Expedient placement of two fluorescent dyes for investigating dynamic DNA protein interactions in real time

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Abstract

Many questions in molecular and cellular biology can be reduced to questions about ‘who talks to whom, when and how frequently’. Here, we review approaches we have used with single-pair fluorescence resonance energy transfer (spFRET) to follow the motions between two well-placed fluorescent probes to ask similar questions. We describe two systems. We have used a nucleosomal system in which the naked DNA molecule has the acceptor and donor dyes too far apart for FRET to occur whereas the dyes are close enough in the reconstituted nucleosome for FRET. As these individual nucleosomes were tethered on a surface, we could follow dynamics in the repositioning of these two dyes, inferring that nucleosomes stochastically and reversibly open and close. These results imply that most of the DNA on the nucleosome can be sporadically accessible to regulatory proteins and proteins that track the DNA double helix. In the case of following the binding of recombination protein RecA to double-stranded DNA (dsDNA) and the RecA filament displacement by DNA helicase motor PcrA, the dsDNA template is prepared with the two dyes close enough to each other to generate high FRET. Binding of the RecA molecules to form a filament lengthens the dsDNA molecule 1.5-fold and reduces the FRET accordingly. Once added, DNA motor protein helicase PcrA can displace the RecA filament with concomitant return of the DNA molecule to its original B-form and high FRET state. Thus, appropriately placed fluorescent dyes can be used to monitor conformational changes occurring in DNA and or proteins and provide increased sensitivity for investigating dynamic DNA–protein interactions in real time.

Introduction

The combination of physics and biology has led to single-molecule approaches in which one macromolecule is studied (Science 1999, JBC 1999, Leuba & Zlatanova 2001, Rigler *et al.* 2002, Zander *et al.* 2002). Asynchronous processes such as transcription can be followed one-molecule-at-a-time (Zlatanova

et al. 2006). Single-molecule approaches including electron microscopy and atomic force microscopy and magnetic and optical tweezers have already clarified chromatin structure and dynamics (Leuba & Zlatanova 2002, Zlatanova & Leuba 2002, 2003a,b, 2004b, Zlatanova 2003, Leuba *et al.* 2004, Claudet & Bednar 2006, van Holde & Zlatanova 2006, Zlatanova & van Holde 2006), such as the

three-dimensional arrangement of nucleosomes in a chromatin fiber, the piconewton forces needed to unravel individual nucleosomes and the stretching forces applied to DNA needed to prevent nucleosome assembly.

Movements in molecular complexes can be mapped to three-dimensional distance changes between any two points over time. The change in the distance between two attached fluorescent dyes serves as an indicator of larger-scale conformational changes. Expedient placement of two fluorescent dyes, a donor and acceptor dye pair, facilitates measurement of dynamic DNA and/or protein conformations through single-pair fluorescence resonance energy transfer (spFRET). spFRET is FRET from one donor dye to one acceptor dye molecule (recently reviewed in Zheng *et al.* 2007, Deniz *et al.* 2008). The following formula indicates the distance relationship of FRET: $E_{\text{FRET}} = 1/(1+(R/R_0)^6)$ (Clegg 1992), where E_{FRET} is the efficiency of FRET (Förster 1959), R is the radius between the acceptor and donor dye and R_0 is the distance at which 50% FRET occurs between the two dyes (Förster 1959). Reversible conformational transitions that bring the two covalently attached dyes close together and then farther apart on the same molecule will result in anti-correlated intensities of the two independent dyes, confirming that they are on the same molecule. The transfer of energy is affected also by the orientation of the two dyes. Most spFRET studies rely on the relative changes in FRET rather than absolute distance changes (Ha 2001). spFRET occurs within ~2 to ~8 nm in a commonly used dye pair, cyanine dyes Cy3 and Cy5 (Mujumdar *et al.* 1993). The R_0 for this dye pair is 6 nm (Tan *et al.* 2003). With the quality of fluorescent dye pairs presently available (Mujumdar *et al.* 1993) and recent developments in spFRET (Weiss 1999, Selvin 2000, Ha 2001, Zheng *et al.* 2007, Deniz *et al.* 2008), it should be possible to follow these nanometer motions in the millisecond timescale range.

In this review, we will describe two approaches based on site-specific fluorescently labeled dsDNA templates that we have used to follow nucleosome opening, recombination protein assembly and disassembly, and DNA helicase translocation. The purpose of this review is not to give a comprehensive coverage of the progress made in these two model systems, but to give the reader an idea of how different dye placements can be used to follow dynamics in DNA–protein interactions.

Fluorescent labeling of proteins or DNA molecules

In proteins deficient in cysteine, an introduced cysteine can be used as a handle for the maleimide of the fluorescent dye. Nucleic acid suppliers can prepare oligonucleotides with a fluorescent dye at the 5' end, the 3' end, and/or some dyes internally such as tetramethylrhodamine (e.g. www.IDTDNA.com). Alternatively, nucleic acid companies can produce oligonucleotides with internal amino linkers, which can be labeled chemically with the *N*-hydroxysuccinimide ester of the fluorescent dye (e.g., Zheng *et al.* 2005). Another method to label one strand of dsDNA is to use the other strand as a template containing two or three out of four nucleotides. Then, using Klenow polymerase, the other strand is polymerized using one or two nucleotides that are fluorescently labeled internally, e.g., (Anand *et al.* 2007). Highly fluorescently labeled commercial oligonucleotides can be expensive, whereas Klenow polymerase can perform the labeling specifically at a much lower cost.

Nucleosomes

The nucleosome is the fundamental DNA–protein portion of a chromosome. Eukaryotic DNA is packaged in chromatin, which poses a problem to DNA transactions (transcription, replication, repair, recombination) that must access the underlying DNA template (van Holde 1988, Wolffe 1998, Zlatanova & Leuba 2004a). Some 150 bp of DNA are wrapped almost twice around an octamer of core histones H2A, H2B, H3 and H4 to form the nucleosome. A fifth histone, linker histone H1, binds to the DNA entering and exiting nucleosomes and to the ensuing linker DNA connecting adjacent nucleosomes.

The positions of 70% of the core histone amino acids have been mapped with exquisite detail in the crystal structures of the histone octamer (Arents *et al.* 1991) and the 146 bp or 147 bp nucleosome (Luger *et al.* 1997, Harp *et al.* 2000, Davey *et al.* 2002). The remaining ~30% of the core histone amino acids are in the histone tail termini where most of the posttranslational modifications such as acetylation, methylation and phosphorylation occur. Histone posttranslational modifications as well as modifications to the DNA structure itself (e.g. DNA methylation) are thought to assist with regulation of nucleosomal and chromatin fiber stability. Additionally, the exchange between the

canonical histones and their evolutionarily conserved variants are thought to assist with regulation. Finally, ATP-dependent nucleosome remodeling proteins and complexes contribute to regulation (for a review see Travers & Owen-Hughes 2004). It is not well understood mechanistically how these modifications may assist with dynamic regulation of access to the underlying DNA templates for processes such as transcription, replication, repair and recombination.

Regulation of access to the underlying DNA templates

Dynamic changes in nucleosomes regulate genetic responses in the cell. Current assays used to study spontaneous or factor-induced dynamic changes in chromatin structure rely on population-averaged measurements that may not be sensitive enough to detect fast reversible changes. Moreover, the disparate biological events that can occur in one molecule are lost in the average of the population of many molecules.

Nucleosomes stochastically open and close

In the case of the nucleosome, a dsDNA template is prepared with the two dyes too far away for FRET to occur as naked DNA (Figure 1A), but when the DNA is folded around the histone octamer to form a nucleosome, the two dyes come into close enough proximity for FRET (Figure 1B) (Tomschik *et al.* 2005). Note that the sites for the two dyes are on the opposite side of the nucleosome from the pseudodyad of the nucleosomal DNA positioning sequence. In our experiments, in order for FRET to decrease from the high-FRET canonical nucleosomal state, the DNA has to unwrap from the histone octamer core by at least 30–40 bp before FRET can even begin to decrease. We have estimated that the methyl carbons of the pyrimidines upon which the Cy3 and Cy5 dyes are mounted are 2.8 nm apart in the formed nucleosome.

In our application, we use an evanescent-field fluorescent microscope (e.g., Zheng *et al.* 2005), otherwise known as total internal reflection fluorescence microscope (Axelrod 1981), with a wide field illumination from a laser. The evanescent field generated by the laser excites donor fluorescent dyes on DNA molecules tethered to a surface. The fluorescence image of these excited molecules is split into two equal

images that are filtered separately with either a green (donor dye signal) or a red filter (acceptor dye signal) and the resulting images are collected side by side, left and right, simultaneously with a video camera that has the sensitivity to detect the intensity of single fluorescent dyes. This approach is analogous to what astronomers did over 40 years ago to measure red shifts in stars (Baum 1962). The actual data from imaging of single fluorescent dye molecules attached to a surface, which appear as scattered white spots of intensity against a black background, does in fact resemble looking at a constellation of stars at night. From the constellation of intensities of the two images, an anti-correlation of intensities of the same spot in the left and right images over time indicates the dyes are on the same DNA molecule.

In the case of imaging a population of individual naked nucleosomal positioning DNA molecules, the donor and acceptor dyes are too far apart for FRET to occur and the intensity is concentrated in the green channel (Figure 2A). The apparent efficiency of FRET (E_{app}) is the fraction of the total amount of absorbed energy that is transferred from the donor dye to the acceptor dye and can be roughly determined from the data as the ratio of the acceptor intensity divided by the sum of the intensities of the acceptor and donor dyes (Clegg 1992, 1995). Additionally, one has to take into account spectral crosstalk of the donor signal into the acceptor channel. In the case of naked DNA molecules, the average E_{app} after correction for spectral crosstalk is 0.13 (Figure 2A histogram). Once the DNA molecules are reconstituted into nucleosomes, the two dyes are in close proximity for energy transfer to occur; a strong register of intensities is observed in the right image (Figure 2B) and the histogram is shifted to 0.87 E_{app} units. Rinsing the reconstituted nucleosome slide with 2 M NaCl removes all of the proteins, and the naked DNA molecules left behind regain the low-FRET signal of 0.11 E_{app} units (Figure 2C).

There are several methods for reconstituting nucleosomes, including (i) salt dialysis (Tatchell & Van Holde 1977) and salt jump (Zivanovic *et al.* 1990) and (ii) reconstitution using histone chaperones such as nucleosome assembly protein Nap1 (Ishimi *et al.* 1983). Nucleosomes first reconstituted by salt dialysis or salt jump beginning from a high salt and dialyzing or diluting to a low salt are then attached to the quartz surface by biotin–streptavidin linkages. On the other hand, chaperone-assembled

Figure 1. Fluorophore positions on naked DNA and nucleosomes (Tomschik *et al.* 2005). (A) Model of a 164 bp dsDNA molecule with covalently bound cyanine dyes (Mujumdar *et al.* 1993). Donor Cy3 dye is at position 122 and acceptor Cy5 dye is at position 47. The dyad of the nucleosome positioning sequence is at position 84. (B) Model face and side views of a nucleosome (1kx5) (Davey *et al.* 2002) with the attached cyanine dye pair. Attachment of dyes was performed using Xfit (McRee 1993) and models displayed in PyMOL (DeLano 2002).

nucleosomes can be assembled *in situ* on the quartz glass. We have used both methods.

The images in Figure 2 are snapshots from a video. Hence, we can follow the intensity of the two dyes attached to one DNA molecule over time to view a time trajectory (Figure 3). Here, the red trace is the intensity of the acceptor dye and the green trace is the intensity of the donor dye. The anti-correlated intensities ensure that we are following the stochastic behavior of a single nucleosome. The two dyes reversibly revert between high and low FRET states before the acceptor dye photobleaches. The photobleached state is significantly lower than the low state, indicating that the low state does not represent photoblinking events of the acceptor dye molecule. Schematics of the two states, a closed canonical state and open states either with symmetrical or asymmetrical DNA opening, are indicated either below or above the time trajectory, respectively, correlating with the observed FRET signals.

Based on the specific placement of the two dyes (deep within the nucleosomal particle), we observed long-range conformational fluctuations, with almost half of the nucleosomal DNA being unwrapped within 100 ms (Tomschik *et al.* 2005). This interpretation was supported by fiber stretching experiments performed with optical tweezers (Brower-Toland *et al.* 2002), and by theory (Marky & Manning 1991, 1995). These results implied that most of the DNA on the nucleosome can be sporadically accessible to regulatory proteins and proteins that track the DNA double helix. We suggest that histone modifications or variant replacement and/or ATP-dependent nucleosome remodeling activities may change either the frequency or duration of the open periods (Tomschik *et al.* 2005).

Other groups have recently studied nucleosomes using FRET (Tóth *et al.* 2001, 2006, Bao *et al.* 2004, 2006, Li & Widom 2004, Park *et al.* 2004, White & Luger 2004, Li *et al.* 2005, Lovullo *et al.* 2005,

Figure 2. Evanescent-field fluorescence microscopy and FRET of naked DNA and reconstituted nucleosomes on fluorescently labeled DNA (Tomschik *et al.* 2005). (A) In naked DNA molecules, the two dyes are ~25 nm apart and only the Cy3 filtered image exhibits much fluorescence intensity. The accompanying histogram centers at 0.13 apparent efficiency of FRET (E_{app}) units. (B) In the reconstituted octasomes, the two dyes are within ~3 nm and the Cy5 filtered image exhibits strong intensities with an accompanying histogram centered at 0.87 E_{app} units. (C) After a 2 M NaCl wash of the reconstituted nucleosomes, the remaining naked DNA exhibits high fluorescence intensities predominantly in the Cy3 filtered image with an accompanying histogram centered at 0.11 E_{app} units. The Cy3 and Cy5 images are shown in inverted contrast to better display the individual intensities (dark spots) against a white background.

Bussiek *et al.* 2006, Yang *et al.* 2006, Gansen *et al.* 2007, Hoch *et al.* 2007, Kelbauskas *et al.* 2007, 2008, Koopmans *et al.* 2007), although most experiments have been at the population level. In bulk FRET, an overall FRET signal is observed from a population of molecules. Addition of a factor or a change in conditions such as a change in overall ionic strength is measured by an irreversible shift in the total FRET signal. A limitation of experiments at the population level is that they do not allow observations of spontaneous, asynchronous, reversible events occurring in individual molecules.

Pioneers of the use of FRET to study nucleosomes (Tóth *et al.* 2001, 2006, Bussiek *et al.* 2006), Tóth and Langowski and co-workers have recently used spFRET with solution fluorescence correlation spectroscopy (FCS) to distinguish between two different histone octamer positions on the same DNA molecule (Gansen *et al.* 2007). Positioning the donor dye near the dyad and, one wrap away, placing the acceptor dye on the DNA leaving the nucleosome, van Noort and colleagues observed 3% of their surface-tethered nucleosomes stochastically breathing (Koopmans *et al.* 2007).

Figure 3. Example time trajectories of individual fluorophores from the image in Figure 2B (Tomschik *et al.* 2005). Green trajectory represents the intensities of donor Cy3 dye and the red trajectory represents the intensities of the cognate acceptor Cy5 dye (100 ms time points). The anti-correlation of the two trajectories indicates they come from the same DNA molecule. The intensities revert back and forth between two states, a high and low FRET state, before the Cy5 dye photobleaches. The intensity of Cy5 photobleaching is lower than the low state intensity, indicating that the observed low states are not from Cy5 photobleaching events. The length of time that the nucleosome resides or dwells in a low state or high state is indicated. Schematics of how the nucleosome might appear in the high state or the low state are shown above and below the time trajectories, respectively.

The PcrA helicase of Gram-positive bacteria

Destabilization of dsDNA is an essential step in DNA transactions such as replication, repair and recombination. DNA helicases are enzymes that catalyze the physical separation of the two strands of DNA required for processes where single-stranded DNA (ssDNA) is an intermediate (Lohman 1993). PcrA is a conserved helicase present in all Gram-positive bacteria sequenced so far. It belongs to the SF1 superfamily of helicases (Hall & Matson 1999), which also includes the UvrD and Rep helicases of *E. coli*. It was first identified by Iordanescu in a

genetic screen for chromosomally encoded genes that affected the rolling-circle replication of plasmid pT181 (Iordanescu 1993). Homologues of PcrA from various important pathogenic bacteria such as *Staphylococcus aureus*, *Bacillus anthracis* and *Streptococcus pneumoniae* have since been overexpressed as recombinant proteins in *E. coli* and biochemically characterized (Chang *et al.* 2002, Naqvi *et al.* 2003, Anand *et al.* 2004, Ruiz-Maso *et al.* 2006). The PcrA proteins from various bacteria share approximately 60% identity. Gene knockout studies in *B. subtilis* and *S. pneumoniae* have shown that the helicase is essential (Petit *et al.* 1998, Ruiz-Maso *et al.* 2006).

Anti-sense inhibition studies have shown that *pcrA* knockout in *S. aureus* is not viable (Ji *et al.* 2001). The helicase fulfills functions in UV-repair as well as plasmid rolling-circle replication (Petit *et al.* 1998, Chang *et al.* 2002). The crystal structures of PcrA (Subramanya *et al.* 1996, Velankar *et al.* 1999), UvrD (Lee & Yang 2006) and Rep (Korolev *et al.* 1997) are available. The crystal structure of the PcrA homologue of *Bacillus stearothermophilus* (PcrA_{Bst}) shows that the protein has four structural domains, termed 1A, 1B, 2A and 2B (Figure 4A) (Subramanya *et al.* 1996, Velankar *et al.* 1999). The conserved motifs involved in ATPase and ssDNA translocation are present in domains 1A and 2A (Velankar *et al.* 1999). PcrA_{Bst} prefers duplex DNA substrates containing a 3' ss tail (Bird *et al.* 1998, Dillingham *et al.* 2000, 2002), whereas mesophilic PcrA homologues that have been studied so far prefer a 5' ss tail folded into stem-loop structures (Naqvi *et al.* 2003, Anand & Khan 2004, Ruiz-Maso *et al.* 2006). Replication of chromosomal DNA is only slightly affected in

conditional knockouts of *pcrA* in *B. subtilis*, suggesting that it is not the major replicative helicase (Petit *et al.* 1998). Suppressor mutants for PcrA knockouts in *B. subtilis* map in *recF*, *recO* and *recR* genes whose products are involved in promoting *recA*-dependent homologous recombination (Shan *et al.* 1997, Bork *et al.* 2001b, Petit & Ehrlich 2002, Morimatsu & Kowalczykowski 2003). In the absence of PcrA, unregulated formation of recombination intermediates may be toxic to the cell (Petit & Ehrlich 2002). This suggested that an interaction between PcrA and these proteins is essential for supporting cell viability.

The Rep helicase of *E. coli*

The Rep protein of *E. coli* was the first helicase to be characterized (Denhardt *et al.* 1967). This helicase is required for the replication of single-stranded DNA bacteriophages M13 and ϕ X174 in *E. coli* (Denhardt

Figure 4. Structure (3PJR) (Velankar *et al.* 1999) of DNA helicase PcrA_{Bst} (A) and zoomed views of sites of PcrA mutants T65 (B) and Q254 K37 (C). We made the corresponding changes (T61I for PcrA3, Q250R K33A for PcrAH') in PcrA_{Sau}. Models displayed in PyMOL (DeLano 2002). Experiments performed on either of the mutants do not detect ATPase hydrolysis and helicase activity, indicating that the mutations interfere with these functions (Anand *et al.* 2007).

et al. 1972, Eisenberg *et al.* 1977, Scott *et al.* 1977). The precise role of Rep helicase in host DNA metabolism remains to be fully elucidated. However, replication fork progression is slowed in *rep* mutants, suggesting a role for Rep helicase in DNA replication (Lane & Denhardt 1975) in spite of not being the major replicative helicase. The Rep helicase shares 40% identity with the *E. coli* UvrD helicase. *E. coli rep* mutants are viable (Colasanti & Denhardt 1987), whereas *E. coli rep uvrD* double mutants are not (Taucher-Scholtz *et al.* 1983). Moreover, *E. coli rep uvrD* double mutants can be complemented by *pcrA*, suggesting a common role for these helicases in DNA transactions (Petit *et al.* 1998). Despite some similarities, there are also differences among these three helicases. For example, the UV repair defect of *E. coli uvrD* mutant is complemented by *B. subtilis pcrA* but not by *E. coli rep* (Petit *et al.* 1998). Similarly, UvrD efficiently dismantles RecA-DNA filaments, whereas Rep has only a marginal activity (Veaute *et al.* 2005). Moreover, UvrD but not Rep prevents homologous recombination *in vivo* (Veaute *et al.* 2005). As observed in an spFRET-based assay, Rep undergoes repetitive shuttling on ssDNA close to blockades such as ssDNA/dsDNA junctions (Myong *et al.* 2005). Curiously, the biological relevance of this activity remains unknown. Preliminary evidence suggests that Rep could reduce DNA binding efficiencies by proteins such as RecA by this activity as described later (Myong *et al.* 2005).

The bacterial recombination protein RecA

RecA is a conserved protein present in bacteria and its homologues are ubiquitous (Roca & Cox 1997, Cox 2003). Clark and Margulies discovered RecA in a genetic screen for mutants defective in recombination and UV repair (Clark & Margulies 1965). RecA is essential for DNA recombination and mediates the SOS response upon DNA damage (Kowalczykowski 1991, Cox 2003, 2007, McGrew & Knight 2003). A nucleoprotein complex formed by RecA catalyzes DNA strand exchange (Kowalczykowski 1991, Cox 2003, McGrew & Knight 2003). Assembly of *E. coli* RecA protein (RecA_{Eco}) on DNA is favored by ATP, dATP and ATP γ S (Kowalczykowski 1991, Cox 2003, McGrew & Knight 2003). RecA_{Eco} polymerizes in the 5' to 3' direction on DNA and depolymerizes predominantly in the same direction

(Register & Griffith 1985, Bork *et al.* 2001a). The crystal structures of RecA from *E. coli* and various bacterial homologues are known (Story *et al.* 1992, Datta *et al.* 2000, Rajan & Bell 2004, Krishna *et al.* 2006), and they highlight the importance of inter-subunit contacts in the RecA filament. Unregulated recombination is harmful and, therefore, various mechanisms exist to regulate recombination inside the cells (Watt *et al.* 1995, Chanet *et al.* 1996, Aguilera 2001, Myung *et al.* 2001, Petit & Ehrlich 2002, Traverso *et al.* 2003, Veaute *et al.* 2005, Kang & Blaser 2006). RecA forms characteristic striated filaments in negatively stained electron micrographs (Stasiak *et al.* 1981). Thus, the displacement of RecA from ssDNA by UvrD helicase has been demonstrated by electron microscopy (Veaute *et al.* 2005). However, the advent of sensitive single-molecule approaches has allowed measurements of RecA dynamics in real time (Leger *et al.* 1998, Bennink *et al.* 1999, Hegner *et al.* 1999, Shivashankar *et al.* 1999, Fulconis *et al.* 2004, 2006, Galletto *et al.* 2006, Joo *et al.* 2006, Li *et al.* 2006) without the need to fix the samples. Here we will describe two spFRET-based methods for studying RecA dynamics on ssDNA and dsDNA. Single-molecule imaging allowed testing of these hypotheses.

An spFRET-based assay to monitor RecA binding and its displacement from ssDNA

Rep helicase harbors high affinity for 3' tailed duplex substrates. Therefore, the Ha group designed a duplex DNA substrate (20 bases) that had a 3' overhang (dT₄₀). They placed a donor dye (Cy3) at the 3' end of the overhang and an acceptor dye (Cy5) at the ss/ds junction of the substrate (Figure 5A). Because ssDNA is much more flexible than dsDNA, the free substrate exhibits high FRET as the donor and acceptor dyes are often in close proximity (Figure 5B). RecA-DNA filaments are rigid. Therefore, formation of RecA-DNA complex at the ss portion of the substrate would make the DNA rigid and straight (Figure 5C) and keep the dyes apart. As a result, in the presence of the RecA protein, nearly all of the fluorescently labeled tailed duplex substrate would exhibit a low FRET (Figure 5D, compare the upper panel versus the lower panel) (Myong *et al.* 2005). Thus, the binding of RecA to ssDNA can be followed using a simple substrate dual-labeled

Figure 5. Rep helicase inhibits assembly of RecA on ssDNA. (A) Schematic of experimental system. A dsDNA molecule with a 3' dT₄₀ tail is secured via a 3' biotinylated end/streptavidin to a polyethylene glycol surface, which promotes reduction of nonspecific protein binding. The shorter oligonucleotide is labeled with an acceptor Cy5 dye and the 3' end of the dT₄₀ tail has a donor Cy3 dye. (B and C) Upon binding of RecA, the protein forms a filament on the ssDNA molecule with concomitant increasing distance between the two dyes leading to low FRET. Added Rep DNA helicase can displace the RecA filament and maintain the higher FRET signal. (C) Time dependent experiment upon the addition of RecA. At zero time, the upper histogram centers at 0.3 FRET units, whereas by 30–35 seconds the lower histogram centers at a lower FRET peak. (D) Time dependent experiment upon the addition of RecA and Rep. At zero time, the upper histogram centers at 0.3 FRET units whereas by 30–35 seconds, the lower histogram has a lower FRET peak (as in C, lower histogram) and, additionally, a second peak with even higher FRET. The occurrence of the second peak suggests that Rep helicase inhibits RecA filament formation on the DNA molecule. (A–C) redrawn from Figure 3 of Myong *et al.* 2005; (D, E) drawn from data from Figure 3 of Myong *et al.* 2005 with permission.

with fluorescent dyes. When RecA assembly was carried out in the presence of the Rep helicase, the proportion of DNA molecules that showed a conversion from high FRET to low FRET was low (Figure 5E). Since Rep helicase harbored the ability to

undergo repetitive shuttling on ssDNA, these results suggested that the Rep helicase was probably preventing the assembly of RecA on the DNA by continuously translocating back and forth on the ssDNA substrate (Myong *et al.* 2005). Thus, dual-labeled

DNA substrates can be successfully used to demonstrate assembly and removal of RecA from ssDNA by following FRET signals.

PcrA inhibits RecA mediated DNA strand exchange

Biochemical studies showed that PcrA inhibits RecA-mediated DNA strand exchange and displaces RecA from both ssDNA and dsDNA (Anand *et al.* 2007). Additionally, these studies showed that the helicase activity of PcrA is not required for these functions (Anand *et al.* 2007). These studies used two well-defined site-directed mutants of PcrA. In one mutant, PcrAH⁻, the alteration of two amino acids (Q250R, K33A) in the Walker Box I and II motifs adversely affected its ATPase and helicase activities (Anand *et al.* 2004, 2007) (Figure 4B). The second mutant, *pcrA3* (PcrA T61I), was able to support cell survival but was unable to support the rolling circle (RC) replication of the pT181 plasmid in *S. aureus* (Iordanescu 1993) (Figure 4C). Interestingly, the recombinant PcrA3_{Sau} protein was defective in its ATPase and helicase activities, and predicted to be impaired in its DNA translocation activity, which requires ATP hydrolysis (Anand *et al.* 2005). Thus, we hypothesized that the helicase/translocation activity of PcrA may be dispensable for the survival of *S. aureus*. These results were contrary to what is known for some other helicases belonging to superfamily 1 (SF1) such as the *Escherichia coli* UvrD and Rep helicases and the yeast Srs2 helicase, which have been shown to displace RecA or its eukaryotic homologue Rad51 from the DNA (Krejci *et al.* 2003, Veaute *et al.* 2003, 2005, Myong *et al.* 2005, Anand *et al.* 2007). There were two plausible explanations for helicase-independent displacement of RecA by PcrA. One obvious explanation was direct competition for DNA binding sites between PcrA and RecA. However, in all biochemical assays PcrA was added after formation of the RecA filaments, thus ruling out this possibility. The second explanation was direct protein-protein interactions between PcrA and RecA somehow leading to the destabilization of RecA from the DNA. However, the nonstoichiometric nature of RecA inhibition (around one molecule of PcrA for ten molecules of RecA) argued for a catalytic mechanism.

A spFRET based assay to monitor RecA assembly and displacement from dsDNA

PcrA is an ssDNA translocase (Velankar *et al.* 1999, Dillingham *et al.* 2000), and it cannot access ds regions of a substrate without unwinding DNA. RecA can extend its polymerization into neighboring dsDNA in the 5' to 3' direction (Lindsley & Cox 1990). Thus, we needed an approach to test destabilization of RecA from ds regions of the DNA not directly accessible by the helicase mutants of PcrA, which do not unwind dsDNA. We developed a novel spFRET based assay to monitor assembly of RecA on dsDNA and its subsequent displacement by PcrA. We utilized the principle of binding of RecA to dsDNA that is known to increase the length of dsDNA 1.5-fold (Stasiak *et al.* 1981). The high FRET signal of an appropriately placed dye pair in B-DNA would decrease upon lengthening of the DNA brought about by the binding of RecA. Likewise, displacement of RecA from the DNA would return the DNA molecule to its original naked B-form and regain the higher FRET signal. We used a synthetic tailed duplex substrate (134 bp with a (dT)₂₀ ss tail) containing four equally placed Cy3/Cy5 FRET pairs (Anand *et al.* 2007). The 20 nt ssDNA tail served as the major site for nucleation of RecA, after which it could extend into the neighboring ds region as the polymerization of RecA proceeds in a 5' to 3' direction. A single biotin at the 5' end of the tailed duplex allowed the substrate to be immobilized on the quartz surface. The assay was performed in quartz flow-cells coated with streptavidin as described (Zheng *et al.* 2005, Anand *et al.* 2007). The individual FRET pairs were 33 bp (~11 nm) apart to prevent FRET among the pairs. Additionally, the order of the dyes forming the individual FRET pairs was reversed to increase the effective physical separation of the donor dyes from any acceptor dye other than the intended one. The molecules were visualized with an evanescent-field fluorescent microscope (Zheng *et al.* 2005), and analysis of the data was carried out using the Origin software (Zheng *et al.* 2005, Anand *et al.* 2007).

Naked DNA molecules exhibited high FRET (Figure 6). A video snapshot showing several individual molecules of DNA from the actual experiment is shown in Figure 6. RecA binding to the substrate decreased the mean apparent efficiency of FRET (E_{app}) from 0.8 to 0.35 owing to lengthening of the

Figure 6. PcrA DNA helicase reverses RecA induced dsDNA lengthening. The chematics between the images and the histograms indicate the order of the reactions. (A) Donor Cy3/acceptor Cy5 filtered wide-field evanescent-field fluorescence microscope images of naked DNA molecules. The two dyes are 13 bp (13 bp $\times$ 0.34 nm/bp $\nless 4.42$ nm) apart and exhibit high FRET with mean E_{app} of ~ 0.8 units in the accompanying histogram. (B) Related images of the DNA molecules in (A) mixed with RecA which form filaments on the doubled-stranded molecule and lengthen it 1.5-fold. Now the dyes are about 6.63 nm apart with a corresponding drop in FRET in the accompanying histogram with a mean of ~ 0.3 E_{app} units. (C) Related images of the DNA/RecA complexes in (B) mixed with PcrA DNA helicase. The accompanying histogram has a mean of 0.8 E_{app} units, indicating that the helicase has displaced the RecA proteins and the DNA molecules have regained their original B-form structure of 0.34 nm/bp. Intriguingly, using the formula $E_{FRET} = 1/(1 + (R/R_0)^6)$, we can calculate an E_{FRET} value of 0.86 for the 4.42 nm (13 bp) high FRET distance and E_{FRET} of 0.35 for the 6.63 nm low FRET distance, and these calculated values are in general agreement with the measured mean E_{FRET} values of ~ 0.8 and ~ 0.3 despite the fact that the dyes are attached to the bases on carbon linkers. Histograms and order of addition schematic adapted from (Anand *et al.* 2007).

DNA and the concomitant increase in distance separating the individual FRET pairs (from ~ 4.4 nm to ~ 6.6 nm). In the image, one can readily visualize this by the dramatic reduction in fluorescence arising from the Cy5 channel. This reduction in FRET was stable for the period of the experiment (at least up to

30 min). Upon addition of PcrA, there was a reversal in the mean E_{app} to ~ 0.8 (Figure 6), indicating removal of RecA by PcrA. Removal of RecA and concomitant reversal of FRET values can also be readily visualized on the screen as Cy5 fluorescence intensities increase again. We used a similar assay to

compare the efficiency of RecA displacement by wild-type PcrA_{Sau} and its helicase mutants. We observed no noticeable differences in the RecA displacement activity of PcrA_{Sau}, PcrA3 and PcrAH⁻. Thus, the spFRET-based assay helped us to demonstrate that the helicase activity of PcrA is not required for displacing RecA from dsDNA. Further, the assay conveniently demonstrated displacement of RecA from the ds portion of the substrate, which is not physically accessible to the helicase mutants of PcrA.

Mechanism of RecA displacement from DNA

The results of RecA displacement by PcrA, UvrD and Rep suggest that bacterial helicases can utilize both translocation-dependent and translocation-independent mechanisms to regulate the function of the bacterial recombination protein RecA (Krejci *et al.* 2003, Veaute *et al.* 2003, 2005, Myong *et al.* 2005, Anand *et al.* 2007). Since the ATPase mutants of PcrA, which lack helicase and translocation activities, displace RecA from the DNA, we hypothesize

Figure 7. (A) Hydrolysis of ATP occurs in waves in the RecA-DNA filament. In this image, the first, seventh and thirteenth RecA monomers have ADP in red, and the remainder have ATP in blue. The RecA subunits are colored alternately with yellow and salmon to distinguish them. ATP hydrolysis within a RecA filament is coordinated such that the first, seventh and thirteenth RecA subunits hydrolyze ATP at the same time. (B) The upper schematic shows the subunit organization of the RecA filament depicted in 7 Å. The red and blue spheres indicate RecA-ADP and RecA-ATP, respectively. The lower schematic indicates the subunit organization of the RecA filament viewed along its vertical axis as shown in Figure 7C. There are 6 RecA subunits per turn of the RecA-DNA helical filament. Only one-in-six RecA subunits in a filament hydrolyze ATP at the same time. These subunits align along the same face of the RecA filament. Figure inspired by and adapted from Cox *et al.* (2005). (C) The structure of a RecA filament viewed along its vertical axis. ADP is depicted with red spheres, ATP with blue spheres, and the RecA monomers are ribbon figures alternating in color between yellow and salmon. Models in (A) and (C) displayed in PyMOL (DeLano 2002).

that PcrA may utilize the inherent cooperativity of RecA monomers within its filament to cause its disassembly from the DNA. The speculated mechanism would increase the efficiency of RecA displacement by PcrA, which is a non-processive monomeric helicase. Several biochemical studies have shown that the ATPase activity of RecA is highly cooperative (Cox *et al.* 2005). The residues responsible for propagating intersubunit cooperativity have been mapped (Kelley De Zutter *et al.* 2001, McGrew & Knight 2003). The Cox group has shown that RecA hydrolyzes ATP in waves with the subunits registered on the same surface of the filament hydrolyzing ATP at the same time (Figure 7). These results are captured in the elegant model first described by Cox (Cox *et al.* 2005) and recapitulated here for discussion.

RecA/DNA complexes assembled in the presence of ATP γ S and dATP are highly stable and also resistant to displacement by helicase mutants of PcrA (Anand *et al.* 2007), lending credence to the possibility that PcrA could utilize the ATPase activity of RecA to trigger its own collapse from the DNA. Thus, in one model of RecA displacement, PcrA could trigger an allosteric switch on RecA, which would be propagated to neighboring subunits of RecA through the cooperativity that exists in RecA filaments. Contact of one molecule of PcrA with one RecA molecule could trigger the collapse of an entire RecA filament. This model can easily be tested by a spFRET-based assay described earlier by studying the kinetics of reappearance of FRET from RecA-DNA complexes in the presence of PcrA and its helicase mutants. Polymerization of the RecA filament along the DNA molecule or the displacement of the RecA monomers by a processive DNA helicase can be observed as the change in four FRET pairs in real time; the distance between two independent changes in FRET occurring at the same microscopic location could indicate the duration of time for one process as it proceeds from one FRET pair to the next FRET pair on the same DNA molecule. These experiments are currently in progress in our laboratory.

spFRET-based studies on helicases will increase the sensitivity of methods used to study helicases and dramatically increase our understanding of the behavior of helicases. These experiments should also lead to the discovery of new behaviors, which could

otherwise go unnoticed using conventional methods such as traditional biochemistry and electron microscopy. However, understanding of the biological relevance of the observations made using powerful biophysical methods requires testing of the models generated from such studies *in vivo* using genetic approaches. For example, current biochemical evidence suggests that the helicase activity of PcrA is not essential for the regulation of recombination in *S. aureus* (Anand *et al.* 2007). However, more genetic studies are required to prove unequivocally that this is indeed the case *in vivo*. Likewise, genetic studies have shown that the ability of the *E. coli* Rep helicase to regulate RecA function is not essential for *E. coli* (Colasanti & Denhardt 1987). Thus, the biological function of prevention of RecA assembly on DNA by Rep helicase requires further investigation.

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