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Visualization of Rad54, a Chromatin Remodeling Protein, Translocating on Single DNA Molecules

Short Article

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Summary

Rad54 protein plays an important role in the recombinational repair of double-strand DNA (dsDNA) breaks. It is a dsDNA-dependent ATPase that belongs to the Swi2/Snf2 family of chromatin-remodeling proteins. Rad54 remodels (1) DNA structure, (2) chromatin structure, and (3) Rad51-dsDNA complexes. These abilities imply that Rad54 moves along DNA. Here, we provide direct evidence of Rad54 translocation by visualizing its movement along single molecules of dsDNA. When compared to the remodeling processes, translocation is unexpectedly rapid, occurring at 301 ± 22 bp/s at 25°C. Rad54 binds randomly along the dsDNA and moves in either of the two possible directions with a velocity dependent on ATP concentration ($K_m = 97 \pm 28$ μM). Movement is also surprisingly processive: the average distance traveled is ~11,500 bp, with molecules traversing up to 32,000 bp before stopping. The mechanistic implications of this vigorous Rad54 translocase activity in chromatin and protein-DNA complex remodeling are discussed.

Introduction

Rad54 is a dsDNA-dependent ATPase that plays an important role in homologous recombination and the repair of dsDNA breaks in eukaryotes (Petukhova et al., 1998; Swagemakers et al., 1998; Symington, 2002). It binds to and stabilizes Rad51-ssDNA filaments to facilitate homologous pairing of DNA at an early step in the repair process (Mazin et al., 2000, 2003; Petukhova et al., 1998). Rad54 stimulates DNA pairing (Mazin et al., 2000; Petukhova et al., 1998), DNA heteroduplex extension (Solinger and Heyer, 2001; Solinger et al., 2001), and the dissociation of Rad51 from the heteroduplex DNA (Solinger et al., 2002)—all of which require ATP hydrolysis by Rad54. Rad54 belongs to the Swi2/Snf2 family of chromatin-remodeling proteins (Eisen et al., 1995). Although Rad54 is a dsDNA-dependent ATPase, it does not display any helicase activity (Petukhova et al., 1998; Swagemakers et al., 1998). Rather, Rad54 produces topological changes in DNA structure, introducing equivalent positive and negative supercoiled domains into closed circular dsDNA (Petukhova et al., 1999; Tan et al., 1999). This change in DNA topology by Rad54 also requires ATP hydrolysis and is greater than can be explained by DNA wrapping, suggesting that Rad54 entraps a domain and translocates along the DNA (Ristic et al., 2001). In vitro, Rad54 also stimulates DNA pairing when the target duplex is chromatin (Alexeev et al., 2003; Alexiadis and Kadonaga, 2002; Jaskelioff et al., 2003; Wolner and Peterson, 2005). Furthermore, Rad54 promotes ATP-dependent remodeling of nucleosomes by sliding the nucleosome along dsDNA (Alexeev et al., 2003). These activities of Rad54 suggest that Rad54 translocates along dsDNA (Alexeev et al., 2003; Petukhova et al., 1999; Ristic et al., 2001; Tan et al., 1999), but translocation behavior has not been quantified. Therefore, we sought a direct and quantitative assay of translocation. Here, we report the imaging of individual molecules of Rad54 on single molecules of dsDNA and show that translocation by Rad54 is both rapid and processive.

Results and Discussion

The *Saccharomyces cerevisiae* Rad54 used in these experiments was purified as a glutathione S-transferase (GST) fusion protein, previously shown to be fully functional in vivo (Solinger et al., 2001) and in vitro (Mazin et al., 2000). The Rad54 protein was visualized by binding a fluorescein (FITC)-labeled anti-GST antibody to the GST moiety of the Rad54-GST fusion protein to produce an FITC-antibody-GST-Rad54 complex (FITC-Rad54). To visualize translocation, the instrument described previously (Bianco et al., 2001; Handa et al., 2005; Spies et al., 2003) was used: briefly, it consists of an epifluorescence microscope, an optical trap, and a multichannel laminar flow cell. The FITC-Rad54, in the absence of ATP, was bound to λ DNA (48,502 bp) that had been labeled at one end with biotin and attached to a 1 μm streptavidin-coated polystyrene bead (Figure 1A). A single FITC-Rad54-DNA-bead complex was captured by the laser trap in the first channel of the flow cell and then moved to the second channel of the flow cell, which contained the ATP, to initiate the enzymatic reaction. This strategy permitted visualization of individual FITC-Rad54 entities because the protein-DNA complex is moved to a channel lacking protein and, hence, devoid of background fluorescence; in addition, this procedure ensured that no subsequent binding (or rebinding) of protein occurred during the course of the reaction.

Figure 1A shows sequential frames, as vertical strips, from a video recording of two such reactions; flow, in each case, is from left to right. On the left side of each strip, a bright spot is evident: this is the 1 μm bead to which the FITC-labeled antibody, or the FITC-Rad54, binds nonspecifically. To the right of this fixed fluorescent spot, another fainter spot is evident. This spot, which is downstream of the optically trapped bead, is an FITC-Rad54 complex bound to the tethered DNA, which is unlabeled and, hence, invisible. With time, the FITC-Rad54 molecule in each strip is seen to move: in the left strip, the FITC-Rad54 is visibly moving toward the bead (upstream; see Movie S1 available in the Supplemental Data with this article online), whereas in the right strip, the FITC-Rad54 is moving away from the bead (downstream; Movie S2). These observations,

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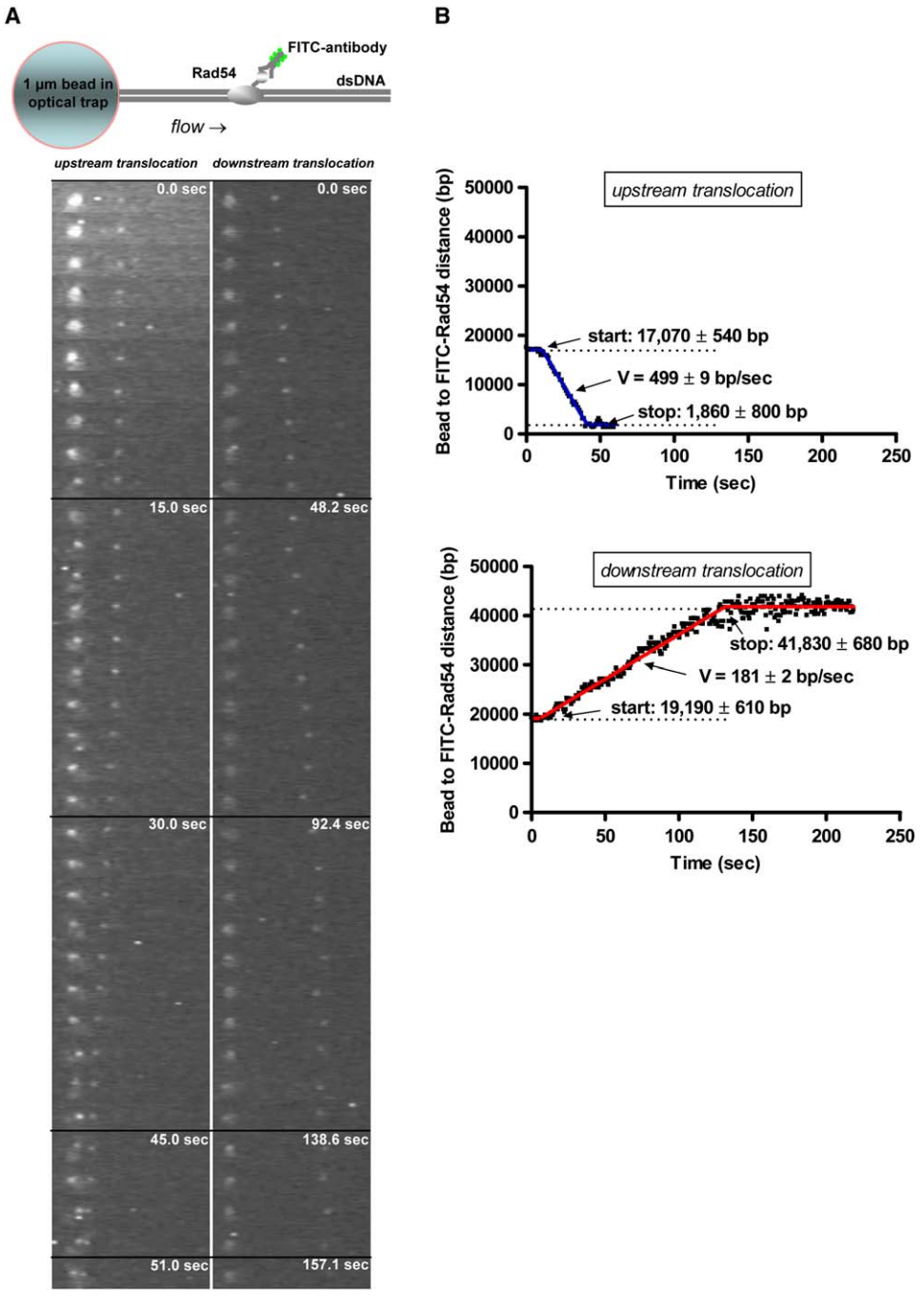

Figure 1. Direct Imaging of Rad54 Reveals Translocation along dsDNA

(A) An illustration of the optically trapped DNA-bead complex and a complex of the Rad54-GST fusion protein and FITC-anti-GST antibody. Below the illustration, sequential images of the labeled Rad54 translocating along dsDNA are shown. The vertical strip on the left side shows movement toward the bead (upstream), whereas the strip on the right side shows movement away from the bead (downstream). Each repeating rectangular frame in each strip is 3.5 × 17.5 μm, and the time for every tenth frame and the last is indicated. (See Movies S1 and S2 for the videos of upstream and downstream translocation, respectively.)

(B) For the two molecules in (A), the position of Rad54 was plotted relative to the 1 μm bead. The λ DNA is 48,502 base pairs in length, and the end attached to the bead is denoted as zero base pairs. The translocation profiles were fit to a three segment line; the start position, stop position, and velocity, as well as the respective standard deviations, are indicated.

particularly the upstream movement, show that Rad54 is clearly a DNA translocase.

Measurement of FITC-Rad54 position, relative to the fixed position of the optical trap, permits quantification of the movement (Figure 1B). The Rad54 in the left strip bound the λ DNA at about 1/3 of its length from the bead (17,070 ± 540 bp); after a delay of 11 s, it moved monotonically at 499 ± 9 bp/s and traveled just over 15 Kbp (15,210 ± 1340 bp) in 30 s before stopping at or near the microsphere (1860 ± 800 bp). The Rad54 in

the right strip started from approximately the middle (19,190 ± 610 bp) of the λ DNA molecule after a brief 6 s delay but moved in the opposite direction at 181 ± 2 bp/s and traveled almost 23 Kbp (22,640 ± 1490 bp) in about 2 min before stopping near the DNA end (41,830 ± 680 bp). Thus, Rad54 is capable of rapid and processive movement.

To determine whether Rad54 has any preferred translocation start sites or a directional bias, the starting position and direction of travel were plotted (Figure 2A). This analysis includes all molecules that translocated at all of the ATP concentrations examined (see below); the initial binding sites of the Rad54 molecules that traveled upstream are indicated with a blue vertical tick above the horizontal line that represents the λ DNA, whereas those that translocated downstream are indicated below with a red tick. The graph shows that the Rad54 initiation sites are widely and randomly distributed on the DNA and that about half of the molecules traveled upstream whereas the other half traveled downstream; due to difficulties associated with the detection of movement at either end of the DNA, initiation at the ends is likely to be underrepresented in our sample set. Regardless, these results show that the initial binding of Rad54 to dsDNA is largely random with regard to both position and translocation direction.

Unidirectional translocation must be ATP dependent so, to establish the validity of our observations, Rad54 translocation was measured as a function of ATP concentration (Figure 2B). To ensure that the translocation velocities were not being biased by solution flow, the molecules were first segregated into two groups: those that moved upstream (blue triangles) and those that moved downstream (red, inverted triangles). In both groups, the Rad54 molecules displayed a hyperbolic increase in translocation rate with increasing ATP concentration. The Michaelis constants for each group were the same (see figure legend), within experimental error, demonstrating that translocation velocities were independent of flow. At any given ATP concentration, the translocation velocities could vary by as much as 10-fold between the fastest and slowest molecules; a comparable variation was also observed for RecBCD helicase (Bianco et al., 2001; Handa et al., 2005; Spies et al., 2003). Fitting the mean translocation rate (black circles) for all of the Rad54 molecules at each ATP concentration yielded the following Michaelis-Menten parameters: V_{max} = 301 ± 22 bp/s and K_m = 97 ± 28 μM. The dependence of Rad54 translocation on ATP concentration confirms that the observed movement of Rad54 is driven by free energy changes associated with ATP hydrolysis.

Our experiments also permit determination of the processivity of translocation, i.e., the distance translocated prior to dissociation. Measurement of this distance, from data such as those shown in Figure 1, is a direct measure of the processivity. The average distance traveled, N, is plotted for each ATP concentration in Figure 3A; remarkably, the average Rad54 molecule could translocate more than 11 Kbp before stopping. After stopping, most of the Rad54 molecules remained bound to the DNA for at least 10–20 s, and sometimes longer; however, we did not systematically continue the observation because we could not discriminate

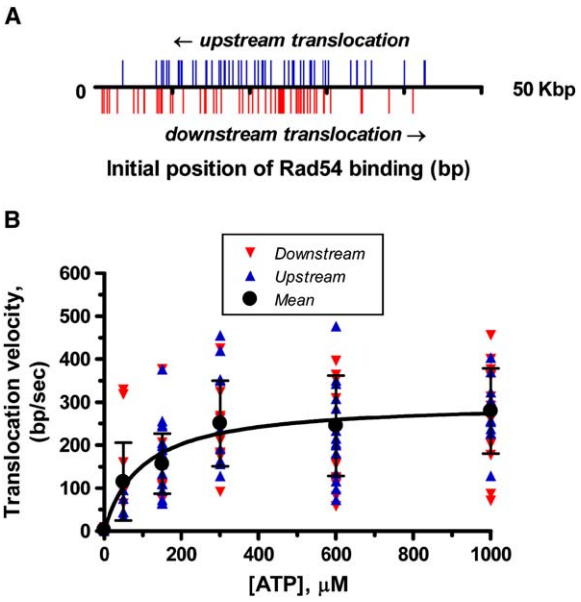

Figure 2. Rad54 Binds to dsDNA Randomly and Translocates at a Rate that Is Dependent on ATP Concentration

(A) The initial position of Rad54 binding on the λ DNA and the direction of travel are shown. Molecules that moved upstream are represented by the blue ticks above the horizontal line that represents the λ DNA, whereas the molecules that moved downstream are represented by the red ticks that are below the line.

(B) The rate of translocation was determined at various ATP concentrations as described in Figure 1B. Individual Rad54 molecules that traveled downstream (∇) or upstream (Δ) are plotted separately, as well as the mean value for all molecules (•), with its standard deviation. Fitting the downstream (n = 63) and the upstream (n = 69) data separately to the Michaelis-Menten equation yielded V_{max} values of 264 ± 25 and 301 ± 29 bp/s, respectively and K_m values of 50 ± 29 and 112 ± 42 μM, respectively; fitting the mean for all of the molecules yielded a V_{max} of 301 ± 22 bp/s and K_m of 97 ± 28 μM ATP.

photobleaching from dissociation. Unexpectedly, we saw no dependence of the average distance traveled on ATP concentration; thus, the processivity is independent of ATP (above 50 μM). This observation was surprising, because for most DNA motor proteins, such as RecBCD helicase (Bianco et al., 2001; Roman et al., 1992), processivity is dependent on ATP concentration. The mean value from Figure 3A is 11,250 bp. The processivity, P, can be defined as the probability of advancing at each translocation step relative to the probability of dissociating (McClure and Chow, 1980). Assuming a step size of 1 bp, P is given by (N − 1) / N; therefore, P is 0.99991. Alternatively, the processivity can be obtained from the distribution of distances translocated before stopping (Figure 3B): the distribution should be exponential (von Hippel et al., 1994). Given that we likely miss molecules that travel less that a few kilobase pairs, the distribution should deviate from exponential for short distances traveled; at and above 4000 bp translocated, however, our observed distribution is exponential with an exponential decay constant of 0.00007, which is the probability of dissociating relative to translocating. Thus, P is 0.99993, which is in good agreement with the data derived from Figure 3A. Our finding that P is independent of ATP concentration is interesting. This finding may suggest, in turn, that the affinities of the various liganded and unliganded states of Rad54 for dsDNA are

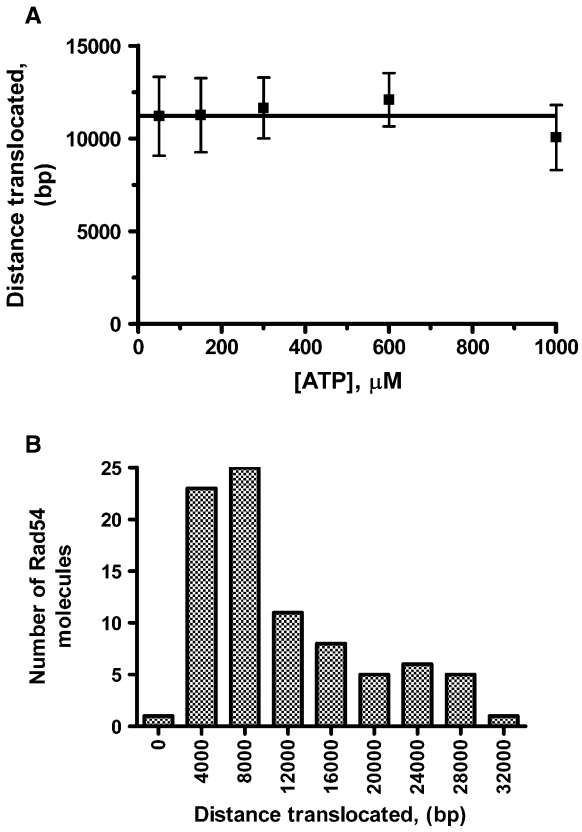

Figure 3. Rad54 Translocation Is Highly Processive

(A) The distance traveled by each Rad54 molecule was calculated from the start and stop positions, as determined in Figure 1B. The average value and its standard deviation at each ATP concentration are plotted. The horizontal line shows the mean for all five values.

(B) The distribution of distances traveled by the entire collection of molecules is shown in a histogram. The data at and above 4000 bp translocated can be fit with a single exponential decay constant of $7.1 \pm 4.7 \times 10^{-5}$.

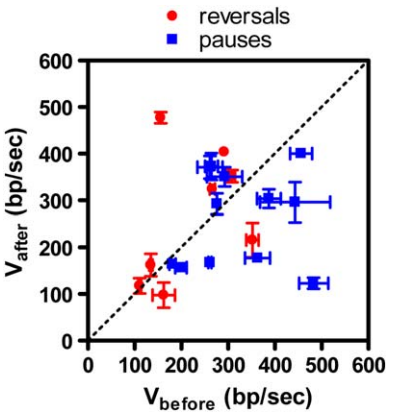

Figure 4. Rad54 Translocation Velocities before and after Both Pauses and Reversals Are Largely Uncorrelated

The translocation velocity before a Rad54 pause (blue squares) is plotted versus its velocity after resuming translocation. The translocation velocity prior to a Rad54 reversal in translocation direction (red circles) is plotted versus its velocity in the new direction. The diagonal broken line shows the trend expected if the velocities before and after the interruption were identical. The standard deviation for each velocity is indicated by the error bars; where the bars are not evident, the standard deviation is smaller than the symbol size.

comparable and high; thus, the bound lifetime is unaffected by translocation speed. This conclusion is also supported by our observation that, after stopping, most Rad54 molecules remained bound to the DNA for at least 20 s.

Although the behavior of the majority (80%) of Rad54 molecules was simple in that translocation was monotonic, complex behavior was also observed. Some Rad54 molecules were seen to pause (11%), reverse direction (6%), or change velocity (3%); examples of this complex behavior are provided in the Supplemental Data (Figure S1). There are no discernable specific DNA locations for pausing, reversing, or changing velocity (Figure S2), but the sample size is small, precluding a firm conclusion about the randomness of these phenomena. To determine whether the same molecular entity was translocating before and after a pause or reversal, we plotted the translocation velocity before a pause or reversal versus the velocity afterward (Figure 4). If the same molecular species was translocating before or after the discontinuity, then the velocities would be identical and all would fall along the diagonal. This is the case for several Rad54 molecules, but most show no correlation in velocity before or after a pause or reversal. Thus, we conclude that there is usually a change in the molecular species responsible for translocation after pausing or reversing; perhaps a different subunit in the functional Rad54 complex is used after translocation resumes. This conclusion, in turn, suggests that the translocating form of Rad54 is a multimer, perhaps a hexamer or dodecamer as for some other translocases. In addition, ~50% of the Rad54 molecules displayed a stationary "lag" phase prior to the start of translocation. The length of lag phase was reduced when the ATP concentration was increased (Figure S3), suggesting that a rate-limiting ATP-dependent structural transition is required before translocation can commence.

In this paper, we have shown visually that Rad54 is a translocase that travels on dsDNA both rapidly and processively. Rad54 binds randomly to the dsDNA and is equally likely to move in either of the two possible directions, consistent with its binding to one of the DNA strands and unidirectional translocation along that strand (Durr et al., 2005; Thoma et al., 2005). Both the speed and distance traveled were unexpected, given the in vitro properties of Rad54. For example, when chromatin remodeling was studied (Alexeev et al., 2003), the time required to move a nucleosome ~150 base pairs was at least 10 min. In comparison, Rad54 can translocate 150 base pairs in less than 1 s. Thus, based on this simple comparison, our work now makes it clear that the rate of chromatin remodeling is not limited by the rate of Rad54 translocation. In fact, our results imply that Rad54 translocates rapidly up to a nucleosome, only to be limited by the slower rate of nucleosome sliding. Furthermore, given that the processivity is high, and the lifetime of the Rad54-dsDNA complex is several minutes, it seems plausible that Rad54 can exert a continuous force at the boundary of the histone-DNA complex, thus exerting stress on the dsDNA between the histone and Rad54. Although the force generated by Rad54 is unknown, if it is at least 6 pN, then reversible

modification of chromatin structure results (Brower-Toland et al., 2002; Cui and Bustamante, 2000). Furthermore, if the force generated were higher (10–20 pN), then the DNA will stretch to some extent (Smith et al., 1996): RNA polymerase (Wang et al., 1998) and the φ29 DNA-packaging protein (Smith et al., 2001) are motor proteins that generate in excess of 20 pN of force. This continued nonequilibrium situation affords another mechanism of chromatin remodeling: namely, the generation of partially stretched DNA, which can be relieved only if successive contacts on the protein-DNA interface of the nucleosome are broken, likely only one contact at a time, or if the Rad54 dissociates. This protein-promoted "stress-dislocation" model would be independent of, but could be associated with, any accompanying changes in DNA twist (Kulic and Schiessel, 2003). This model of nucleosome sliding offers a simple and intuitive explanation for the slow nucleosome sliding by Rad54 and is consistent with its high processivity at all translocation velocities. Relaxation of DNA stress by successive small relaxations around the surface of the histone octamer is an uncomplicated way to explain nucleosome sliding and mobilization, as well as nucleoprotein complex remodeling in general.

We note that our model does not invoke looping as an intrinsic feature of remodeling, and in fact, our chromatin-remodeling experiments had been designed using short linear DNA substrates to minimize supercoiling-mediated remodeling; consequently, DNA looping may not be required for Rad54-mediated remodeling. Nevertheless, preliminary experiments with fluorescently end-labeled DNA show that sometimes DNA loops can form in a Rad54-dependent manner; the frequency and function, if any, of this looping is under investigation (our unpublished data). However, looping is not required to detect translocation. Furthermore, those experiments, using end-labeled DNA, reveal translocation rates comparable to those reported here, despite our observation that the bulk ATPase activity of Rad54 is reduced 30%–70% by the anti-GST antibody. Finally, similar to the chromatin-remodeling kinetics, the rate at which Rad54 disassembles Rad51-dsDNA complex is also slow (taking at least 1 hr to dissociate about 4 Kb of complex [Solinger et al., 2002]) relative to Rad54 translocation over the same distance (10–15 min). Thus, displacement of Rad51 from dsDNA is the rate-limiting step rather than Rad54 movement along the DNA. A model similar to that described above for nucleosome remodeling can be invoked for Rad51-DNA filament disassembly, where the force exerted by the continuous presence of Rad54 at the filament end can displace the terminal Rad51 monomer when it is in its most weakly DNA bound state during ATP turnover. Although speculative at present, measurements of force generation, nucleosome movement, and Rad51 nucleoprotein filament disassembly by Rad54 can resolve the validity of these models.

Experimental Procedures

DNA-Bead Complex Preparation

One of the cohesive ends (*cosR*) of bacteriophage λ DNA (New England Biolabs) was biotinylated by ligating a complementary 3′-biotinylated 12-mer oligonucleotide (Bianco et al., 2001). The excess biotinylated oligonucleotide and ATP were removed by using a MicroSpin S-400 HR spin column (Amersham) equilibrated with TE buffer (pH 7.6). An equal volume of biotinylated λ DNA (15 pM) was incubated with 1 μm streptavidin-coated polystyrene beads (35 pM; Bangs Laboratories) in 100 mM borate (pH 8.5), 0.1% BSA, 0.05% Tween 20, and 10 mM EDTA on ice for 1 hr.

FITC-Rad54-DNA-Bead Complex Preparation

Rad54-GST protein was purified as described (Solinger et al., 2001). The purified Rad54 protein exhibits a range of particle sizes seen by electron microscopy, suggesting an oligomer with varying subunit numbers (Kiianitsa et al., 2006). The FITC-Rad54-DNA-bead complex was assembled by mixing 0.5 μl of Rad54-GST (690 nM in 20 mM Tris-HCl [pH 7.5], 1 M NaCl, 60% glycerol, 0.1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) with 5 μl of DNA-bead complex. Subsequently, 0.5 μl of FITC-labeled anti-GST antibody (6.7 μM, Immunology Consultants Laboratory; average labeling of six fluorophores per antibody reported) in phosphate-buffered saline solution containing 0.2% BSA was added. The mixture was incubated at room temperature for 10 min and immediately transferred to 400 μl of a degassed solution containing 40 mM Tris-acetate (pH 8.2), 30 mM dithiothreitol, 15% sucrose, 100 μg/ml BSA, and 100 μg/ml casein; the final concentration of Rad54 was 850 pM. Incubation of the FITC antibody with Rad54 reduces bulk phase ATPase activity by 30%–70%.

Single-Molecule Rad54 Translocation Assay

The instrument described previously (Bianco et al., 2001) was used with a few minor modifications. The FITC-labeled Rad54-DNA-bead complex was excited with a high-pressure mercury lamp using the appropriate filter set and imaged with an EB-CCD camera (C7190-23; Hamamatsu Photonics, Hamamatsu, Japan). The images were recorded on S-VHS tape. Translocation of Rad54 along dsDNA was initiated by moving the trapped FITC-Rad54-DNA-bead complex to the second channel of the flow cell containing a degassed solution of 40 mM Tris-acetate (pH 8.2), 30 mM dithiothreitol, 15% sucrose, 2 mM magnesium acetate, and the indicated concentration of ATP. After observing Rad54 translocation, the light source was switched from the high pressure mercury lamp to a xenon lamp for bright field imaging to determine the bead position. The flow rate at the trap position was 100 μm/s. Because the ATPase activity of Rad54 is quickly inactivated at 37°C (Solinger et al., 2001), all measurements were performed at 25°C. Rad54 translocation could be observed for several minutes possibly because Rad54 functions in oligomeric state and is, therefore, labeled with multiple antibodies; however, the protein is not aggregated to such an extent that it scatters light, because when observed under bright-field illumination we did not observe any large objects on the DNA.

Data Analysis

Images were digitized by using an LG-3 frame-grabber card on a PC controlled by Scion Image Beta 4.02 (Scion Corporation). The frame rate for digitization was varied depending on the translocation rate of Rad54. Captured images were averaged to reduce background noise for measurement. The position of the fluorescent FITC-Rad54 relative to the trapped bead was determined by visual inspection of individual video frames. Because the fluorescent light is emitted isotropically, the center of each fluorescent object was determined manually and plotted as a function of time. Under our conditions, the fluctuation of a stationary fluorescent spot is about six pixels; this value corresponds to 840 nm (2470 bp), which represents the noise level in our analysis. Data were fit by nonlinear least squares to a multisegment line using Prism v. 4 for Windows (GraphPad Software). The rate of Rad54 translocation was calculated from the slope of the middle segment.

Supplemental Data

Supplemental Data include three figures and two movies and can be found with this article online at http://www.molecule.org/cgi/content/full/23/1/143/DC1/.

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