DNA complexes (Figure 7a, lane 3). When preincubated with RNAP alone, however, before addition of promoter DNA, gp2 prevents RP₀ formation (Figure 7a, lane 4). These results are in agreement with published results (44) and indicate that the fluorophores do not interfere with gp2 activity.

To test whether quFRET can identify the gp2 mode of action, we examined the effect of gp2 addition using quFRET (Figure 7b). Free dsDNA (lacCy3B−5/ATTO647N−3) shows the broad featureless FRET distribution (Figure 7b, top panel) seen in Figures 1a and 5. As expected, RP₀ formation is evident as a distinct FRET peak at 0.68 (Figure 7b, second panel); an identical FRET signature is obtained when we first form RP₀ via incubation of RNAP with dsDNA and then addition of gp2. In contrast, no RP₀ formation is seen when gp2 and RNAP are preincubated before the addition of promoter DNA (Figure 7b, bottom panel; conditions comparable to those in lane 4 of Figure 7a). This is evident by the very small number of molecules with Eᵣ values of ≈0.7, i.e., the region populated by the RP₀ for lacCy3B−5/ATTO647N−3 (Figures 1 and 2). Our results provide strong support for recent work showing that gp2 prevents bacterial transcription by blocking steps along the formation of RP₀ (44).

Comparison of quFRET and Conventional smFRET. What are the studies that will benefit from the unique features of quFRET versus conventional smFRET? First, one should consider that in cases of donor–acceptor proximity (either due to constraints in experimental design or due to limited information about molecular conformations), quenching is a very real possibility that has to be characterized (32–35), understood, and, if possible, turned into a tool. If such quenching remains undetected, it is likely that a significant fraction of donor–acceptor molecules may be absent from the FRET histograms; FRET will hence not be a reliable ruler, and FRET monitoring cannot be used for kinetic studies without modification of standard analysis methods.

Second, the proximity afforded by quFRET fluorophore pairs results in a very large signal during processes such as DNA opening; this large signal of quFRET is accompanied by the ability to monitor conformational changes within DNA (which relies on conventional smFRET) and on top of changes in the local environment of the fluorophores [e.g., due to PIFE (17)], effects that can be monitored using ALEX-based direct monitoring of both fluorophores.

Finally, the suppression of the fluorescence signal in the absence of DNA opening allows studies of rare events that can be observed in the presence of high concentrations of a DNA probe. The quFRET assay also provides a no-background scenario that can be used to look at rare promoter opening events in constrained situations, such as in bacterial cells loaded with quFRET sensors (a measurement that is very difficult using standard smFRET techniques).

CONCLUSIONS

We introduced a FRET-based assay capable of monitoring the local state of DNA (single- vs double-stranded) both at the ensemble and single-molecule levels. The assay requires the
attachment of two fluorophores in proximity, one on each strand of the dsDNA under study. While quFRET is unlikely to be compatible with every FRET pair, we have shown that the pair of Cy3B and ATTO647N (which are excellent fluorophores for single-molecule experiments) is a robust pair for this new assay. We anticipate that future work will identify additional fluorophore pairs for quFRET; in particular, it would be desirable to identify quFRET-compatible fluorophores in the blue spectral range, because they are smaller moieties and, as such, less likely to interfere with biological function.

We presented different applications and a photophysical rationale for quFRET’s working principle. We showed that quFRET can monitor initial transcription, e.g., RP, formation, and bubble expansion and compaction during initial transcription. The quFRET assay allowed us to monitor the kinetics of binding of bacterial RNAP to lac promoter DNA and to study the mode of action of small proteins and antibiotics that block different steps in transcription initiation and initial transcription. The latter results established that quFRET will find numerous applications not only in mechanistic studies but also in the development of new antibacterial therapies. This work also lays the foundation for using quFRET for the real-time detection of abortive initiation and promoter escape using surface-immobilized transcription complexes.

**SUPPORTING INFORMATION AVAILABLE**

DNA oligonucleotide sequences and additional experimental results. This material is available free of charge via the Internet at http://pubs.acs.org.

**REFERENCES**


