LETTERS

Direct observation of individual RecA filaments assembling on single DNA molecules

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Escherichia coli **RecA is essential for the repair of DNA double-strand breaks by homologous recombination[1]. Repair requires the formation of a RecA nucleoprotein filament. Previous studies have indicated a mechanism of filament assembly whereby slow nucleation of RecA protein on DNA is followed by rapid growth[2–7]. However, many aspects of this process remain unclear, including the rates of nucleation and growth and the involvement of ATP hydrolysis, largely because visualization at the single-filament level is lacking. Here we report the direct observation of filament assembly on individual double-stranded DNA molecules using fluorescently modified RecA. The nucleoprotein filaments saturate the DNA and extend it 1.6-fold. At early time points, discrete RecA clusters are seen, permitting analysis of single-filament growth from individual nuclei. Formation of nascent RecA filaments is independent of ATP hydrolysis but is dependent on the type of nucleotide cofactor and the RecA concentration, suggesting that nucleation involves binding of 4–5 ATP–RecA monomers to DNA. Individual RecA filaments grow at rates of 3–10 nm s^{-1}. Growth is bidirectional and, in contrast to nucleation, independent of nucleotide cofactor, suggesting addition of 2–7 monomers s^{-1}. These results are in accord with extensive genetic and biochemical studies, and indicate that assembly *in vivo* is controlled at the nucleation step. We anticipate that our approach and conclusions can be extended to the related eukaryotic counterpart, Rad51 (ref. 8), and to regulation by assembly mediators[9–11].**

Recombinational DNA repair involves the formation of a RecA nucleoprotein filament typically on single-stranded DNA (ssDNA) for pairing and exchange with homologous double-stranded DNA (dsDNA)[8]. Although the kinetically preferred substrate is ssDNA, RecA can form filaments on dsDNA[2,3] that are active in 'inverse' DNA strand exchange with complementary ssDNA[12,13]. Previous studies indicated that filament assembly involves nucleation of RecA onto DNA followed by elongation (Supplementary Fig. 1), but direct visualization of the time-dependent evolution of an individual RecA nucleoprotein filament has been lacking. To visualize RecA filament formation directly, we prepared a fluorescently modified RecA protein that retains all biochemical activity (Supplementary Figs 2–4). This protein was used to monitor RecA filament formation on dsDNA using the following strategy[14]. A flow cell is used to generate three separate laminar flow channels (Fig. 1a). Biotinylated bacteriophage λ DNA, attached to streptavidin-coated beads (1 μm), is optically trapped in the capture channel. To ensure that the single DNA molecule is at full length, trapping is performed using the fluorescent DNA-binding dye YOYO-1. The trapped bead–YOYO-1–DNA complex is then moved into a second channel (the observation channel) under conditions that dissociate the dye from DNA. Figure 1b (panels 1 and 2) shows a λ DNA molecule in the observation channel before and after dissociation of YOYO-1, respectively. This dye-free DNA is subsequently moved to a third channel (RecA channel) containing the fluorescently modified RecA. After incubation to allow for the formation of a RecA filament,

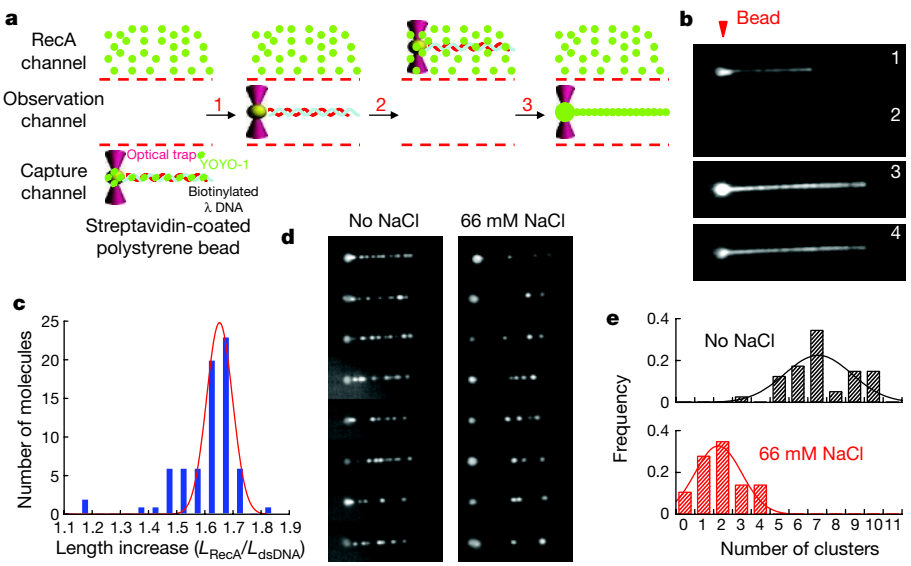

Figure 1 | Nucleation and growth of RecA filaments visualized on individual dsDNA molecules. **a**, Experimental approach (see the text for details). **b**, Panels 1 and 2 show a bead–DNA complex trapped before (panel 1) and after (panel 2) dissociation of YOYO-1. Panel 3 shows a fluorescent filament in the observation channel after 5 min in the RecA channel with 500 nM RecA, 0.5 mM ATP-γS and 1 mM $Mg(OAc)_2$. Panel 4 shows the same RecA filament after ~20 min in the observation channel with continued flow, no illumination. **c**, Relative extension for RecA filaments ($n = 72$); the line is a gaussian fit. **d**, Eight different nascent RecA filaments after incubation for 1 min with 200 nM RecA, 0.5 mM ATP-γS, 1 mM $Mg(OAc)_2$ and indicated NaCl concentration. **e**, Number of clusters observed; lines are gaussian fits.

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the trapped DNA is moved back into the observation channel where the bound fluorescent RecA is visualized directly.

After incubation in the RecA channel for 5 min in the presence of ATP-γS, a complete RecA nucleoprotein filament is visible in the observation channel (Fig. 1b, panel 3). The nucleoprotein filament is extended relative to naked dsDNA, consistent with the extension induced by RecA[15]. The size distribution for the relative extension of DNA by RecA (Fig. 1c) is approximately gaussian, with an average extension of 1.65 ± 0.05 ($n = 72$). Given that the persistence lengths of dsDNA and RecA nucleoprotein filaments differ greatly[4,5,15], the observed extension is consistent with the polymorphic ~1.5-fold increase in dsDNA length reported, and indicates that complete RecA filaments are formed. The length of the RecA nucleoprotein filament does not change after 20 min of continued flow in the RecA- and ATP-γS-free observation channel (Fig. 1b, panel 4), even in the presence of NaCl or ATP (Supplementary Fig. 5), indicating that dissociation of the ATP-γS–RecA–dsDNA complex is negligible.

Although our protocol does not permit the monitoring of RecA filament formation in real time due to the high background fluorescence in the RecA channel, snapshots of filament formation on the same DNA molecule can be obtained by incubating the DNA in the RecA channel for a short time (dipping), and then moving the DNA to the observation channel. Examples of eight partial filaments formed by dipping in the RecA channel for 1 min are shown in Fig. 1d. It is evident that multiple RecA clusters have formed on the dsDNA, showing directly that RecA filaments form from multiple nucleation events. The frequency of RecA nucleation is strongly dependent on salt concentration (Fig. 1e). From 20 to 80 mM NaCl, the average rate of RecA cluster formation decreased from ~8 to ~1 min^{-1} (Supplementary Fig. 6); given that λ DNA is 48,502 base pairs (bp), the average nucleation frequency ranged from $\sim 1.6 \times 10^{-4}$ to 2×10^{-5} nuclei $min^{-1}\,bp^{-1}$. Previous measurements, at different conditions, produced comparable values[4,16,17], but, as shown below, nucleation frequency is strongly influenced by reaction conditions. The affinity of RecA for DNA is highly sensitive to salt concentration[18,19], suggesting that nucleation is occurring on the DNA. This conclusion is in agreement with a previous ensemble study[7] and is consistent with the DNA composition-dependence and nucleotide-dependence presented below. Furthermore, even though nucleation was seemingly equally likely along the length of the dsDNA at the lowest NaCl concentration (Supplementary Fig. 7), nucleation occurred more frequently and preferentially at (A+T)-rich regions of the λ DNA when the NaCl concentration was elevated (Supplementary Figs 8–10). Ensemble studies showed that (A+T)-rich duplex DNA stimulates nucleoprotein filament assembly, which was attributed to a rate-limiting nucleation of RecA onto one strand of the dsDNA[2].

The affinity of RecA for DNA is strongly influenced by the nature of the bound nucleotide cofactor[18,19]. Although ATP-γS–RecA clusters are stable for long times in the observation channel, filaments formed with ATP completely dissociated in ~6 min after a lag of ~10–20 s (our own unpublished observations). However, if the ATP–RecA clusters are moved into an observation channel containing ATP-γS, then they are completely stabilized (Fig. 2a). The average number of clusters formed with ATP-γS or ATP, under otherwise identical experimental conditions, is shown in Fig. 2b. With ATP-γS, RecA nucleates ~5-fold faster than with ATP (10.2 ± 0.6 min^{-1} compared with 1.86 ± 0.06 min^{-1}), consistent with previous modelling[4]. The difference in nucleation rate cannot be attributed to a different degree of saturation of RecA with ATP or ATP-γS due to differences in affinity, because identical rates are obtained when a fourfold higher concentration of ATP is used (Fig. 2b, orange diamonds). However, the difference might be the consequence of ATP-hydrolysis-induced dissociation. To address this point, Ca^{2+} was substituted for Mg^{2+} in the RecA channel, because Ca^{2+} reduces the rate of ATP hydrolysis by 96%[18]. Although Ca^{2+} decreases the rate of nucleation, the same ~5-fold difference in rate is maintained when ATP-γS replaces ATP (Fig. 2b). Thus, the nucleation frequency is not sensitive to ATP hydrolysis but rather is sensitive to the differences in affinity for DNA that are induced by nucleotide cofactors and metal ions[18,19]. This conclusion, together with the increased nucleation frequency in (A+T)-rich regions (Supplementary Figs 7–10), further implies that nucleation occurs on the DNA.

The effect of RecA concentration on nucleus formation is shown in Fig. 2c. The rate of cluster formation, determined from the linear increase in nucleation frequency with time, is shown as a function of RecA concentration in Fig. 2d. A pronounced nonlinear dependence on RecA concentration is evident. The solid line is the fit according to the relationship $k_{obs} = c[\mathrm{RecA}]^n$, where n represents the minimum number of RecA molecules involved in the cooperative process and c is a constant[20]. This simplistic approach suggests that formation of a stable nucleus on dsDNA requires at least four RecA molecules ($n = 4 \pm 0.4$). Notably, the same dependence ($n = 4.5 \pm 0.5$) is observed with ATP (Fig. 2d). Although the same power dependence is maintained, the entire curve is shifted towards higher RecA concentrations, consistent with the slower nucleation rate of ATP–RecA. This simple analysis suggests that 4–5 RecA molecules are needed to form a stable nucleus.

At high concentrations of salt, the nucleation frequency is strongly limited and growth from an individual cluster can be monitored via successive incubations in the RecA channel (Figs 3 and 4). An individual DNA molecule was dipped for 1 min in the ATP-γS–RecA channel until clusters were detected (Fig. 3a, 0 min). Then, the molecule was repeatedly moved into the RecA channel for a fixed time, and back out to the observation channel to follow growth of the initial clusters (Fig. 3a, 1–9 min). The size of the two clusters in the top panel (0 min) increases linearly with observed rates of 3.3 ± 0.1

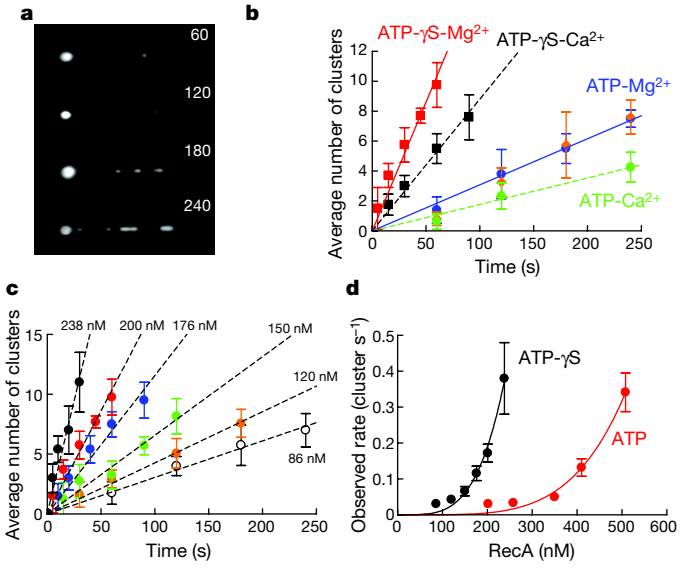

Figure 2 | Nucleation of RecA filaments is independent of ATP hydrolysis but dependent on the type of nucleoside triphosphate and the RecA concentration. **a**, Nascent RecA filaments formed by incubation of different DNA molecules with 176 nM RecA, 0.5 mM ATP and 1 mM $Mg(OAc)_2$ for the indicated times (seconds), and visualized in the observation channel containing 0.5 mM ATP-γS. **b**, Time dependence of cluster appearance after incubation in the RecA channel with 176 nM RecA and: (red squares) 0.5 mM ATP-γS, 1 mM $Mg(OAc)_2$; (black squares) 0.5 mM ATP-γS, 1 mM $CaCl_2$; (blue circles) 0.5 mM ATP, 1 mM $Mg(OAc)_2$; (green circles) 0.5 mM ATP, 1 mM $CaCl_2$; and (orange diamonds) 2 mM ATP, 2.5 mM $Mg(OAc)_2$. Each time point is the average of 4–12 individual molecules. **c**, Time dependence of cluster appearance in 0.5 mM ATP-γS and 1 mM $Mg(OAc)_2$ at different RecA concentrations. **d**, Rate of cluster formation as a function of RecA concentration for either ATP-γS (black circles) or ATP (red circles); the standard deviation for some points is smaller than the symbol size. Error bars in **b**–**d** indicate standard deviation.

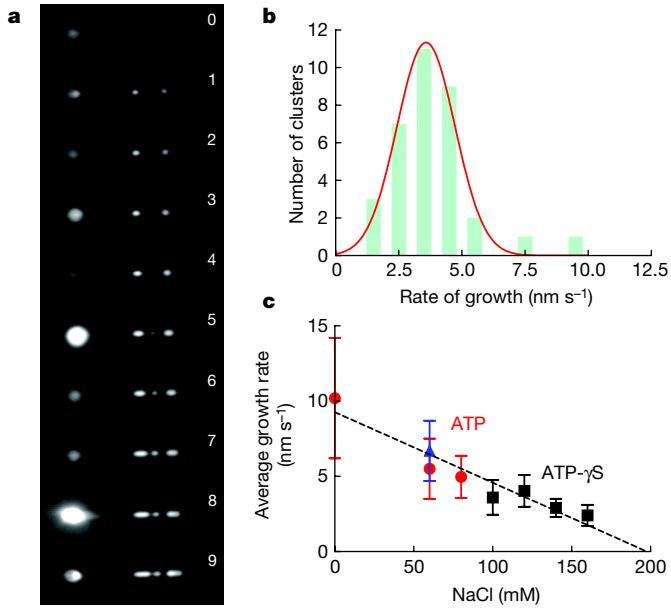

Figure 3 | Growth of individual RecA filaments is independent of nucleoside triphosphate. **a**, One DNA molecule after successive incubations with 170 nM RecA, 0.5 mM ATP-γS, 1 mM $Mg(OAc)_2$ and 100 mM NaCl. Time in minutes is shown. **b**, Distribution of growth rates. **c**, Salt dependence for the average rate of filament growth observed for 170 nM RecA and: (black squares) 0.5 mM ATP-γS, 1 mM $Mg(OAc)_2$; (red circles) 0.5 mM ATP, 1 mM $Mg(OAc)_2$ and stabilized with 5 mM ATP and 10 mM $CaCl_2$; and (blue triangle) 0.5 mM ATP, 1 mM $Mg(OAc)_2$ and stabilized with 0.5 mM ATP-γS (see the text). Error bars indicate standard deviation.

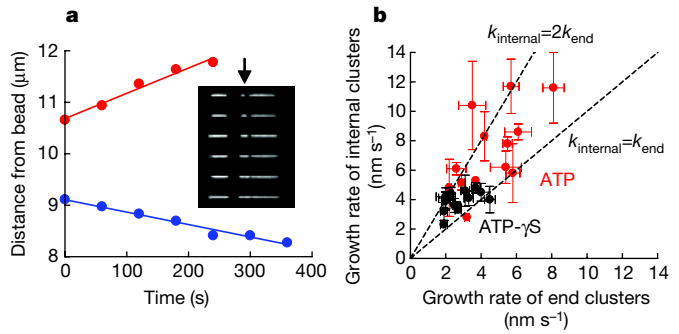

Figure 4 | RecA filaments can grow bidirectionally in the presence of ATP. **a**, Red and blue symbols represent the growing right and left edges of the clusters, respectively; lines are linear fits. The arrow in **a** points to the cluster measured. **b**, Growth rate for RecA filaments that nucleate at an internal DNA location versus the growth rate for filaments that nucleate at the ssDNA end on the same DNA molecule, for all of the salt concentrations examined, for either ATP (red circles) or ATP-γS (black circles). Error bars indicate standard deviation.

and $2.7 \pm 0.2\,nm\,s^{-1}$. The distribution of growth rates from individual clusters (Fig. 3b) is $3.6 \pm 1.1\,nm\ s^{-1}$ ($n_{clusters} = 34$) at 100 mM NaCl. When we consider a stoichiometry of 3 bp per RecA monomer and a correction factor of 1.6 due to extension of dsDNA by RecA, the data suggest that filament growth occurs at an apparent rate of ~2 RecA monomers s^{-1} under these conditions. Our single-filament rates are comparable to model-derived estimates from single-DNA extension studies when those are normalized for the number of nuclei on a DNA molecule[4,17,21] (Supplementary Fig. 11).

With ATP, RecA nucleation occurred at a ~5-fold slower rate than with ATP-γS. Consequently, the optimal conditions for monitoring growth from individual ATP clusters are at different salt concentration regimes. However, the salt dependence of growth rates (Fig. 3c) permits a comparison of growth from individual clusters, with either ATP-γS or ATP. The rates range from 3 to $10\,nm\,s^{-1}$ (~2–7 monomers s^{-1}) and follow a linear dependence on NaCl concentration. The collinearity of the ATP and ATP-γS rates indicates that the nature of the nucleotide bound to RecA protein has no effect on the elongation phase. Thus, ATP hydrolysis is not involved in filament growth.

When ATP–RecA clusters are stabilized in the observation channel by Ca^{2+} (Supplementary Fig. 12), the subsequent addition of ATP–RecA occurs on nascent filaments that are homogeneous with respect to the bound nucleotide. Alternatively, when the RecA clusters are stabilized by ATP-γS, addition of ATP–RecA to the filament occurs on clusters that are heterogeneous with respect to the bound nucleotide (ATP-γS) (Fig. 3c, blue triangle). The data show that the growth rate is the same whether the RecA clusters are stabilized by Ca^{2+}-ATP or ATP-γS. Thus, this heterogeneous extension experiment shows that both ATP and ATP-γS induce RecA nucleoprotein conformations that are indistinguishable with regard to their capacity to add new RecA molecules.

Assembly of the RecA filament on ssDNA occurs with a net 5′→3′ polarity[22]. However, the rates of addition at 5′- versus 3′-ends are undefined. When a 5′ or 3′ ssDNA tail is attached to dsDNA, polymerization onto the dsDNA of 3′-tailed dsDNA is slower than for 5′-tailed dsDNA, suggesting that growth on duplex DNA follows the same net 5′→3′ polarity[23,24]. Notably, the very fact that RecA can extend in the 3′→5′ direction suggests that growth can occur at both ends of the filament[23,24], albeit at different rates. To address this issue, we analysed molecules where cluster growth was not complicated by the presence of additional clusters growing at neighbouring positions. Of the ~140 clusters analysed, only 21 could be unequivocally examined for bidirectionality. A cluster that formed with ATP in the middle of the DNA molecule grew with a strong unidirectional bias (Supplementary Fig. 13); however, we found ATP–nucleoprotein filaments that clearly grew in both directions (Fig. 4a and Supplementary Fig. 13). Although the sample size of these completely isolated clusters is too small to provide meaningful statistics on net polarity of assembly, 19% of the RecA filaments nonetheless assembled bidirectionally with ATP. Although we believe that visualization of bidirectional growth reflects RecA addition to each cluster end, we could not completely exclude the possibility that bidirectional growth originated from clusters bound in opposite orientations but too close to distinguish experimentally. To address this issue further, we compared the rate of growth of clusters that formed with either ATP or ATP-γS at the DNA end and which could only grow from one end in one direction (towards the bead), with clusters that formed internally on the same DNA and, hence, could potentially grow from both ends. We found that the growth rate for single-ended filaments is always lower than or equal to the growth rate for most of the double-ended filaments (Fig. 4b). The higher growth rate for clusters that can extend in both directions provides a kinetic argument for bidirectional filament growth[23,24].

Our single-filament observations provide visible evidence that RecA stochastically forms multiple nuclei, which are subsequently extended in the growth phase. ATP hydrolysis is not required for either nucleation or growth, corroborating the proposition that only ATP binding is needed for filament assembly, whereas ATP hydrolysis is needed to facilitate dissociation of RecA either from ssDNA to permit contiguous filament formation or from the heteroduplex DNA product upon completion of DNA strand exchange[25]. Our analysis of the nucleation and growth phases reveals important differences in these processes. Although neither involves ATP hydrolysis, nucleation is affected by the chemical nature of the nucleotide cofactor, whereas growth is insensitive to the nature of the nucleoside triphosphate bound to either the filament or to the free RecA. Because the affinity of RecA for DNA is greater as a complex with ATP-γS than with ATP[18], this result also supports the idea that nucleation occurs on the DNA molecule. Also, the nucleation rate is profoundly sensitive to the free RecA concentration, suggesting that

4–5 RecA molecules are required to generate a stable cluster on DNA that is extended in the growth phase. The filament is seen to grow at 3–10 nm s^{-1}. Although our results cannot determine whether growth occurs by addition of RecA monomers, if we assume the simplest case of growth by monomer addition, then growth occurs at rates of ~2–7 monomers s^{-1}. This growth can proceed via addition of RecA to both ends of the filament. Ensemble studies suggest that, despite a net growth in the 5′→3′ direction, growth in the opposite direction is possible[23,24]. Given that RecA makes contact with only one of the strands of the duplex[2,23,26], our findings imply that RecA polymerizes predominantly in the direction imposed by the polarity of the bound ssDNA, with growth in the opposite direction occurring but at a slower rate.

Given the sensitivity of the nucleation phase to solution variables, it is most likely that RecA function is controlled *in vivo* at the nucleation step of filament assembly. This conclusion is in accord with extensive studies that have shown that proteins such as ssDNA-binding protein restrict nucleation[27], whereas mediator proteins such as RecBCD and RecFOR promote nucleation[28,29]. These studies established that mutant RecA proteins, which suppress loss of *recF* function, possess enhanced rates of filament nucleation[27]. By controlling nucleation, the assembly of RecA on 'normal' cellular ssDNA, such as the lagging strand gaps formed during DNA replication, would be restricted; hence, chromosomal crossovers would be limited. Finally, given the similarity between bacterial RecA and its eukaryotic counterpart, Rad51 (ref. 8), we expect that our approach, results and conclusions regarding the control of filament assembly by nucleation[30] apply to mechanistically related processes such as the control of human RAD51 assembly by BRCA2 and other mediators[9–11].

METHODS

Visualization of RecA nucleoprotein filaments. The experimental protocol was similar to that reported previously[14]. A three-channel flow cell 4.5 mm wide and 70 μm deep generated three 1.5-mm laminar flow paths. Bacteriophage λ DNA, ligated to a 3′-biotinylated oligonucleotide complementary to *cosL*, was attached to a streptavidin-coated 1-μm polystyrene bead (Bangs Laboratories). The bead–DNA complex in 20 mM Tris-OAc (pH 8.2), 20% sucrose and 30 mM dithiothreitol (DTT), containing 10–20 nM YOYO-1, was trapped at a linear flow rate of 100–130 μm s^{-1} at 25 °C for nucleation experiments, or 37 °C for growth experiments. The trapped bead–λ DNA complex was then moved to the second, observation, channel containing 20 mM Tris-OAc (pH 8.2), 20% sucrose, 30 mM DTT, 0.2 mg ml^{-1} catalase, 0.4 mg ml^{-1} glucose oxidase, 4.5 mg ml^{-1} glucose, 5 mM $Mg(OAc)_2$, and the indicated concentration of NaCl, ATP or $CaCl_2$; for experiments with $CaCl_2$ in the observation channel, the scavengers and $Mg(OAc)_2$ were omitted. The YOYO-1 dye allowed measurement of DNA length and verification that a single DNA molecule was captured. In 5 mM $Mg(OAc)_2$, YOYO-1 dissociated from the DNA after several minutes. The presence of YOYO-1 in this protocol had no effect on RecA nucleoprotein filament properties, because its omission did not change nucleation frequency. The bead–λ DNA complex was then moved to the third channel (RecA channel) containing fluorescent RecA (see Supplementary Information) in 20 mM MES (pH 6.2), 20% sucrose, 30 mM DTT, plus the indicated concentration of NaCl, $Mg(OAc)_2$, $CaCl_2$, ATP-γS or ATP. Finally, after the indicated incubation time in the third channel, the bead–DNA–RecA complex was moved back into the observation channel. Illumination was with a mercury lamp and the fluorescence was observed using an FITC filter (Chroma Technology Corp., number 41001). Analysis is described in Supplementary Information.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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