Real-Time Observation of RecA Filament Dynamics with Single Monomer Resolution

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SUMMARY

RecA and its homologs help maintain genomic integrity through recombination. Using single-molecule fluorescence assays and hidden Markov modeling, we show the most direct evidence that a RecA filament grows and shrinks primarily one monomer at a time and only at the extremities. Both ends grow and shrink, contrary to expectation, but a higher binding rate at one end is responsible for directional filament growth. Quantitative rate determination also provides insights into how RecA might control DNA accessibility in vivo. We find that about five monomers are sufficient for filament nucleation. Although ordinarily single-stranded DNA binding protein (SSB) prevents filament nucleation, single RecA monomers can easily be added to an existing filament and displace SSB from DNA at the rate of filament extension. This supports the proposal for a passive role of RecA-loading machineries in SSB removal.

INTRODUCTION

RecA functions in homologous recombination, SOS response, and mutagenic DNA repair, which are essential for maintaining the integrity of genetic information (Cox, 1999; Kowalczykowski, 2000). A human homolog, Rad51, interacts with BRCA2, whose mutation increases susceptibility to breast and ovarian cancers (Venkitaraman, 2004). The copy number of RecA in E. coli is less than 10,000 in the basal level but increases by more than an order of magnitude during SOS response (Cox, 2003; Sommer et al., 1998); thus, the physiologically relevant concentration of RecA is in the micromolar range. The protein binds to single-stranded (ss) DNA to form a nucleoprotein filament that exhibits a helical structure (Story et al., 1992) with each pitch consisting of six RecA monomers and each monomer occupying approximately three nucleotides (nt) of the ssDNA (Egelman and Yu, 1989; Zlotnick et al., 1993). The ssDNA within the filament is stretched along the longitudinal axis of the filament and has the capability of seeking out and recognizing a double-stranded (ds) DNA of a homologous sequence to initiate a strand-exchange reaction during homologous recombination.

The formation of a RecA filament is highly cooperative and utilizes ATP as a cofactor (Menetski and Kowalczykowski, 1985). A number of monomers bind to ssDNA simultaneously during nucleation, but this crucial event is poorly understood because it has been difficult to separate nucleation from the subsequent filament extension that immediately follows. After nucleation, additional RecA units bind and extend the filament in the 3’ direction of the ssDNA. For this reason, we will refer to the 3’ end of ssDNA-RecA filament as the “3’-extending end.” The unit of filament extension, whether it is the RecA monomer or higher-order complexes, is currently unknown for E. coli RecA. If the filament encounters an ss-dsDNA junction during extension, it continues to extend into the dsDNA (Register and Griffith, 1985). However, filament formation on dsDNA alone is extremely slow (Pugh and Cox, 1988) unless a 5’-ssDNA tail is present or the dsDNA is melted by applying strong force (Hegner et al., 1999) or torque (Fulconis et al., 2004). The rate of filament extension on dsDNA was determined by a single-molecule measurement (Shivashankar et al., 1999; van der Heijden et al., 2005), but the extension rate on ssDNA has been difficult to measure because of complications arising from multiple nucleation events.

RecA is a DNA-dependent ATPase and hydrolyzes ATP at the rate of 0.5 s⁻¹ at 36°C (Bedale and Cox, 1996), regardless of where it is located on the filament (Brenner et al., 1987). When a monomer at the 5’ end of the filament, termed here “5’-disassembly end,” hydrolyzes ATP, it dissociates from the filament (Lindsley and Cox, 1990; Register and Griffith, 1985) in a pH-dependent manner (Arenson et al., 1999). If RecOR interacts with the 5’-disassembly end (Shan et al., 1997), or if a slowly hydrolyzing ATP analog, ATPγS, is used, RecA dissociation becomes insignificant.
The difference between the two ends in terms of apparent stability may play a role during strand exchange via a treadmilling process, in which monomers dissociating from the 5' -disassembly end can rebind to the 3' -extending end so that a finite number of RecA monomers can be recycled efficiently and any discontinuity in the filament can be fixed (Menetski et al., 1990). A similar mechanism has been proposed for actin filaments, which show a large difference in dissociation constants between the two ends (Wegner, 1982). However, the rates with which RecA binds to the two ends and dissociates from the 3'-extending end are not known yet. Thus, the molecular mechanism for the directional growth of a RecA filament has not been clear.

In E. coli, most of a nascent ssDNA becomes occupied by SSB (ssDNA binding protein), preventing other proteins from accessing the ssDNA. Because of the highly cooperative nature of RecA binding, absence of a naked ssDNA precludes RecA-filament nucleation on SSB-coated ssDNA. Therefore, for RecA-mediated DNA repair to initiate on SSB-coated ssDNA, accessory proteins are necessary to help load RecA (Amundsen and Smith, 2003). Once RecA is loaded, SSB should no longer present a barrier to the 3'-extending end RecA filament (Kowalczykowski and Krupp, 1987; Thresher et al., 1988). To comprehend these two different modes of SSB function in relation to RecA, we need techniques that can observe the dynamic interactions between RecA and SSB in real time.

In the double-strand break repair (Cox et al., 2000; Kowalczykowski, 2000; Smith, 2001), a blunt-ended dsDNA is processed into a partial dsDNA with a 3'-tail, which becomes coated with SSB. After RecFOR occupies the ss-dsDNA junction (Morimatsu and Kowalczykowski, 2003), RecA displaces SSB assisted by RecOR (Bork et al., 2001; Umezuruike et al., 1993). How RecFOR helps load RecA on SSB-coated ssDNA is not yet clear. Likewise, RecA displaces SSB assisted by χ-modified RecBCD (Anderson and Kowalczykowski, 1997) and, in eukaryotes, Rad51 removes RPA supported by Brh2, a BRCA2 homolog (Yang et al., 2005).

Single-molecule FRET (Förster Resonance Energy Transfer) (Ha, 2001; Ha et al., 1996; Weiss, 1999) is a powerful method for observing the real-time dynamics of DNA (Lee et al., 2005; McKinney et al., 2005), RNA (Zhuang, 2005), protein-DNA interactions, and conformational changes of proteins (Diez et al., 2004; Myong et al., 2005). Here, we present single-molecule FRET assays that enabled us to watch binding and dissociation of individual RecA monomers in real-time. Combined with a novel analytic tool based on hidden Markov modeling, our work provides a new approach of determining not only the nature of the RecA binding and dissociation unit but also kinetic rates of the dynamics at both ends of the filament for the first time. Furthermore, by visualizing the dynamics of the filament near the ss-dsDNA junction, we provide insight into the role of RecA and its interactions with other proteins. In addition, we examine the filament nucleation process and determine its kinetic properties, as well as the minimum number of monomers required for nucleating a RecA filament. Finally, based on an assay in which a cluster of the filament is prenucleated near the ss-dsDNA junction, we find that RecA, once nucleated, is able to replace SSB almost as quickly as filament extension.

RESULTS

Single-Molecule FRET Assay
As illustrated in Figure 1A, a biotinylated dsDNA (18 base pairs) with a 3'-ssDNA tail is immobilized on a surface (Experimental Procedures). The donor (Cy3) and acceptor (Cy5) fluorophores are attached at the end of the ssDNA tail and the ss-dsDNA junction to report on changes in the end-to-end distance of the ssDNA through FRET. The ssDNA by itself is highly flexible (permeance length of ~1.5–3 nm), such that its conformational fluctuation is averaged out on a much faster time scale than our time resolution (Murphy et al., 2004). For the (dT)21 tail, two dyes are in close proximity on average giving relatively high FRET efficiency, E ~ 0.55 (Figures 1A, left and 1Ca, white), in our standard solution condition (Experimental Procedures). Addition of 1 μM RecA and 1 mM ATP/S, our standard RecA and cofactor concentrations, leads to the filament formation and straightening of the ssDNA (Figure 1A, right). This results in low FRET, E ~ 0.1 (Figure 1Ca, green; different lengths of ssDNA in Figure S3B; different cofactors in Figure S4), distinguishable from acceptor blinking (E = 0) (Ha et al., 1999). Likewise, the DNA in bulk solution shows high FRET without RecA and low FRET when the filament forms with ATP (Figure 1B).

Nucleation of a Filament
Whereas RecA forms a stable filament on the (dT)21 tail with ATP/S, it does not form a filament stably when ATP is used as the cofactor; low FRET (E ~ 0.1) shows up only a small fraction of the time (6%) (Figure 1Cb). Likewise, the fluorescence spectrum in bulk does not show any significant change when RecA is added with ATP (Figure 1B). This observation confirms previous measurements, which found that RecA, together with ATP, does not form a stable filament on ssDNA lengths shorter than ~30 nt (Bianco and Weinstock, 1996; Brenner et al., 1987).

Examination of individual single-molecule traces reveals transient RecA binding, as evinced by occasional and brief transitions from high FRET (E ~ 0.55) to low (E ~ 0.1) (Figures 1D and S3C). Since the transient low-FRET state has an identical value to that observed with the stable ATP/S-RecA filament (Figure 1Ca, green), we interpret it as representing brief formation of a RecA filament. While there must be several monomers in the filament formed on the (dT)21 tail, the transitions between the two FRET states display only a few data points at 100 ms time resolution, much faster than expected from binding and dissociation of a monomer at an established filament (see Figure 2C; binding and dissociation of monomers take several seconds under the same condition). This was also true of (dT)17 and (dT)19. Therefore, there must be either simultaneous binding of multiple monomers or...
binding of a preassembled oligomer in order for RecA to form a cluster of a filament on such short ssDNA.

We then determined the average dwell time of the low-FRET state with different lengths of ssDNA tail and found that shorter ssDNA yields a shorter average dwell time (Figure 1Ea). Meanwhile, the average dwell time decreases as the temperature increases (Figure 1Fa), and this is inversely proportional to the increase in the ATP hydrolysis rate (Bedale and Cox, 1996). These dependences suggest that the stability of the cluster is correlated with the number of monomers bound to ssDNA and ATP hydrolysis. Next, we measured how frequently the cluster forms. The frequency sharply drops when shorter lengths of ssDNA than (dT)21 are used (Figure 1Eb). Furthermore, there is no filament formation observed with (dT)13 and (dT)15. Finally, the frequency of cluster formation depends only weakly on the temperature (Figure 1Fb). These collective findings suggest that the transition observed here represents the formation/dissociation of a nucleation cluster that would likely lead to filament extension on a longer ssDNA.

**Dynamics at the 5'-Disassembly End**

For an ssDNA longer than ~30 nt, a RecA filament forms stably even with ATP. In this case, FRET changes would reflect the assembly/disassembly occurring at the ends of the filament.
of the filament rather than the formation/dissociation of the nucleation cluster. Since FRET between the two ends of such a long naked ssDNA is relatively low, we sought to achieve high resolving power in FRET by placing the donor internally on the ssDNA tail without disrupting DNA backbone or perturbing RecA activity (Figure 2A; Supplemental Experimental Procedures; further discussion in the Supplemental Data). The ssDNA of (dT)13+46 has the donor and acceptor separated by 13 nt, followed by 46 nt of 3′ tail so that FRET changes reflect the dynamics at the 5′-disassembly end. This DNA shows high FRET ($E \sim 0.65$) by itself but exhibits low FRET ($E \sim 0.15$) in the presence of RecA and ATP;S (Figure 2Ba) due to stable filament formation.

In contrast, a broad FRET histogram spanning from 0.15 to 0.7 is observed with RecA and ATP (Figure 2Bb). Examining the single-molecule traces, we find that they exhibit stepping between well-defined FRET levels (Figure 2C). Traces no longer exhibit the simple two-state dynamics found in shorter tails, suggesting that different binding modes (i.e., different numbers of RecA monomers on the 13 nt region between donor and acceptor) are being observed. If these different FRET values correspond to different assembly states of the filament, we may expect to see up to five different FRET values (0, 1, 2, 3, and 4 monomers) because up to four monomers could bind to the 13 nt region. However, not only is it challenging to identify five states given the limited signal-to-noise ratio, but it could also bias the analysis to presume that there exist five states. Therefore, we employed a newly developed form of hidden Markov modeling (HMM) to analyze the data statistically without imposing a preconceived model (See Experimental Procedures) (McKinney et al., 2006). HMM analysis identifies distinct FRET states in each trace (Figure 2C, the fit in green) and determines the number of states, their FRET values, and the transition rates between them (Figure 2D).

Figure 2Da shows the result of HMM analysis as a transition density plot (TDP) of 17,203 transitions from 165 DNA molecules. A TDP is effectively a two-dimensional histogram that shows how frequent transitions are between different states. They are presented with the FRET levels prior to and after the transition on the horizontal and vertical axes respectively. It is evident that there are four distinct FRET values (0.15, 0.3, 0.5, and 0.7) along each axis, in addition to the no-FRET peaks due to acceptor blinking. The highest FRET value ($E \sim 0.7$) agrees with the DNA-only value (Figure 2Ba, green), therefore the state with $E \sim 0.7$ is designated M0. The lowest FRET value ($E \sim 0.15$) is close to the value observed with RecA and ATP;S (Figure 2Ba, white). Thus, the remaining FRET values ($E \sim 0.5, 0.3,$ and $0.15$) are termed M1, M2, and M3, respectively, with each number representing the number of monomers bound contiguously, starting from the donor location to the 13 nt segment (Figure 2A). Noncontiguous binding of RecA was not considered because we did not detect any binding of RecA to (dT)13+46 with ATP (see the previous section); de novo nucleation of a filament in such a short ssDNA segment is extremely unlikely, and the binding transitions here represent extension from the existing filament only.

It is not clear why only three monomers appear to bind instead of four. When we shortened the ssDNA segment between the donor and acceptor using (dT)4+46 and (dT)10+49, we observed that transitions occur primarily between two and three states, indicating that one and two monomers can access the 5 and 10 nt segments respectively (TDP in Figure 2F and time traces in Figure S5). The TDP in Figures 2D and 2F show that the most frequent transitions are of the type $M_i \rightarrow M_{i+1}$, indicating that the majority, $>78\%$ for (dT)13+46 and $>93\%$ for (dT)10+49, of binding and dissociation events occurs one monomer at a time. Note that the mirror symmetry in the TDPs comes from the fact that the majority of transitions are of the type $M_i \rightarrow M_{i-1}$ and that the system under observation is in equilibrium, when the number of transitions of $M_i \rightarrow M_{i+1}$ is the same as that of $M_{i-1} \rightarrow M_i$.

Once every transition in the plot is classified according to which $M_i \rightarrow M_j$ pair it belongs to, kinetic rates can be determined for each pair (Figure 2Db). For example, the binding rates ($k_{on\text{obs}}$) are largely independent of RecA concentration, as we would expect (Figure 2Eb), but vary slightly with the position at the two highest RecA concentrations: 1 m$M_i$ and 10 m$M_i$. This effect may be due to the reduction in the amount of naked ssDNA available for binding as the number of bound monomers increases.

The dissociation rates ($k_{off}$) are largely independent of RecA concentration, as one would expect (Figure 2Eb), but vary slightly with the position at the two highest RecA concentrations, 1 m$M_i$ and 0.25 m$M_i$. We should point out that the average $k_{off}$ (averaged over all three dissociation rates) is not necessarily equal to or lower than the ATPase rate ($k_{cat}$). In fact, it was shown that when a monomer leaves the 5′-disassembly end, $k_{off}$ of the adjacent monomer is twice $k_{cat}$ (Arenson et al., 1999). Thus, such an effect should be considered when $M_i$ has been arrived at via RecA dissociation. When the probability for $M_i$ to have come about via RecA dissociation is $p$ and the coupling efficiency of ATP hydrolysis to dissociation is $\varphi$, $k_{off} = \varphi \times (2k_{cat} \times p + k_{cat} \times (1 - p))$ :  

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Then, from $p$ and $k_{off}$ values determined by HMM analysis and TDP (Figure 2Db), we can calculate the “corrected” dissociation rate [$k_{off}' = \varphi \times k_{cat}$ (Figure S6A)]. This rate of 0.12 ± 0.02 (s$^{-1}$), independent of the position of the dissociating monomer and RecA concentration, is comparable to the ATPase rate of 0.18 s$^{-1}$ at 22°C, estimated from Bedale and Cox by extrapolation (Bedale and Cox, 1996). This comparison indicates that the coupling efficiency $\varphi$ is close to 1 in accord with the previous estimate at neutral pH (Arenson et al., 1999). Finally, the value of $k_{off}'$ is similar to $k_{on\text{obs}}$, 0.11 ± 0.02 (s$^{-1}$) in the presence of 100 nM
Figure 2. Dynamics at the 5’-Disassembly End

(A) FRET reports on the dynamics at the 5’-disassembly end.
(B) FRET histograms (a) from DNA only (E ~ 0.85, green) and with RecA and ATPγS (E ~ 0.15, white, after 2 min incubation) and (b) with RecA and ATP (after 30 min incubation). Vertical gray lines mark $E = 0$ due to donor-only species.
(C) Time traces with RecA and ATP (100 ms time resolution). The FRET trace (black) is fitted by HMM (green); then, four states are assigned (M₀, M₁, M₂, and M₃, based on the TDP in [Da]). Donor photobleaching was at t ~ 255 s (arrow).
(D) Transition density plot (TDP). (a) This pseudo-3D plot is constructed by adding a Gaussian peak for each transition. There are four states with $E$ ~ 0.15, 0.3, 0.5, and 0.7. (b) Rates and frequencies (inside circles) of each transition, $M_i$ → $M_{i+1}$, are presented in the same scheme.
(E) (a) Binding and (b) dissociation rates versus RecA concentration for different transitions (based on 12,204 transitions from 172 molecules at 0.1 μM, 23,580 from 196 at 0.25 μM, and 17,203 from 165 at 1 μM). The errors are SDs of three datasets each.
(F) TDP and kinetic rates (a and b) with (dT)₁₀+₄⁹ tail based on 8637 transitions from 104 molecules (E ~ 0.35, 0.5, and 0.6) (c and d) and with (dT)₅+₅⁴ based on 5917 transitions from 92 molecules (E ~ 0.8 and 0.9).
RecA, so we estimate that the dissociation constant ($K_D$) is about 100 nM at the 5′-disassembly end.

**Filament Formation with ATPγS**

Next, we attempted to observe the filament dynamics at the 3′-extending end using a ssDNA tail of (dT)$_{13-16}$, the same DNA as (dT)$_{13-46}$ but labeled differently so that FRET reports on the dynamics of the 13 nt segment at the 3′-extending end. At 1 μM RecA, this DNA gave stable low FRET, indicating filament formation with no discernible dynamics at the 3′-extending end (data not shown). At 100 nM, we observed irregular FRET fluctuations, likely as a result of both formation/dissociation of the nucleation cluster and the assembly/dissociation at the 3′-extending end (data not shown), precluding detailed analysis. We realized that what we needed was a method to separate the nucleation step from the filament extension. We could achieve just that, based on an unexpected finding that we will discuss in this section.

As described earlier, stable filaments form on short ssDNA tails, from (dT)$_{13}$ to (dT)$_{21}$ (Figure 1Ca) if ATPγS is used as a cofactor (Leahy and Radding, 1986). Here, we will refer to such filament as <ssDNA/ATPγS>. With (dT)$_{13}$, <ssDNA/ATPγS> results in low FRET ($E \approx 0.2$) (Figure 3Ab, white). If RecA is subsequently removed from solution via flow while keeping the magnesium concentration constant (Figures 3Ac and 3Ad), the single-molecule FRET histogram is restored to that of DNA only within 5 min (Figure 3Ab, white), indicating that the ssDNA tail becomes free of RecA. Single-molecule traces recorded during this disassembly reveal individual states ($M_i$) of (dT)$_{13}$ (Figure S8). While the disassembly was also observed with (dT)$_{15}$–(dT)$_{21}$ tails (data not shown), longer tails, (dT)$_{30}$ and (dT)$_{70}$, however, supported stable <ssDNA/ATPγS> even after removing free RecA and ATPγS (data not shown) and required SSB for RecA removal from ssDNA (Figure 5B).

To study a possible filament formation of <dsDNA|ATPγS>, we attached the donor and acceptor fluorophores at the ends of dsDNA, which has a 3′ tail of (dT)$_{15}$ (Figure 5Ba). $E$ decreases from 0.25 to 0.1 when RecA and ATPγS are added, indicating the formation of <dsDNA|ATPγS>. Since it cannot form in the absence of an ssDNA tail (Figure S9C), its formation here must be by utilizing a different configuration of labeling (Figure S9A) and by a footprinting assay (Figure S9B), we found that the filament forms over the entire length of dsDNA.

Next, when RecA and ATPγS are removed from solution, no significant change in FRET is observed for 1 hr, while it slowly starts to disassemble in 2 hr (Figure 3Bb), suggesting unexpectedly high stability of <dsDNA|ATPγS> ($>1$ hr) compared to <ssDNA|ATPγS> formed on 13–21 nt ssDNA (<5 min). A similar property was observed at high temperature (35°C) (Figure S10B) and also with dsDNA that has 5′-ssDNA tail (Figure S11).

**Dynamics at the 3′-Extending End**

The difference in stability between <ssDNA|ATPγS> and <dsDNA|ATPγS> in the absence of free RecA results in a unique configuration in which a filament is present only around the dsDNA portion of a partial duplex DNA (Figure 3Ac). We suspected that the <dsDNA|ATPγS> in such
a construct might act as a nucleation cluster, thus facilitating the formation of ssDNA:ATP when RecA is added with ATP. Indeed, we observe stable filament formation on (dT)13 within 1 s of adding RecA and ATP (Figures 3Ae and 3Af), whereas there was no or rare filament formation observed with 13–21 nt ssDNA without dsDNA:ATP (Figure 1Eb). The similar observation was made also at a higher temperature (35°C) (Figure S10A). This unexpected finding allows us to decouple filament extension from nucleation and enables the observation of filament dynamics at very low RecA concentrations.

Figure 4B shows FRET fluctuations after the addition of 8 nM RecA with ATP, indicative of filament dynamics at the 3'-extending end (Figure 4A). Transitions between multiple FRET states detected in single-molecule time traces are presented in the TDP (Figure 4Ca; 82 molecules and 4635 transitions), where five different states are clearly discerned (E \sim 0.2, 0.3, 0.55, 0.75, and 0.85; M4, M3, M2, M1, and M0, respectively), consistent with the maximum number of states counted in Figure S8Ba. Almost all transitions occur between the nearest neighbors, M4 \leftrightarrow M3, M3 \leftrightarrow M2, M2 \leftrightarrow M1, and M1 \leftrightarrow M0, again suggesting that the unit of binding and dissociation is a monomer also for the 3'-extending end. It also provides the most direct evidence thus far that RecA cannot dissociate from the middle of the filament.

$\kappa_{\text{on}}^{\text{obs}}$ is significantly lower than those at other positions (Figure 4Cc, left). Excluding $\kappa_{\text{on}}^{\text{obs}}$ M0 \leftrightarrow M1, the average $\kappa_{\text{on}}^{\text{obs}}$ is 0.18 \pm 0.03 s^{-1}. $\kappa_{\text{off}}^{M2 \leftrightarrow M1}$, $\kappa_{\text{off}}^{M3 \leftrightarrow M2}$, and $\kappa_{\text{off}}^{M4 \leftrightarrow M3}$ vary with respect to one another but the corrected dissociation rates are nearly identical with the exception of $\kappa_{\text{off}}^{M1 \leftrightarrow M0}$ (Figure S6B). There appears to be a high-energy barrier between M0 and M1, that slows both binding and dissociation, probably due to the junction of heterofilaments, ssDNA:ATP and dsDNA:GyS (Prevost and Takahashi, 2003). The average $\kappa_{\text{on}}^{\text{obs}}$ (excluding $\kappa_{\text{off}}^{M1 \leftrightarrow M0}$) of 0.16 \pm 0.04 (s^{-1}) is close to the average $\kappa_{\text{on}}^{\text{obs}}$ in the presence of 8 nM RecA; thus, K0 must be about 8 nM in the 3'-extending end.

RecA Displaces SSB Easily when Supported by a Preformed Nucleation Cluster at an ss-dsDNA Junction

The binding of an SSB tetramer to the 3’ (dT)70 ssDNA tail of a partial dsDNA, a structure that would be produced in the double-strand break repair, results in high FRET (E \sim 0.7) in our solution condition (Figure 5A). This is likely caused by the wrapping of ssDNA around SSB, observed both in crystallography and ensemble FRET studies (Kozlov and Lohman, 2002; Raghunathan et al., 2000). In comparison, FRET is very low in the absence of any protein (E \sim 0.1) and in the presence of RecA (E \sim 0) (Figures 5Ab and 5Bc). Once SSB binds to the ssDNA, it does not come off appreciably even after 1 hr in the absence of free SSB in solution (data not shown). Upon addition of RecA
and ATP, SSB remains even after 1 hr (Figure 5Ad), indicating that SSB is extremely difficult to be displaced by RecA.

We wondered whether RecA might form a filament by replacing SSB if \(<\text{dsDNA}\rangle\text{ATP}g\text{S}\rangle\), the preassembled nucleation cluster at the junction, is present. As illustrated in Figure 5B, adding RecA and ATP to the partial dsDNA results in very low FRET (Figure 5Bc). If we then add 10 nM SSB and remove RecA and ATP\text{g}S from solution, SSB slowly binds to ssDNA by disassembly of \(<\text{ssDNA}\rangle\text{ATP}g\text{S}\rangle\) (Figure 5Bd), eventually returning to the single-molecule FRET histogram identical to what was obtained with SSB alone (Figure 5Ac) with a time scale of ~5 min (Figure S13), suggesting that SSB has replaced the RecA filament on the ssDNA. Strikingly, upon subsequent addition of RecA and ATP, the FRET peak shifts to a very low FRET within seconds (Figure 5Be), indicating efficient formation of a stable filament. Such rapid filament formation is in sharp contrast to the data obtained without the preassembled nucleation cluster (Figure 5Ad), where RecA and ATP could not displace SSB during a 1 hr observation window.

The detailed process by which RecA replaces SSB is shown in single-molecule time traces (Figures 5Ca–5Cc). After solution exchange (t = 0), E drops through diverse pathways: FRET changes (a) without any intermediate state and (b) with one or (c) more intermediate states were found with comparable frequencies. To distinguish the low FRET (E ~ 0) from fluorescence signal with an inactive acceptor, the activity of the acceptor was checked by directly exciting the acceptor using a 633 nm laser at t > 38 s (arrows). (d) A group of FRET traces (57 molecules for 1 \(\mu\)M RecA; 26 for 0.1 \(\mu\)M) are averaged and fitted by exponential decay curves.
DISCUSSION

In this work, we have established new single-molecule FRET assays capable of observing binding and dissociation of individual RecA monomers in the nucleoprotein filament. The kinetic rates of the filament dynamics have been determined via a novel analysis based on the hidden Markov model. Below, we summarize the most important conclusions that can be drawn from our studies.

The Minimum Size of the Nucleation Cluster
A RecA-nucleation cluster forms briefly and infrequently on 17–21 nt-long ssDNA. The actual processes of formation and dissociation of the cluster were faster than we could resolve clearly with the time resolution of 100 ms. The dwell time of the cluster formed on 17 and 19 nt tails were 4.3 and 6.0 s, respectively (Figure 1Ea), similar to the dissociation time of a single RecA monomer ($K_{\text{on}}$)$^{-1}$, 8.3 s. This coincidence implies that dissociation of even a single monomer makes the cluster bound to 17–19 nt very unstable and induces rapid dissociation of the cluster. Furthermore, the nucleation frequency dropped precipitously when the ssDNA length was below 21 nt (Figure 1Eb), and no nucleation was observed with 13 and 15 nt. Therefore, ~5 monomers bound to 17 nt could be considered the minimum unit for nucleation.

The Unit of Binding and Dissociation
Since free E. coli RecA exists in solution in diverse assembly states, including a monomer, trimer, hexamer, and so on (Brenner et al., 1990), there has been no definitive way to determine the size of a binding unit in the filament assembly. Because the filament binding and dissociation transitions occurred predominantly between nearest neighbor states, as revealed in the TDPs (Figures 2D, 2F, and 4C), we conclude that this unit of binding as well as of dissociation is primarily a single monomer. The data further indicate that dissociation occurs from the extremities of the filament because extensive dissociation from the internal sites on the filament would have yielded a much more significant amount of nonnearest neighbor transitions.

Binding Rates at Each End
The individual monomer binding rates $k_{\text{on}}$(obs) have been directly measured at both 3’-extending and 5’-disassembly ends. In previous works, binding of RecA to the 5’-disassembly end had been largely ignored in describing the dynamics of a filament, but we found that the $K_{\text{on}}$(obs) was even higher than $k_{\text{off}}$ at the 5’-disassembly end at 1 μM RecA. The RecA filament was thought to exhibit a monotonic disassembly followed by a rapid recovering assembly in a previous analysis, which neglected RecA binding at the 5’-disassembly end (Tlusty et al., 2004), but we observed continual binding and dissociation of monomers (Figure 2C). Thus, RecA binding at the 5’-disassembly end should be included in the description of filament dynamics.

We also determined the heretofore immeasurable $k_{\text{on}}$(obs) at the 3’-extending end (0.18 s$^{-1}$ at 8 nM RecA). As a rudimentary comparison, if we extrapolate to 1 μM, $k_{\text{on}}$(obs) becomes 23 s$^{-1}$, which coincides with the rate determined with dsDNA (20 s$^{-1}$ in the same RecA concentration [van der Heijden et al., 2005]).

Dissociation Rates at Either End
The dissociation rates $k_{\text{off}}$ were also measured at both ends. $k_{\text{off}}$ at the 5’-disassembly end was consistent with the previous report (Arenson et al., 1999), and $k_{\text{off}}$ at the 3’-extending end was determined for the first time. $k_{\text{off}}$ at the 5’-disassembly end and at the 3’-extending end were essentially the same. Meanwhile, there is about an order of magnitude difference in the dissociation constant of ~100 nM at the 5’-disassembly end and ~8 nM at the 3’-extending end. Therefore, the directionality in the filament growth is mainly due to the difference in the binding rates.

The dissociation rate after correction ($K_{\text{on}}$)$^{-1}$ was close to the ATPase rate, showing that disassembly of a filament is tightly coupled with ATPase activity. $K_{\text{off}}$ was independent of the position of the dissociating monomer, indicating that there is no significant coupling between neighbor monomers in terms of ATPase activity (Shan et al., 1996).

RecA Replaces SSB if Given a Nucleation Site
It has been long known that RecA can displace SSB from ssDNA (Kowalczykowski and Krupp, 1987). Using a preassembled nucleation cluster, here we provided the most comprehensive evidence that SSB is removed by an extending RecA filament. The rates of SSB removal and the unhindered extension rate of the RecA filament were found to be almost identical, suggesting that the hindrance provided by SSB to a growing filament is minimal. Even though an unraveled section of ssDNA between the nucleation cluster and SSB must be very small, RecA could still bind and extend the filament with ease. Therefore, how SSB can function in two contrasting modes in relation to RecA is clearly explained; the ssDNA binding energy of SSB is smaller than that of the RecA filament so that SSB can be easily removed by an extending filament, but the exposed length in SSB-saturated ssDNA is shorter than ~17 nt, such that a de novo nucleation of a filament is inhibited.

Our result supports the proposal that a sufficient role for the RecA-loading machineries in helping RecA replace SSB is to provide a nucleation cluster-like structure to initiate RecA-filament extension (Anderson and Kowalczykowski, 1997). If so, no active SSB-clearing mechanism is required for the RecA-loading machineries (Kowalczykowski, 2005). Crystallography revealed that the interface between BRCA2 and Rad51 bears a clear resemblance to the interface between neighbor Rad51 monomers in Rad51 filaments (Conway et al., 2004; Pellegrini et al., 2002). It is possible that the RecA-loading machineries, RecBCD (Spies and Kowalczykowski, 2006) and RecFOR, might have adopted the same strategy by emulating the
RecA Filament near the Junction

There were no dissociation events observed from <ssDNA>ATP> when its 5'-disassembly end was stabilized by <dsDNA>ATPγS> at 1 μM RecA, analogous to the stabilization of the 5'-disassembly end by RecOR (Shan et al., 1997). We therefore expect that once a RecA filament forms assisted by RecA-loading proteins, other proteins would not be able to access the ssDNA or the junction (Figure 6B).

In the absence of such stabilizing factors, for example, after the RecA-loading proteins have left the junction, a stretch of ssDNA next to the junction would become exposed due to the intrinsic instability of the 5'-disassembly end (Figures 6C and 6D). Since the FRET histogram of (dT)13+46 was broadly biased toward low FRET at 1 μM RecA (Figure 2Bb), the exposed ssDNA is shorter than ~13 nt on average. Stronger bias toward low FRET was observed with a longer segment between donor and acceptor as expected (Figure S7). When RecA concentration was lowered to 0.1 μM, the histogram of (dT)13+46 shifted toward high FRET (data not shown), indicating that more than 13 nt is routinely exposed.

Our data therefore show that ssDNA accessibility at the junction of a 3'-tailed dsDNA is modulated by RecA concentration. When the RecA concentration is above ~1 μM, the filament would not be immediately destroyed but would go through constant fluctuations of limited range (Figure 6C). Therefore, RecA would still retain to a large extent the capacity to perform its role, the degree of which would be higher than previously thought since the binding at the 5'-disassembly end is significant. On the contrary, at submicromolar concentrations, the filament would disassemble swiftly (Figure 6D). Indeed, we found that the activity of Rep helicase that unwinds 3'-tailed dsDNA was affected by RecA concentration and that SSB binding to ssDNA was greatly delayed by the presence of 1 μM RecA in solution (unpublished data).

RecA Filament In Vivo

Based on our findings, we propose the following scenarios of protein interactions during the first steps of the double-strand break repair (Figure 6). When a blunt-ended dsDNA is processed into a partial dsDNA, SSB binds to the 3' ssDNA tail generated. Then, the RecA-loading machinery binds to the junction and recruits and holds RecA (Anderson and Kowalczykowski, 1997; Bork et al., 2001; Kowalczykowski, 2005; Morimatsu and Kowalczykowski, 2003; Pellegrini et al., 2002; Spies and Kowalczykowski, 2006; Umezu et al., 1993; Yang et al., 2005), nucleating a RecA filament. Additional RecA monomers bind successively to extend the filament in the 3' direction, displacing SSB (Kowalczykowski and Krupp, 1987; Thresher et al., 1988). The filament is very stable not only because the 5'-disassembly end is held by the RecA-loading machinery (Shan et al., 1997) but also because the K_{ss} of the 3'-extending end is much lower than the concentration of RecA in the basal level. Therefore, neither the ssDNA tail nor the junction is easily accessible for binding by other proteins. The 5'-disassembly end becomes unstable if the RecA-loading machinery leaves the junction, but because the basal level of RecA is still higher than the K_{ss} of the 5'-disassembly end, only a small region of ssDNA near the junction becomes accessible to other proteins. Future experiments, including the RecA-loading machineries, may test these ideas directly.
EXPERIMENTAL PROCEDURES

Reaction Condition for Single-Molecule Assay
As shown in Figure S1, DNA is immobilized on a quartz surface (Finkenbeiner), which is coated with polyethylene glycol (a method in the Supplemental Experimental Procedures) in order to eliminate nonspecific surface adsorption of proteins (Ha et al., 2002). The immobilization was mediated by biotin-Neutravidin binding between biotinylated DNA (i.e., DNA) Neutravidin (Pierce), and biotinylated polymer (PEG-MW 5,000, Nektar Therapeutics). About 100 pM of DNA molecules are immobilized and observed in our standard solution condition: 1 mM ATP (Sigma) or ATP-γ-S (Calbiochem), 10 mM MgAc, 100 mM NaAc, 25 mM Tris-Ac at pH 7.5 in the presence of oxygen scavenging system of 1 mg/ml glucose oxidase (Sigma), 0.4% (w/v) glucose (Sigma), 0.04 mg/ml catalase (Roche), and 1% v/v 2-mercaptoethanol (Acros) at room temperature (22 ± 1°C) unless otherwise specified. The concentration of RecA (New England Biolabs) is 1 μM unless otherwise specified. SSB (10 mM, the standard concentration) (a gift from Dr. T.M. Lohman) was not added unless mentioned. A prepared solution ~100 μl was injected into an assembled chamber that holds ~20 μl. Therefore, the injection results in a very efficient change in chemical environments around immobilized DNA. When RecA and ATP are in the injected solution, the system inside the chamber reaches equilibrium in less than a minute.

Single-Molecule Data Acquisition
Cy3 on DNA is excited by an Nd:YAG laser (532 nm, 75 mW. CrystaLase) via total internal reflection (Figure S1). The fluorescence signal from Cy3 and Cy5 that is collected by a water immersion objective lens (60×, Olympus) goes through a 550 nm long-pass filter to block out laser scattering. It is separated by a 630 nm dichroic mirror and is detected by EMCCD camera (Andor) with a time resolution of 30–200 ns. Fluorescence signal of donor and acceptor molecules is amplified before camera readout; therefore, the recorded fluorescence intensity is in an arbitrary unit. The signal was recorded using software written in Visual C++. Single-molecule traces were extracted from the recorded video file by IDL software.

Single-Molecule Data Analysis
Basic data analysis on the single-molecule traces were carried out by Origin and software written in MatLab, with which FRET efficiency, E, is calculated as “acceptor intensity” divided by the sum of “donor and acceptor intensities” after correcting for the crosstalk between the donor and acceptor signals, ~15%.

Single-molecule FRET histograms in Figures 2, 3, and 5 are from more than 1000 molecules from multiple imaging areas. The width of individual peaks in the histograms is typically about 0.1 (full width at half maximum) and is mainly due to statistical and instrumental noise. The standard error is typically less than 0.01. E = 0 peak in FRET histograms (marked by vertical lines) is due to “donor-only” molecules for which the acceptor is not present or inactive.

The complex single-molecule traces arising from RecA binding and dissociation were analyzed by hidden Markov modeling, which is more reliable, reproducible, and less susceptible to human bias than traditional thresholding algorithms (McKinney et al., 2006). Recognizing that our system can be recast as a hidden Markov model, we used an optimizing Viterbi algorithm to determine the most likely combination of FRET states, noise, and transition rates for each trace. Though a certain number of distinct FRET values were expected, given our system, we did not impose it in our analysis. Instead, the algorithm was asked to find more than the number of expected states (for example, twice) and to determine those states’ noise (assumed to be a Gaussian sigma), their state-to-state transition probabilities, and the number of each transition type occurring in that trace. To speed up analysis, the Turing CPU cluster was used.

Supplemental Data
Supplemental Data include thirteen figures and experimental procedures and can be found with this article online at http://www.cell.com/cgi/content/full/126/3/515/DC1/.

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REFERENCES
Diez, M., Zimmermann, B., Borsch, M., Konig, M., Schweinberger, E., Steigmiller, S., Reuter, R., Felekyan, S., Kudryavtsev, V., Seidel, C.A.,


Real-Time Observation of RecA Filament Dynamics with Single Monomer Resolution

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Supplemental Experimental Procedures

DNA Preparation

ssDNA molecules were purchased from IDT DNA or were provided as a generous gift from Dr. T. Lohman. Labeling of dye and biotin at 5’ or 3’ end of ssDNA was done during DNA synthesis. In case of internally-labeled ssDNA, Cy3 (NHS-ester from GE Healthcare) was attached to the base of a dT through a C6 amino linker in order to keep the DNA backbone intact. Annealing of dsDNA was performed by heating a sample that contained two complementary ssDNA molecules at micromolar concentrations to 90 °C and slowly cooling for 2 hours at the room temperature. The sequence of biotinylated ssDNA forming the dsDNA is: 5’-GCC TCG CTG CCG TCG CCA-biotin-3’ and that of ssDNA with 3’ tail is 5’- TGG CGA CGG CAG CGA GGC -(tail)- 3’. The sequences of (dT)_{5+54}, (dT)_{10+49} and (dT)_{13+46} are 5’ TGG CGA CGG CAG CGA GGC-T5-T*-T53-3’, 5’ TGG CGA CGG CAG CGA GGC-T10-T*-T48-3’ and 5’ TGG CGA CGG CAG CGA GGC-T13-T*-T46-3’ where T* stands for an amine-modified dT with Cy3 labeled.

Protein-Repelling Surface

All the experiments were carried out on quartz slides, which were coated by polyethylene glycol (PEG) in order to minimize any possible interaction between a protein and a surface. Quartz slides and coverslips were cleaned as follows. The slides were sonicated in a glass staining dish for 20 min in 10 % alconox, 5 min in water, 15 min in acetone and 20 min in 1 M KOH. The coverslips were sonicated in another glass staining dish for 20 min in 1 M KOH. Then, the slides and the coverslips were burned by a propane torch for one minute and a few seconds, respectively. Finally, both were placed back in the previous glass staining dishes and stored in methanol until the next step.

The slides and coverslips were next amino-modified as follows. In a flask which was cleaned with methanol in 5 minute sonication, 100 ml of methanol (Fisher, ACS grade), 5 ml of acetic acid (Fisher, ACS grade) and 1 ml of aminosilane (A0700, UCT) were mixed. The methanol in the glass staining dishes was replaced by the mixture. They were incubated for 20 minutes at room temperature and sonicated for 1 minute during the incubation. Then, the solution was replaced by methanol and the slides and the coverslips were stored under the condition until next step.

The amine-modified surface of the slides and the coverslips was polymer-coated by amine-reactive PEG as follows. The slides and the coverslips were rinsed by water (MilliQ, 18.5 MΩ) and placed well-leveled. 70 µl of the following solution was dropped on each slide and the coverslips were laid over them; the solution was made by dissolving 0.2 mg of biotin-PEG (BIONHS-5000, MW 5000, Nektar Therapeutics) and 16mg of mPEG (M-SPA-5000, MW 5000,
Nektar Therapeutics) in 64 µl of freshly made 0.1 M sodium bicarbonate, pH 8.5 and by centrifuging it for a minute to remove bubbles. Then the overlapped slides and coverslips were incubated in dark and humid for 2–3 hr. Finally, they were disassembled, rinsed with water (MilliQ) and stored in dark and dry at −20°C until use.

**Fluorescence and Absorption Spectra**
The spectra of 50 nM partial dsDNA molecules with (dT)$_{21}$ and (dT)$_{13}$ tail labeled with Cy3 and/or Cy5; and 10 nM 77mer ssDNA (5’ TGG CGA CGG CAG CGA GGC-T13-T*-T45 3’, T* is amine-modified dT with Cy3 labeled) were measured by Cary Eclipse (Varian) in the identical chemical condition as single-molecule measurements after 10 min incubation at the room temperature. The fluorescence signal was recorded by exciting with 540 nm (640 nm in case of directly exciting Cy5) light and by collecting signal from 550 to 750 nm (650 to 750 in case of directly exciting Cy5). The recorded fluorescence signal was corrected for the wavelength-dependent sensitivity of the detector. The absorption spectrum was measured by Cary Bio 100 (Varian) under the identical condition.

**ATPase Rate Measurement**
50 nM of labeled 77mer ssDNA molecules (5’ TGG CGA CGG CAG CGA GGC-T13-T*-T45 3’, T* is amine-modified dT with Cy3 labeled) and unlabeled DNA (5’ TGG CGA CGG CAG CGA GGC -T59 3’) were, respectively, mixed with 1.3 µM RecA and 2 mM ATP in the identical chemical condition as single-molecule measurements at room temperature. ATPase activity was measured by EnzChek kit (Invitrogen) based on the increase in absorption at 360 nm, which was detected by Cary Bio 100 (Varian). The rates were determined by fitting the change of absorption in the first minute. The measurement was repeated three times for each sample.

**DNase I Footprinting**
600 nM of a partial dsDNA molecules with (dT)$_{13}$ tail was incubated with RecA and 1 mM cofactor (ATP$_{γ}$S or ATP) for a given time, in a buffer that contains 2.5 mM MgCl$_2$, 0.5 mM CaCl$_2$, 1% v/v 2-mercaptoethanol, and 10 mM Tris-HCl (pH 7.6). Next, 10 µl of the solution was cleaved by 0.05 U/µl of DNase I (New England Biolabs) for 20 seconds, which was stopped by adding SDS, EDTA and tRNA (0.3%, 70 mM and 1 µg/ml in final). The solution was ethanol-precipitated, then, the DNA-pellet was dissolved in 10µl formamide. It was run in 12% denaturing PAGE for 2 hr (20W) and imaged in a fluorescence imager (FLA-3000, Fujifilm) with 633 nm light exciting Alexa647 that is attached to the dsDNA.

**Interaction between a Fluorophore and a RecA Filament**

**Effects of a RecA Filament on Fluorescence**
A fluorescence spectrum of a partial dsDNA with (dT)$_{13}$ ssDNA tail that was labeled with Cy3 and Cy5 (Figure S2A) was measured in ensemble (Supplemental Experimental Procedures). While DNA alone shows high FRET (red in Figure S2Ba), introduction of RecA with ATP$_{γ}$S leads to 60% decrease in FRET (green in Figure S2Ba) due to the filament formation.

To study an effect of a RecA filament on Cy3 (donor), the measurement was repeated with Cy3-only sample (Figure S2Bb). Cy3 attached at the end of ssDNA tail became 70% brighter when the RecA filament formed with ATP$_{γ}$S, which has been also observed in single-molecule measurements (Figures S3C-D). The increase in fluorescence signal is likely due to conformational constraint imposed by a protein nearby which decreases the non-radiative decay
rate and consequently increasing the fluorescence quantum yield of Cy3. While the donor quantum yield change does affect the absolute FRET efficiency, it does not affect the apparent FRET efficiency we and other typically use for single molecule FRET analysis as shown below.

The donor quantum yield \( \phi_D \) is given by \( \phi_D = \frac{k_r}{k_r + k_{nr}} \), where \( k_r \) is the radiative decay rate and \( k_{nr} \) is the non-radiative decay rate. The apparent FRET efficiency \( E \) is given by

\[
E \equiv \frac{I_A}{I_A + I_D} = \frac{\eta_A I_A^0}{\eta_A I_A^0 + \eta_D I_D^0},
\]

where \( I_D \) and \( I_A \) are the measured intensities of donor and acceptor molecules, \( I_D^0 \) and \( I_A^0 \) are the true intensities of donor and acceptor emission, \( \eta_D \) and \( \eta_A \) are the detection efficiencies of donor and acceptor signals determined by instrumentation. Since

\[
I_D^0 \propto \frac{k_r}{k_{ET} + k_r + k_{nr}} \quad \text{and} \quad I_A^0 \propto \phi_A \frac{k_{ET}}{k_{ET} + k_r + k_{nr}} \quad \text{where} \ k_{ET} \text{ is the energy transfer rate and}
\]

\( \phi_A \) is the acceptor quantum yield, we obtain

\[
\frac{I_D^0}{I_A^0} = \frac{k_r}{\phi_A k_{ET}}. \]

Finally, inserting this into the formula for \( E \), we obtain

\[
E = \frac{1}{1 + (\eta_D / \eta_A)(k_r / \phi_A k_{ET})} \text{ which is independent of changes in the non-radiative decay rate of the donor, } k_{nr}.
\]

In addition, the RecA filament formation did not cause any change in the fluorescence emission spectrum of Cy3. The same measurement was carried out with a ssDNA internally-labeled with Cy3 (that was used in Figure 2A), with which we again observed the increase in fluorescence signal (50%) but no change in the emission spectrum (Figure S2Eb).

A similar measurement was carried out by directly exciting Cy5 (acceptor) that is positioned at the junction of ss-dsDNA (Figure S2Bc). We observed a decrease of the signal (30%), smaller than that in FRET (60%), when RecA formed a filament with ATP\(_\gamma\)S. One possible reason for the fluorescence decrease is photobleaching of Cy5 which may be enhanced by the filament formation on the ssDNA as well as dsDNA. The photobleaching frequently showed up in single-molecule traces in the same condition (Figures S2C). We can easily distinguish such photobleaching \( (E = 0) \) from a normal active state of Cy5 \( (E > 0) \) in single-molecule traces based on a FRET value. Regardless of the source of Cy5 signal reduction, our data analysis on FRET trajectories do not rely on the absolute values of FRET but rather on its relative changes. Therefore, the kinetic rates measured 1) from all-or-none events of a nucleation cluster (Figure 1), (2) from stepwise change in FRET (Figures 2–4) and (3) from gradual change in FRET (Figure 5) would not be influenced by the decrease in Cy5 signal.

As shown in single-molecule traces taken with (dT)\(_{13}\) and (dT)\(_{21}\) tail (Figures S2C and S2D), the low-FRET state which results from a RecA filament formed with ATP\(_\gamma\)S is distinguished from photobleaching or photobleaching of Cy5 by the difference in FRET \( (E > 0 \text{ versus } E = 0) \). We further checked the activity of Cy5 by exciting Cy5 directly using a 633 nm laser (Figure S2D).

When absorption spectra were measured, there was only slight change observed (Figures S2Bd, Ec), indicating insignificant influence of a RecA filament on the absorption properties.
An Effect of a Dye on a RecA Filament

We have measured ATPase activity with 77mer ssDNA (Supplemental Experimental Procedures) ATP hydrolysis rates were 0.14 ± 0.00 and 0.14 ± 0.01 (sec⁻¹ RecA⁻¹) with labeled and unlabeled ssDNA, respectively, indicating that the dye (Cy3) positioned in the middle of DNA does not affect ATPase activity of a RecA filament. The minimal influence of organic dyes on a RecA filament were reported previously (Bazemore et al., 1997; Gourves et al., 2001; Xiao and Singleton, 2002). We could also carry out RecA-mediated homologous strand exchange with a ssDNA that was internally-labeled in a single-molecule assay (unpublished observation).

Filament Formation on a Short ssDNA
The Shorter the ssDNA, the Higher the FRET Efficiency

When ssDNA tail (10–17 nt) of a partial dsDNA (Figure S3A) is free from a RecA filament, FRET efficiency is high ($E > 0.7$) and slightly depends on the length of the ssDNA (Figure S3B, gray). When a RecA filament forms with ATPγS, FRET efficiency drops sharply (Figure S3B, black). The new FRET values strongly depend on the length of the ssDNA—the longer the ssDNA, the lower the FRET efficiency, reflecting the difference in the contour lengths of the ssDNA.

RecA Filament Formation with ATP

Figure S3C shows traces taken with (dT)₂₁ tail in the same condition as Figure 1D. A fraction of filament assembly/disassembly events are slow enough to display intermediate FRET values during transition (Figures S3Cc and S3Cd).

The change in FRET is well-correlated with the change in total fluorescence intensity as has been discussed above (Figure S2Bb). When a two-dimensional density plot is made for $E$ vs. “fluorescence intensity,” the anticorrelation between FRET and intensity is clearly visualized. This anti-correlated property was carefully considered in analyzing traces, especially if FRET is too low ($0 < E \ll 1$) to distinguish from an inactivated state of acceptor molecules ($E = 0$).

RecA Filament Formation with Different Types of NTP Factors

(dT)₂₁ ssDNA tail free from a RecA filament shows high FRET ($E \sim 0.55$) (Figures S4Ba and 1Ca), while it shows low FRET ($E \sim 0.1$) with the filament (Figures S4Bb and 1Ca). When dATP is used as a NTP factor, a stable filament forms (Figures S4Bc; arrows) in contrast to the brief filament formation with ATP (Figures S4Ba) (Menetski and Kowalczykowski, 1989). The single molecule trace also shows more stable low-FRET states (Figure S4Ca). A similar property was observed with (dT)₁₇ tail (Figure S4Cb) while the filament formation was rarely observed with ATP (Figure 1Eb).

In the absence of NTP factors, RecA still forms a filament but in a collapsed form with a lower pitch than usual (Bell, 2005; Story et al., 1992). The FRET efficiency ($E \sim 0.3$) is higher than that with ATPγS ($E \sim 0.05$) possibly reflecting the lower pitch (Figure S4Bd). The broad distribution in a FRET histogram is due to two-state fluctuation between $E \sim 0.3$ (filament) and ~0.55 (no filament) (Figure S4Cc).

Disassembly of <ssDNA|ATPγS>

After <ssDNA|ATPγS> forms around a short oligonucleotide by incubating with RecA and ATPγS, if we remove RecA and ATPγS from solution, the filament disassembles (Figure 3Ac). We found that, if ATPγS is kept in solution, single-molecule traces recorded during this
disassembly visualize individual states ($M_i$) of $(dT)_{13}$ since monomers dissociate slowly one by one (Figure S8A). Among the molecules that exhibit a monotonic and stepwise change in FRET, we could count up to ~5 states as shown in an example time trace (Figure S8Ba), consistent with expectation from the ratio between RecA and nucleotides. Molecules with 4 states are also observed with a similar frequency (Figure S8Bb). The same experiment was carried out with $(dT)_{10}$ tail that showed four different states in maximum (Figure S8Ca), while there were a similar number of molecules that show three states only (Figure S8Cb). Not all the molecules show a monotonic and/or stepwise change in FRET, therefore, it should be noted that this assay is only for counting the maximum number of FRET states but not for understanding the disassembly process of <ssDNA|ATPγS>.

Filament Formation around a dsDNA Mediated by ssDNA Tail

Filament Formation on dsDNA Is Not Local to the ss-dsDNA Junction

In order to test whether <dsDNA|ATPγS> forms all over the dsDNA or only a part of it (e.g. only near the junction of ss-dsDNA), we relocated Cy5 at the junction (Figure 3B) into the middle of the dsDNA as shown in Figure S9A. (The sequence of the ssDNA that is labeled with biotin and Cy5 is 5’-GCCTCGC T* GCCGTCGCCA-biotin-3’, where T* stands for an amine-modified dT with Cy5 labeled.) While $E \sim 0.6$ without a RecA filament, $E$ becomes as low as ~0.4 when RecA and ATPγS are added (Figure S9Ab). Since the change in FRET reflects a filament formation over a portion of dsDNA that is far from the junction and since <dsDNA|ATPγS> cannot form with a blunt end of the dsDNA (Figure S9C), <dsDNA|ATPγS> is not likely to form only near the junction, but rather to form over the entire length of 18 bp duplex DNA.

<dsDNA|ATPγS> Protects dsDNA from Digestion by an Endonuclease

DNase I footprinting was carried out to check the presence of <dsDNA|ATPγS> using an independent, non-FRET based method. A dsDNA with 3’ (dT)$_{13}$ tail was labeled with a dye at the junction (Figure S9B). The DNA was incubated with RecA and ATPγS, which was followed by cleavage by DNase I (Supplemental Experimental Procedures). When the DNA was incubated with RecA and ATPγS for a different amount of time (lanes 1–5 in Figure S9Bb) before cleavage reaction, a different degree of cleavage activity was observed—the longer incubation, the less cleavage. Similarly, when the DNA was incubated with RecA and ATPγS for 10 minutes but with different RecA-concentrations, the less cleavage reaction was observed with the larger amount of RecA (lanes 8–11). Such protection of dsDNA by RecA was not observed when ATP was used (lane 7). Therefore, this footprinting assay further supports the formation of a filament around dsDNA with ATPγS mediated by 3’ ssDNA tail, which has not been reported previously.

<dsDNA|ATPγS> Cannot Form without ssDNA Tail

When the same DNA as that in Figure 3B but without ssDNA tail (Figure S9C) was incubated with RecA and ATPγS, we could not observe any filament formation around dsDNA for up to 2 hours (Figures S9Cc). This rules out the possibility that <dsDNA|ATPγS> which formed in a 3’ tailed DNA was due to the dyes acting as nucleation sites, as the blunt dsDNA used for the control here also contained the dyes in the same location but did not show evidence of filament formation.
Formation and Disassembly of <dsDNA|ATPγS> at High Temperature (35°C)
The same experiment as Figure 3 was carried out at 35°C instead at the room temperature (Figure S10). The formation of <ssDNA|ATPγS> was observed when RecA and ATPγS were added in the same way as at the room temperature, resulting in a transition from high to low FRET (Figures S10Aa and S10Ab). When RecA and ATPγS were removed from solution, the disassembly of <ssDNA|ATPγS> was observed as at the room temperature but at a faster rate (<1 min) (Figures S10Ac and S10Ad). Finally, when RecA and ATP were added after 10 minute-incubation in the previous condition (no RecA and ATPγS in solution at 35°C), only ~50% of the molecules in the high-FRET state shifted into the low-FRET state (Figures S10Ae and S10Af). Therefore, <dsDNA|ATPγS> is less stable at 35°C than at the room temperature. Note that <dsDNA|ATPγS> is still more stable than <ssDNA|ATPγS> at 35°C for a short 3′ tail (13 nt).

Similarly, the formation of <dsDNA|ATPγS> was directly observed at 35°C (Figure S10Ba). A half of the molecules lost the filament 10 minutes after RecA and ATPγS were removed from solution at 35°C (Figure S10Bb) consistent with the result above (Figure S10Af).

Formation of <dsDNA|ATPγS> Mediated by 5′ ssDNA Tail
The same experiment as shown in Figure 3 was carried out with a dsDNA with 5′ (dT)20 ssDNA tail, instead of 3′ tail (Figure S11). When RecA and ATPγS were added, a filament formed (Figures S11Aa and S11b) in less than half a minute. When RecA and ATPγS were removed from solution, the disassembly of <ssDNA|ATPγS> was observed in a similar time scale as that with 3′ tail (~5 min) (Figures S11Ac, d). Finally, when RecA was added with ATP, most of the population in the high-FRET state was shifted into low FRET (Figures S11Ae and S11Af). Such transition was not observed in the absence of <dsDNA|ATPγS> (Figure S11B).

However, a small population were in high and intermediate FRET (Figure S11f, arrows), the origin of which is revealed when single-molecule traces are analyzed. As shown in Figure S11Ag, E fluctuates between high and low FRET dynamically. This large fluctuation is in clear contrast with a trace recorded with 3′ (dT)19 tail (Figure S11C), where E always stays low. This difference between 3′ and 5′ tails, observed in FRET histograms and single-molecule traces, is anticipated since <ssDNA|ATP> on 5′ tail corresponds to the unstable 5′-disassembly end of a filament ($K_D \sim 100$ nM), while <ssDNA|ATP> on 3′ tail corresponds to the stable 3′-extending end ($K_D \sim 8$ nM). Conversely, it implies that the polarity of <dsDNA|ATPγS>, which appears to act as a nucleation cluster, is not random but rather follows that of <ssDNA|ATPγS>.

Formation of <dsDNA|ATPγS> was directly observed by attaching the dye pair at two ends of the dsDNA (Figure S11Da) and <dsDNA|ATPγS> remained stable in the absence of RecA and ATPγS for ~2 hr until it started to disassemble after ~3 hr (Figure S11Db).

Extension Speed of a RecA Filament

RecA Extends a Filament Swiftly, Regardless of the Presence of SSB
As shown in Figure 5Cd, RecA forms a filament displacing SSB with a rate of 0.55 (sec⁻¹). We carried out a similar experiment as Figure 5B but removed DNA-bound SSB before adding RecA and ATP in order to find out how much SSB influences the formation/extension of a RecA filament. SSB was removed efficiently by adding 100 nM of 80-mer ssDNA (Figure S12Ae), which restored the FRET histogram into that of DNA-only ($E \sim 0.1$) (Figure S12Ab). When RecA and ATP were added, FRET became even lower (Figure S12Af). The transitions are shown in Figure S12B. After RecA was added at t = 0, FRET dropped from $E \sim 0.1$ to 0 through
two different ways; 1) FRET becomes high briefly, right before $E$ becomes 0 (Figure S12Ba), or 2) it changes monotonically (Figure S12Bb). The origin of the brief high-FRET state is not known at this point. The time taken for the transition was measured from 55 molecules, which gave the transition rate of $\sim 0.8$ (sec$^{-1}$) (Figure S12Bc), which is close to the value obtained in the presence of SSB, 0.55 (sec$^{-1}$) (Figure 5Cb). Note that the former rate is over-estimated since it reflects not only the filament extension but also the filament formation de novo, which makes the two values even closer. It suggests that SSB is removed by the extending RecA filament at the rate of regular filament extension, implying that the binding of SSB must be weak compared to the force exerted during extension of a RecA filament.

References
Figure S1. Single-Molecule Spectroscopy for FRET
Figure S2. Fluorescence and Absorption spectra of Cy3 and Cy5 That Are Conjugated to DNA

(A) Formation of a filament on a dsDNA with 3′ ssDNA tail
(B) (a) While (dT)\textsubscript{13} ssDNA tail alone shows high FRET (red), the filament formation with RecA and ATP\textsubscript{γ}S results in 60% decrease in FRET (green, after 10 min incubation) (b) The same DNA but with Cy3 only. Fluorescence of Cy3 becomes enhanced when a filament forms with ATP\textsubscript{γ}S, by 70% (after 10 min incubation). (c) Fluorescence signal of Cy5 decreases when the filament forms with ATP\textsubscript{γ}S, by 30% (after 10 min incubation) (d) Absorption spectra of Cy3 and Cy5 molecules without and with a RecA filament formed, with ATP\textsubscript{γ}S (after 10 min incubation)
(C) Time traces (100 ms time resolution) with RecA and ATP\textsubscript{γ}S shows photobleaching of Cy5 at t ~30–90 and ~95–130 s before photobleaching of Cy3 at t ~ 150 s (arrows).
(D) Time traces (100 ms time resolution) taken by alternative excitation of green and red laser light. A dsDNA with (dT)$_{21}$ tail was excited by 532 nm light for 9 seconds and by 633 nm for 1 second, alternatively. Until photobleaching of Cy5 at t ~ 32 (sec) (arrow), not only there is Cy5 signal by FRET ($E\sim0.1$) but also there is Cy5 signal when directly excited by 633 nm light. After the photobleaching of Cy5, there is neither FRET ($E\sim0$) nor Cy5 signal by direct excitation. 

(E) (a) Formation of a filament on a ssDNA with Cy3-labeled in the middle that was used in Figure 2A (b) Fluorescence of Cy3 becomes enhanced when a filament forms with ATP$_{\gamma}$S, by 50% (after 10 min incubation) (c) Absorption spectra of Cy3 molecule without and with a RecA filament formed, with ATP$_{\gamma}$S (after 10 min incubation).
Figures S3. Filament Formation on Short ssDNA Tail with ATP and ATPγS

(A) Formation of a filament on a dsDNA with 3’ tail.
(B) ssDNA-length dependent FRET efficiency without (gray) and with (black) RecA and ATPγS
(C) FRET and total-intensity trajectories (100 ms time resolution) from (dT)_{21} tail with RecA and ATP in solution. Top: FRET stays high (E ~ 0.55) most of the time but frequently becomes low (E ~ 0.1). Bottom: Accompanying the shift to E ~ 0.1 is an overall increase in the total fluorescence signal.
(D) 2-dimensional density plot of E vs. ‘fluorescence intensity’ from 114 molecules
Figure S4. Filament Formation on Short ssDNA Tail with Different Types of NTP Factors

(A) Formation of a filament on a dsDNA with 3′ tail.

(B) FRET histograms with (dT)$_{21}$ tail in the presence of RecA (a) with ATP (after 1 min incubation), (b) with ATP$_{γ}$S (after 1 min incubation), (c) with dATP (after 1 min incubation), and (d) without any NTP (after 1 min incubation).

(C) Time traces with a 100 msec time resolution (a) (dT)$_{21}$ tail with RecA and dATP in solution. Several low-FRET states are observed until photobleaching of Cy5 (arrow). (b) (dT)$_{17}$ tail with RecA and dATP. Formation of a nucleation cluster is observed several times (arrows). (c) (dT)$_{21}$ tail with RecA only. Frequent two-state transitions between $E \sim 0.3$ and $\sim 0.55$ are observed until Cy3 and Cy5 photobleach, respectively (arrows).
Figure S5. The Dynamics at the 5′-Disassembly End (I)

(A) For better comparison in kinetic rates, Figures 2Db, 2Fb, and 2Fd are re-arranged here. The rates and frequencies of transitions observed with (a) (dT)$_{5+54}$, (b) (dT)$_{10+49}$ and (c) (dT)$_{13+46}$ tails. (B) Time traces with (a) (dT)$_{5+54}$ and (b) (dT)$_{10+49}$ tails with RecA and ATP (200 ms time resolution). The fit by HMM is in green, overlaid on each FRET trajectory in black.
Figure S6. Corrected Dissociation Rates

Corrected dissociation rates based on the data in (A) Figure 2Eb and (B) Figure 4Cc. The errors are standard deviations of (A) three and (B) two data sets each.
Figure S7. The Dynamics at the 5′-Disassembly End (II)

(A) (a) A schematic of a dsDNA with (dT)$_{16+43}$ tail. (b) Time traces with RecA and ATP in solution (200 ms time resolution). They stay in low FRET, most of the time, with infrequent visit to high FRET. (c) A FRET histogram (after 1 min incubation).

(B) (a) A dsDNA with (dT)$_{19+35}$ tail (b) Time traces with RecA and ATP in solution (100 ms time resolution). They stay in low FRET, most of the time, with infrequent visit to high FRET. (c) A FRET histogram display a majority of the population in low FRET (after 1 min incubation).
Figure S8. Disassembly of <ssDNA|ATPγS>

(A) Disassembly of <ssDNA|ATPγS> from (dT)_{13} and (dT)_{10} ssDNA tails.
(B) After 30 min incubation with RecA and ATPγS, upon removal of RecA from solution but with ATPγS kept in solution, monotonic stepwise increase in FRET is observed with (dT)_{13}. Time traces show (a) five and (b) four different FRET states during disassembly.
(C) The same assay as (B) but with (dT)_{10} after 60 min incubation with RecA and ATPγS. Time traces show (a) four and (b) three different states during disassembly.
Figure S9. Formation of $\langle \text{dsDNA} | \text{ATPγS} \rangle$

(A) (a) Filament formation on a dsDNA with 3' (dT)$_{20}$ ssDNA tail. Cy5 is labeled in the middle of a dsDNA instead of at the junction of ss-dsDNA. (b) Change in a FRET histogram when RecA is added with ATPγS indicates a filament formation on the dsDNA (after 15 min incubation).

(B) Protection of dsDNA by $\langle \text{dsDNA} | \text{ATPγS} \rangle$ from an endonuclease (a) dsDNA with 3' (dT)$_{13}$ tail was labeled with Alexa647 at the junction. It is incubated with RecA and ATPγS before cleavage by DNase I. (b) The 600nM DNA molecules were incubated with 10 µM RecA and 1 mM ATPγS for 15, 30, 60, 120, and 300 seconds before cleavage (lanes 1-5). The DNA (600 nM) was incubated with 10 µM RecA and 1 mM ATP (lane 7); and with 0, 2, 6, and 10 µM RecA and 1 mM ATPγS, for 10 minutes before cleavage (lanes 8-11). Lane 6 is for a DNA without any RecA and DNase I.

(C) (a) No filament formation on a dsDNA with blunt ends, which is labeled with Cy3 and Cy5. FRET histograms (b) without RecA and (c) with RecA and ATPγS in solution (after 2 hr incubation).
Figure S10. Formation and Disassembly of $<\text{dsDNA}|\text{ATP}_\gamma\text{S}>$ at 35°C

(A) The same experiment as Figure 3A but at high temperature (35°C). (a and b) Filament formation on a 3′-(dT)$_{13}$ tail of a partial dsDNA results in low FRET (green, $E \sim 0.2$, after 30 min incubation), while DNA-only shows high FRET (white, $E \sim 0.75$). (c and d) $<\text{ssDNA}|\text{ATP}_\gamma\text{S}>$ disassembles after RecA and ATP$_\gamma$S are removed from solution thus FRET is restored to the DNA-only value (Ab, white) (after 10 min incubation). (e and f) After 10 min incubation in the condition of (Ac), RecA and ATP are added and a stable $<\text{ssDNA}|\text{ATP}>$ forms on ~50% of the molecules, assisted by $<\text{dsDNA}|\text{ATP}_\gamma\text{S}>$ (after <1 min incubation).

(B) The same experiment as Figure 3B but at high temperature (35°C). (a) Filament formation on a dsDNA with (dT)$_{20}$ tail results in $E \sim 0.05$ (green, after 30 min incubation) compared to $E \sim 0.2$ of DNA-only (white). (b) About a half of $<\text{dsDNA}|\text{ATP}_\gamma\text{S}>$ disassembles during the first 10 min after removal of RecA and ATP$_\gamma$S from solution (after 10 min incubation).
Figure S11. Formation and Disassembly of \(<\text{dsDNA}|\text{ATPγS}>\) Mediated by 5'-Tail ssDNA

(A) (a and b) Filament formation on 5'-(dT)₂₀ ssDNA tail of a partial dsDNA results in low FRET ($E \approx 0.05$, green, after 30 min incubation), while FRET is high with DNA only ($E \approx 0.6$, white). (c and d) \(<\text{ssDNA}|\text{ATPγS}>\) disassembles in 5 minutes after RecA and ATPγS are removed from solution (after 30 min incubation). (e and f) After 30 min incubation in the condition of (Ac), when RecA and ATP are added, \(<\text{ssDNA}|\text{ATP}>\) forms assisted by \(<\text{dsDNA}|\text{ATPγS}>\) (after <1 min incubation). (g) Time trace (100 ms time resolution) in the condition of (Af). Cy3 photobleached at $t \sim 30$ sec (arrow).
(B) A FRET histogram when RecA and ATP are added, in the absence of $\langle\text{dsDNA}|\text{ATP}\gamma\text{S}\rangle$ (after 1 min incubation). Longer incubation does not change the shape of the distribution.

(C) (a) Time trace (100 ms time resolution) in the same condition and configuration as (Ag) but with $3^\prime$-(dT)$_{19}$ tail. Cy3 photobleached at t $\sim$ 120 s (arrow). (b) A FRET histogram with $3^\prime$ (dT)$_{19}$ tail. In contrast to (Af), no intermediate or high FRET states are observed.

(D) (a) Filament formation on the dsDNA with (dT)$_{20}$ tail results in $E \sim 0.05$ (green, after 30 min incubation) compared to $E \sim 0.2$ of DNA only (white). (b) It takes more than 2 hr for $\langle\text{dsDNA}|\text{ATP}\gamma\text{S}\rangle$ to disassemble after RecA and ATP$\gamma$S are removed from solution.
Figure S12. Formation of \(<\text{ssDNA|ATP}>\) in the Presence of \(<\text{dsDNA|ATPγS}>\)

(A) (a) Extension of a RecA filament from \(<\text{dsDNA|ATPγS}>\) into \(<\text{ssDNA|ATP}>\) in the absence of DNA bound SSB (b–d) The same procedures as Figures 5Bb–5Bd (e) When 100 nM \((\text{dC})_{80}\) is introduced, SSB is transferred to \((\text{dC})_{80}\) within a minute, leaving ssDNA tail naked but keeping \(<\text{dsDNA|ATPγS}>\) intact (after 2 min incubation). (f) When RecA and ATP are added in solution, a filament extends rapidly assisted by \(<\text{dsDNA|ATPγS}>\) (after \(<1\) min incubation).

(B) (a and b) Time traces recorded during the transition from (Ae) to (Af). RecA and ATP are added at \(t = 0\). To distinguish the low FRET \((E \sim 0)\) from photobleaching of Cy5, the activity of acceptor was checked by directly exciting acceptor at \(t > 12\) s (arrows). (c) The transition rate is \(0.8\) (sec\(^{-1}\)), determined from 55 molecules.
Figure S13. <ssDNA|ATPγS> Is Removed by SSB

(A) The same procedures as Figures 5Bb–d. <ssDNA|ATPγS> is displaced by SSB when RecA and ATPγS are removed from solution and 10 nM SSB is added. (B) The change in the population of SSB bound ssDNA is calculated from the change in FRET value. Each data point is based on the normalized average FRET value from more than 100 molecules (recording for 2 seconds). Different areas were imaged for individual data points. t = 0 when SSB is introduced and RecA is removed from solution.