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Visualization of RecA Filaments and DNA by Fluorescence Microscopy

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We have developed two experimental methods for observing *Escherichia coli* RecA-DNA filament under a fluorescence microscope. First, RecA-DNA filaments were visualized by immunofluorescence staining with anti-RecA monoclonal antibody. Although the detailed filament structures below submicron scale were unable to be measured accurately due to optical resolution limit, this method has an advantage to analyse a large number of RecA-DNA filaments in a single experiment. Thus, it provides a reliable statistical distribution of the filament morphology. Moreover, not only RecA filament, but also naked DNA region was visualized separately in combination with immunofluorescence staining using anti-DNA monoclonal antibody. Second, by using cysteine derivative RecA protein, RecA-DNA filament was directly labelled by fluorescent reagent, and was able to observe directly under a fluorescence microscope with its enzymatic activity maintained. We showed that the RecA-DNA filament disassembled in the direction from 5′ to 3′ of ssDNA as dATP hydrolysis proceeded.

Key words: DNA–protein interaction, fluorescence label of protein, homologous recombination, microscopic observations, RecA protein.

Abbreviations: ATPγS, adenosine 5′-*O*-(thiotriphosphate).

Escherichia coli RecA, a DNA-dependent ATPase with a molecular weight of 38 kDa, plays a central role in homologous recombination and DNA repair, and its homologues are present in various biological species in nature (*1–3*). In the presence of ATP, dATP or their poorly hydrolysable analogue, adenosine 5′-*O*-(thiotriphosphate) (ATPγS), the RecA protein polymerizes around a single-stranded DNA (ssDNA), and forms a right-handed helical filamentous complex. This RecA-ssDNA filamentous complex searches for a sequence homology with other double-stranded DNAs (dsDNA), and exchanges their homologous strands between ssDNA and dsDNA (*4,5*). The structure of the RecA filaments has been extensively studied by many measurement methods over the past 25 yr. On the RecA filaments which are complexed with ssDNA or dsDNA, the DNA strands are situated in the central cavity of the RecA helical filament with a pitch of 90–100 Å (*6–11*). The RecA protein readily nucleates the filament formation on ssDNA at a random site, and regularly assembles in the direction from the 5′-end to the 3′-end of ssDNA (*12*). This filament has a dynamic property, in which RecA disassembles from the 5′-side of the filament end upon ATP hydrolysis (*13,14*). In the absence of the nucleotide cofactor the RecA protein also forms helical filaments with ssDNA, and its helical pitch was observed to be 64–76 Å (*11,15–19*). The crystal structures of the RecA protein and its homologues are reported, and their helical pitches vary from 67 to 130 Å (*20–25*). These observations suggest that the RecA filament has a flexible elasticity that can adjust their pitches under environmental conditions. In order to characterize the aspects of the structural and dynamic states of the RecA filament including this helical pitch adjustment and the ATP-dependent filament assembly/disassembly process, the development of experimental measurements that allow observation of their structure in real time is necessary.

In this report, we have visualized RecA filaments under a fluorescence microscope by immunofluorescence staining techniques. The RecA filaments were formed under various nucleotide conditions, and the statistical distribution of the filament length was analysed by fluorescence microscopic images. By using a cysteine derivative RecA protein, the RecA filaments were able to be directly labelled with a fluorescence reagent, and immobilized onto a coverslip while still maintaining its biochemical activity. The location of the ssDNA dissociated from the RecA filament was detected by immunofluorescence staining using the anti-DNA

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monoclonal antibody, and it was shown that the RecA filament was disassembled from the 5′-side of the ssDNA.

MATERIALS AND METHODS

DNA Substrates—For preparation of M13mp18 linear double-stranded DNA (ldsDNA), *E. coli* strain JM109 was grown and infected with the M3mp18 phage. After 5 h the cells were harvested by centrifugation, and the DNA was purified with a QIAGEN plasmid Midi kit (QIAGEN). The DNA solution was loaded onto agarose-gel electrophoresis, and visualized with ethidium bromide. The Form I DNA was recovered using a dialysis membrane (RECOCHIP, TaKaRa, Shiga, Japan). The DNA solution was purified by extraction with phenol/chloroform/isoamyl alcohol and precipitation in 2-propanol. Aliquots of this M13mp18 Form I DNA (15 μg) were incubated with 900 units of *Pst*I (TaKaRa) in the supplied buffer for 2 h at 37°C. The restriction enzyme was inactivated by heat treatment at 65°C for 10 min, and the linearized DNA was purified by extraction with phenol/chloroform/isoamyl alcohol and precipitation in 2-propanol. Purification of the M13mp18 ssDNA was carried out according to the method described in *Molecular Cloning* (J. Sambrook and D. W. Russell, Cold Spring Harbor Laboratory Press). *Escherichia coli* strain XL-1 Blue MRF′ was grown and infected with the M3mp18 phage. After 5 h the cells were centrifuged, and the supernatant solution was treated with 3.6% polyethylene glycol 8000 and 2.7% NaCl. The precipitated bacteriophage particles were recovered by centrifugation, and resuspended in TE buffer. The solution was extracted with TE equilibrated phenol and the cilrcular single-stranded DNA (cssDNA) was precipitated in 2-propanol. For the preparation of the M13mp18 linearized single-stranded DNA (lssDNA), the single-stranded M13mp18 DNA was first annealed with an oligonucleotide d(GGATCCCCGGGTACCG), which has a complementary sequence of an M13mp18 multi-cloning site, and then digested with the *Sma* I endonuclease. The reaction product was loaded onto agarose-gel electrophoresis containing ethidium bromide, and the DNA fragment was recovered using a dialysis membrane (RECOCHIP, TaKaRa). The DNA solution was purified by extraction with phenol/chloroform/isoamyl alcohol and precipitation in 2-propanol. Small oligonucleotide fragments bound to the ssDNA were removed by small-scale gel filtration (Chroma SPIN 200; Clontech). The gel filtration column was first incubated at 65°C. For the preparation of the biotin molecule attached M13mp18 lssDNA, the HPLC-purified oligonucleotide probes, biotin–(spacer18)$_{10}$–d(GGGTAC CGAGCTCGAATTCGTAATCATGGT) and d(ATGCCTG CAGGTCGACTCTAGAGGATCCCC)–(spacer18)$_{10}$–biotin, were supplied by Genset (Paris, France), in which a biotin molecule was attached at the 5′-end or 3′-end of the 30-mer oligonucleotide via a 60 ethylene glycol spacer arm. The DNA sequences were complementary to the 3′-end and the 5′-end regions of the M13mp18 lssDNA. The 333 nM oligonucleotide probe was annealed with 6.7 nM M13mp18 lssDNA under the solution condition of 20 mM Tris-acetate, pH 7.5, 2 mM magnesium acetate, and 50 mM NaCl, and the excess oligonucleotide probe was removed using gel filtration columns (Chroma SPIN 200). Lambda DNA was purchased from TaKaRa.

Purification of RecA Protein—The wild-type *E. coli* RecA protein was purchased from Amersham Biosciences (Uppsala, Sweden), or purified as follows with some modifications. The 353C RecA derivative, in which one cysteine residue was introduced to the C-terminus of a wild-type RecA protein, was prepared and purified according to the method described before (*26*), and further purified by anion exchange column chromatography (DE52, Whatman). *Escherichia coli* strain MV1184 was transformed by plasmid carrying wild-type recA gene or 353C recA gene. The cells were grown to $OD_{600} = \sim 0.7$ in 2 l of LB broth containing 50 μg/ml ampicillin, and then IPTG was added to a final concentration of 1 mM. After further incubation for 4 h, the cells were harvested by centrifugation and suspended in 50 mM Tris-Cl (pH 7.5) and 10% sucrose. A series of 1 s ultrasonic pulses was applied to the cell for 5 min, and then the cell lysate was centrifuged at 100,000*g* for 30 min at 4°C. Ten percent polyethlenimine P-70 solution was added to the supernatant dropwise over a period of 15 min with gentle stirring until a final concentration of 0.5% was reached. After additional stirring for 30 min, the cell lysate was centrifuged at 15,000*g* for 10 min at 4°C. The precipitate was suspended in 20 ml of R-buffer (20 mM Tris-Cl, pH 7.5, 1 mM DTT, 0.1 mM EDTA and 10% glycerol), containing 150 mM ammonium sulphate, stirred with a glass rod for 30 min, and then centrifuged at 15,000*g* for 10 min at 4°C. The precipitate was resuspended in the same buffer, stirred for 30 min and centrifuged again under the same conditions. The precipitate was suspended in 20 ml R-buffer containing 300 mM ammonium sulphate, stirred for 30 min and centrifuged at 15,000*g* for 10 min at 4°C. After re-extraction of the pellets with the same buffer, the supernatants were then combined. Finely grained ammonium sulphate was added to the solution at a final concentration of 0.28 g/ml over a period of 45 min. The precipitate was collected by centrifugation at 15,000*g* for 10 min at 4°C and stored at −20°C. The frozen precipitate was suspended in 5 ml P-buffer (20 mM potassium phosphate, pH 6.8, 1 mM DTT, 0.1 mM EDTA and 10% glycerol), containing 200 mM NaCl, and then dialysed against the same buffer. The solution was applied to a 60 ml phosphor-cellulose column equilibrated with P-buffer containing 200 mM NaCl. The flow-through fraction was collected, and solid ammonium sulphate was added to a final concentration of 0.124 g/ml. Part of the fraction was loaded onto a 10 ml Butyl-Toyopearl column (Tosoh, Tokyo) equilibrated with P-buffer containing 0.124 g/ml ammonium sulphate. The RecA protein was eluted with a stepwise gradient of ammonium sulphate (0.124–0 g/ml). Part of the fraction was loaded onto a 10 ml Hydroxyapatite column (BIO-RAD) equilibrated with P-buffer. The RecA protein was eluted with a gradient of phosphate (0–0.5 M). Part of the fraction was loaded onto a 20 ml DE52 column (Whatman) equilibrated with 50 mM Tris-Cl, pH 7.5, 1 mM

EDTA and 10% Glycerol. The RecA protein was eluted with a gradient of KCl (0–0.5 M). The fraction containing wild-type RecA protein or 353C RecA protein was collected and dialysed against the buffer of 20 mM Tris-Cl, pH 7.5, 1 mM DTT, 0.1 mM EDTA and 10% glycerol (for wild-type RecA protein) or 20 mM Tris–Cl, pH 7.5, 0.1 mM EDTA and 50% glycerol (for 353C RecA protein). The purified protein was stored at 4°C (wild-type RecA) or −20°C (353C RecA). The protein concentration was determined using the molar absorption coefficient, $\varepsilon_{278} = 2.15 \times 10^4\,M^{-1}\,cm^{-1}$ (*27*).

Immunostaining of RecA Filaments—Coverslips were immersed in concentrated KOH solution, washed with MilliQ-water (Millipore), and siliconized with a thiol-functionized silane coupling reagent (TSL 8380, GE Toshiba Silicones). A flow cell for microscopic observation was constructed from two coverslips (bottom 24 mm × 36 mm; top 18 mm × 18 mm), separated by two greased strips of a parafilm cover sheet. Ten $\mu g\,ml^{-1}$ of mouse anti-RecA monoclonal antibody (ARM191, Medical & Biological Laboratory, Nagoya, Japan) in buffer A (10 mM HEPES, pH 7.5, 1 mM EDTA) plus 5 mM magnesium acetate were infused into the flow cell and allowed to adhere to the glass surface for 20 min. The cell was then washed with buffer A, incubated with buffer A containing $1\,mg\,ml^{-1}$ dephosphorylated casein, $80\,\mu g\,ml^{-1}$ heparin and 50 mM potassium glutamate for 20 min, and washed with buffer A. The observation chamber was washed by the reaction buffer without DNA and RecA protein before use. The reaction of the RecA filament formation was carried out at 37°C for 25 min in solutions containing 20 mM Tris-acetate (pH 7.5), 1 mM magnesium acetate, 2 mM ATP, dATP, ATPγS or no nucleotide cofactor, 1.6 μM (for 3:1), 2.4 μM (for 2:1) or 0.8 μM (for 6:1) M13mp18 lssDNA or cssDNA and 4.8 μM RecA. The concentration of DNA is expressed as moles of nucleotide residues. The sample solution was introduced into the flow cell, followed by washing with buffer B (20 mM Tris-acetate, pH 7.5, 1 mM magnesium acetate and 1 mM ATPγS), infusion of $10\,\mu g\,ml^{-1}$ of mouse anti-RecA monoclonal antibody in buffer B, washing with buffer B, infusion of $20\,\mu g\,ml^{-1}$ of fluorescent goat anti-mouse isotype-specific antibody (Alexa Fluor, Molecular Probes) in buffer B and washing with buffer B. When the sample solution contained no nucleotide cofactor, buffer B was replaced by 20 mM Tris-acetate, pH 7.5 and 1 mM magnesium acetate. For DNA labelling, the infusion of the mouse anti-DNA monoclonal antibody (MAB030, Chemicon), washing with buffer B and infusion of fluorescent goat anti-mouse isotype-specific antibody were carried out prior to the RecA labelling. The RecA filament was observed under an inverted fluorescence microscope (IX70, Olympus) equipped with an oil-immersion objective (PlanApo 100, numerical aperture 1.40, Olympus) and a cooled CCD camera (ORCA-ER-1394, Hamamatsu Photonics, or DP70, Olympus). Image processing and the length measurement for the RecA filaments were carried out by ImageJ (W. S. Rasband, National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2005) and its plugin.

Fluorescence Labelling of RecA Protein—The filament formation reactions were carried out at 37°C for 20 min and the reaction solution contained 20 mM Tris-acetate (pH 7.5), 1 mM magnesium acetate, 2 mM dATP, 0.72 μM M13mp18 lssDNA and 2 μM 353C RecA. IC3-PE-maleimido (Dojindo, Kumamoto, Japan) was added to the reaction mixture to a final concentration of $10\,\mu g\,ml^{-1}$. After incubation for 1 min at room temperature, the excess labelling reagents were removed by the gel filtration column (Sepharose 2B; Amersham Biosciences) equilibrated with buffer C (20 mM Tris-acetate, 1 mM magnesium acetate, and 10 mM 2-mercaptoethanol, pH 7.3) containing 1 mM dATP.

ATPase Assay—The ssDNA-dependent ATPase was measured by an enzyme-coupled spectrophotometric assay, modifying the method according to Mikawa *et al.* (*28*). Wild-type or 353C RecA protein at 1.0 μM was incubated with 50 μM M13mp18 cssDNA in a buffer containing 50 mM Tris-acetate, pH 7.5 and 1.2 mM magnesium acetate for 10 min at 37°C. ATP was then added at 1 mM, and incubation at 37°C was continued for 5 min. For the fluorescence labelling, $5\,\mu g\,ml^{-1}$ IC3-PE-maleimido dissolved in dimethyl sulphoxide was added to the sample containing 353C RecA and the sample was incubated for 1 min at room temperature. For control experiments, the IC3-PE-maleimido was replaced by the same volume of dimethyl sulphoxide (final 0.5%). To stop the labelling reaction, mercaptoethanol was added to a final concentration of 25 mM and the sample was incubated for 1 min at room temperature. The magnesium acetate concentration was then increased to 11 mM, and phosphoenolpyruvate, pyruvate kinase, NADH and L-lactate dehydrogenase were added at final concentrations of 2.5 mM, $0.25\,mg\,ml^{-1}$, 0.2 mM and $0.1\,mg\,ml^{-1}$, respectively. After 30 s ATP hydrolysis was monitored at the absorbance of 340 nm using a JASCO spectrophotometer V-570 at room temperature (25°C). Data were taken at every 5 s. The rates of ATP hydrolysis were calculated from $-A_{340}$ per second data using an extinction coefficient of ε_{340} value of $6.22\,mM^{-1}\,cm^{-1}$ for NADH. Phosphoenolpyruvate was purchased from SIGMA. NADH, pyruvate kinase (from rabbit muscle) and L-lactate dehydrogenase (from hog muscle) were purchased from Roche Diagnostics.

Strand Exchange Reaction—For the strand exchange reactions, 20 μM M13mp18 circular ssDNA was preincubated with 6.7 μM wild-type or 353C RecA protein in 25 mM Tris-acetate, pH 7.5 and 1.4 mM magnesium acetate at 37°C for 10 min. ATP was then added at a final concentration of 3 mM, and the sample was further incubated for 10 min. For the fluorescence labelling, $10\,\mu g\,ml^{-1}$ IC3-PE-maleimido dissolved in dimethyl sulphoxide was added to the sample containing 353C RecA and it was incubated for 1 min at room temperature. For control experiments, the IC3-PE-maleimido was replaced by the same volume of dimethyl sulphoxide (final 1%). To stop the labelling reaction, mercaptoethanol was added to a final concentration of 25 mM and the sample was incubated for 1 min at room temperature. Phosphocreatine and creatine phosphokinase were added at final concentrations of 12 mM and $10\,units\,ml^{-1}$,

respectively, and the magnesium acetate concentration was then increased to 11 mM. The reaction was initiated by the addition of the 40 μM linearized M13mp18 dsDNA and 2 μM SSB protein (Amersham Biosciences), and the sample was incubated at 37°C. The final reaction volume was 60 μl. Aliquots of 10 μl were removed at 0, 10, 20, 30 and 45 min, and 5 μl of a gel loading buffer (50% glycerol, 15 mM EDTA, 0.025% BPB and 5% SDS) was then added to each aliquots. The stopped aliquots were stored on ice until the last sample was taken. The samples were subjected to electrophoresis on 0.8% agarose gel in TAE buffer.

Visualization of Filament Dissociation—The flow cell made by superimposing two coverslips was preprared as described earlier, and a slantwise-cut Teflon tube ($\phi 1.0 \times 1.5$ mm) was placed at the entrance of the flow cell and sealed with butyl rubber. The infusion and washing were repeated as follows: infusion of 0.9 mg ml^{-1} biotin-labelled BSA (SIGMA) in buffer A containing 5 mM magnesium acetate (30 min), washing with buffer A, infusion of 0.2 mg ml^{-1} streptavidin in buffer A (2 h), washing with buffer A, infusion of 1 mg ml^{-1} α-casein dephosphorylated, 80 μg ml^{-1} heparin and 50 mM potassium glutamate in buffer A (30 min), and washing with buffer A. The flow chamber was washed with 1 mM dATP in buffer C before infusion of the sample solution. The 353C RecA protein was complexed with the M13mp18 lssDNA, which had been tagged with a biotin molecule through the PEG linker at either the 5′- or 3′-end of ssDNA, and labelled with IC3-PE-maleimido according to the method described earlier. The fluorescently labelled RecA filament was infused into the flow chamber and incubated for 1–2 min. After washing with 1 mM dATP in buffer C, the chamber was further incubated for 10–15 min at room temperature. The reaction was stopped by the addition of 1 mM ATPγS in buffer C, and DNA was immunostained by the anti-DNA monoclonal antibody as described earlier. In order to determine the filament polarity, buffer C containing 1 mM ATPγS was injected from the inlet of the flow chamber at a flow rate of 100 μl min^{-1}.

RESULTS

Visualization of RecA Filaments by Immunofluorescence Staining—In order to visualize the RecA filaments under a fluorescence microscope, we have developed an immunofluorescence staining method for the RecA filament using the anti-RecA monoclonal antibody and the fluorescently-labelled secondary antibody. This method does not require the RecA protein to be genetically or chemically modified and has enabled us to obtain high contrast microscopic images without complicated procedures. The anti-RecA monoclonal antibody, ARM191 (*29,30*), was infused into the flow chamber made by two coverslips, and allowed to adhere to the glass surface. Any excess antibody was removed by washing. The RecA protein was reacted with 20 mM Tris-acetate (pH 7.5), 1 mM magnesium-acetate, and circular or linear ssDNA under various nucleotide conditions. The obtained RecA filaments were infused into the flow chamber and immobilized onto the coverslip via the antigen–antibody interaction. The RecA filaments were immunostained with the anti-RecA antibody and observed under a fluorescence microscope. Figures 1 and 2 show the fluorescence microscopic images of the RecA filaments complexed with the circular (Fig. 1) and linear (Fig. 2) ssDNA. As expected, the contour shape of the RecA filaments was consistent with that of the used DNA substrate. Since the sample was injected from the right to left sides of the microscopic field during the buffer perfusion, the RecA filaments are slightly oriented in the transversal direction. In our experiments, the anti-RecA antibody was first immobilized onto the glass surface before the infusion of RecA filaments. However, these molecules did not interfere with the image of immunostained RecA filaments probably because the density of the antibody on the glass surface was small, and/or the affinity of the glass surface-attached anti-RecA antibody to the secondary antibody was much lower than that of the RecA-bound anti-RecA antibody.

By measuring the contour length of the RecA filaments complexed with the linear ssDNA, we analysed the statistical distribution of the filament formation dependent on the nucleotide conditions. The RecA protein, at a concentration of 4.8 μM, was incubated with 1.6 μM linearized ssDNA under the various nucleotide conditions (2 mM dATP, ATP, ATPγS or no nucleotide cofactor) for 25 min at 37°C in 20 mM Tris-acetate (pH 7.5) and 1 mM magnesium acetate. The linearized ssDNA, derived from M13mp18 ssDNA, is composed of 7249 bases, and its expected length of B-form DNA is about 2.46 μm. The SSB protein, which is used for unfolding the higher-order structure of ssDNA in the RecA filament formation (*31–33*), was not included in these reaction samples. The contour length of the RecA filaments was measured from the fluorescence microscopic images, and its mean values and standard deviations were calculated (Fig. 3 and Table 1). The contour length of the RecA filaments formed in the presence of dATP was found to be 2.19 ± 0.02 μm. (SD = 0.78, $n = 1106$) and that formed in the presence of ATP was found to be 1.65 ± 0.02 μm (SD = 0.68, $n = 948$). The distribution of the RecA filaments is clearly centred on the shorter lengths in the case of ATP. This observation suggests that the dynamic states of the filament assembly and disassembly differ between the filaments formed with dATP and those formed with ATP. Previous biochemical studies showed that the dATP nucleotide induces a higher-affinity ssDNA binding state to RecA protein than ATP, and enhances the enzymatic activity of RecA protein (*34*). The values of the contour length and its deviation were smaller in the presence of ATPγS or in the absence of the nucleotide cofactors, and found to be 1.47 ± 0.01 μm (SD = 0.34, $n = 780$) and 1.09 ± 0.01 μm (SD = 0.30, $n = 1061$), respectively. The narrower distribution suggests the regularity of the binding stoichiometry and/or binding preference and the shorter values of the filament length suggests that the ATP/dATP hydrolysis expands the region of filament formation on ssDNA by changing the binding stoichiometry and/or unfolding of the ssDNA structure.

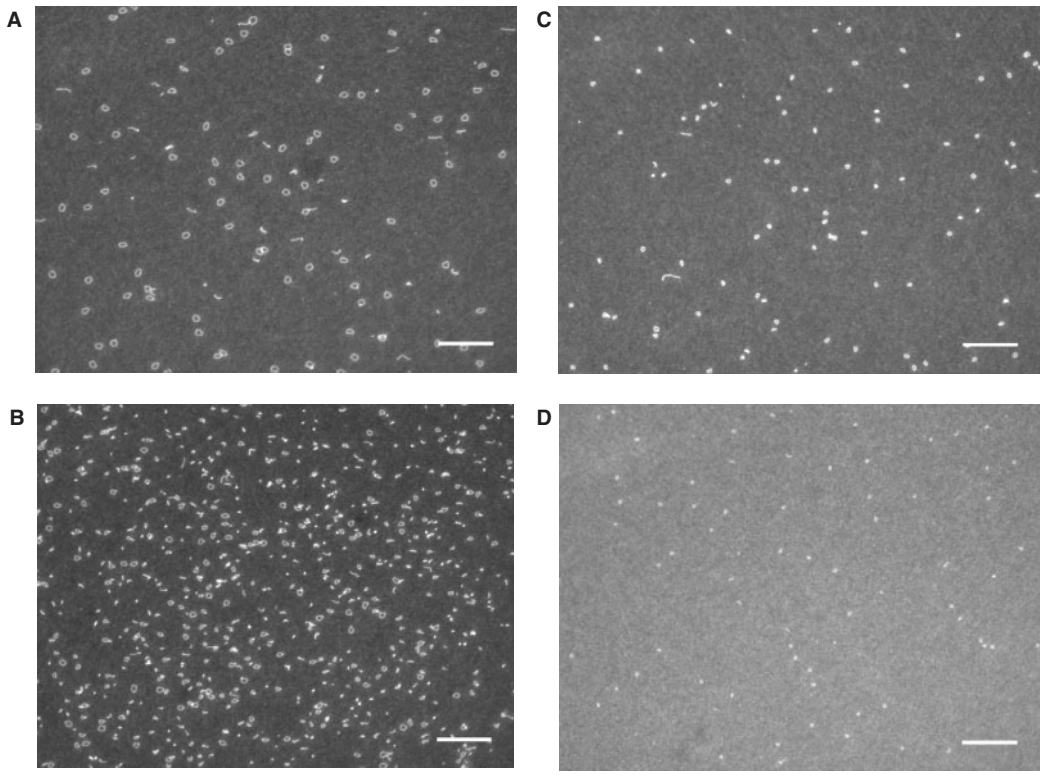

Fig. 1. **Immunofluorescence staining of the RecA filaments formed with M13mp18 circular ssDNA.** The 2.4 μM M13mp18 circular ssDNA was incubated with the 4.8 μM RecA protein for 25 min at 37°C. (A) dATP, (B) ATP, (C) ATPγS and (D) non-nucleotide buffer. Scale bars: 10 μm.

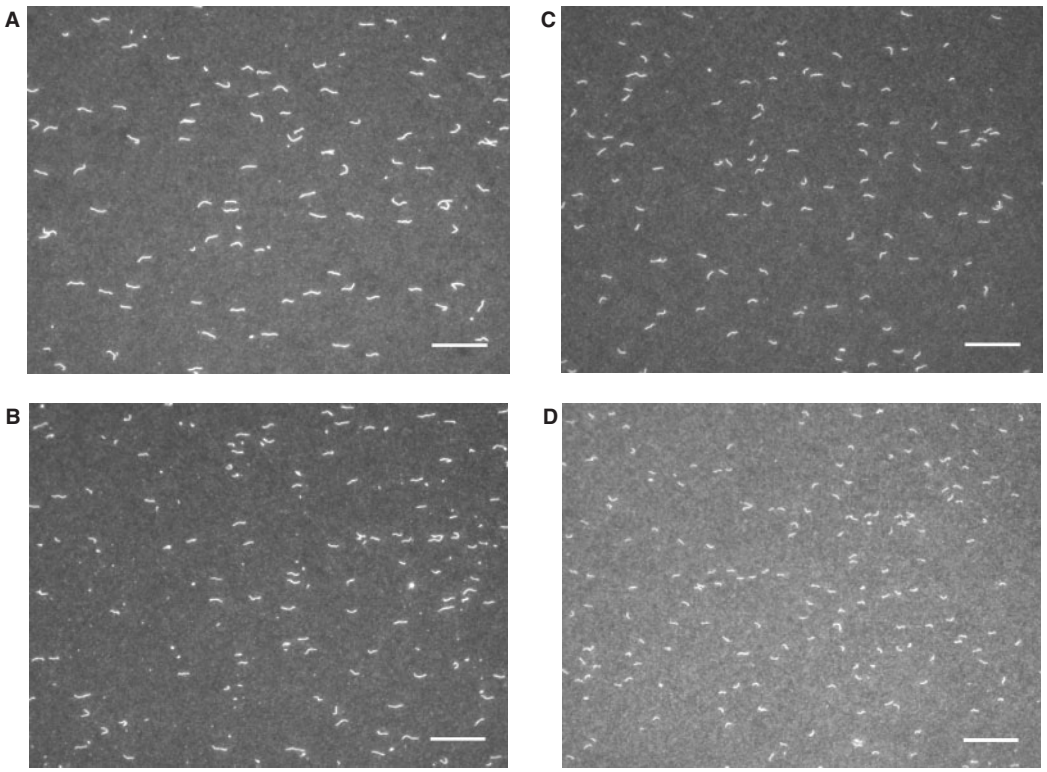

Fig. 2. **Immunofluorescence staining of the RecA filaments formed with M13mp18 linear ssDNA.** The 1.6 μM M13mp18 linear ssDNA was incubated with the 4.8 μM RecA protein for 25 min at 37°C. (A) dATP, (B) ATP, (C) ATPγS and (D) non-nucleotide buffer. Scale bars: 10 μm.

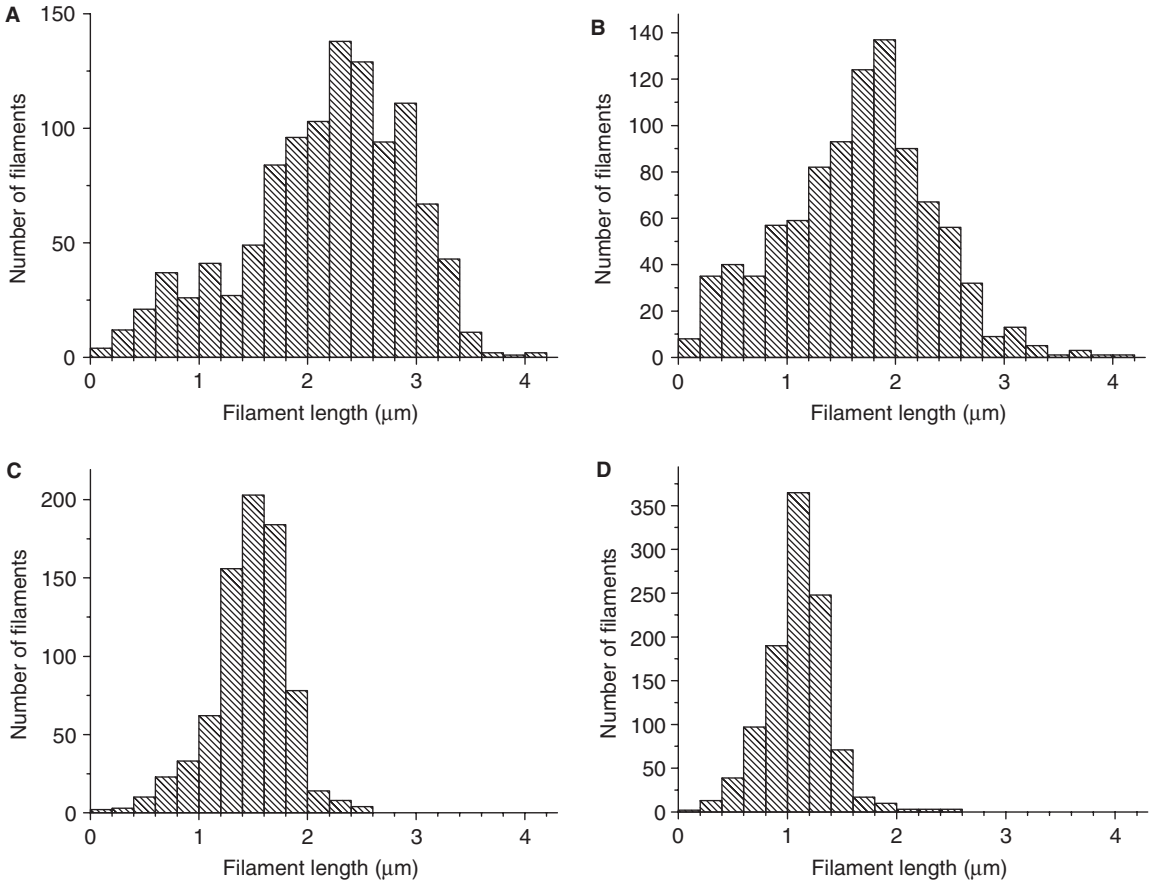

Fig. 3. **Histograms of the filament length of the RecA-linear ssDNA filaments.** The 1.6 μM M13mp18 linear ssDNA was incubated with the 4.8 μM RecA protein for 25 min at 37°C. (A) dATP, (B) ATP, (C) ATPγS and (D) non-nucleotide buffer.

Table 1. **Summary of the statistic distribution of RecA-linear ssDNA filaments formed with various nucleotide cofactors.**

	RecA:DNA	mean ± SEM (μm)	SD (μm)	*n*
dATP	2:1	2.11 ± 0.03	0.50	208
	3:1	2.19 ± 0.02	0.78	1106
ATP	2:1	1.51 ± 0.03	0.62	314
	3:1	1.65 ± 0.02	0.68	948
	6:1	1.85 ± 0.05	0.73	186
ATPγS	2:1	1.66 ± 0.03	0.42	168
	3:1	1.47 ± 0.01	0.34	780
Non-nucleotide	2:1	1.22 ± 0.02	0.25	163
	3:1	1.09 ± 0.01	0.30	1061

Direct Fluorescent Labelling of RecA Filaments—In order to directly label the RecA filament with fluorescent reagents, we prepared a cysteine derivative protein, which had one additional cysteine residue at the C terminus of the RecA protein (353C RecA protein) (*26*). However, the RecA protein has three other cysteine residues in its protein sequence, and the labelling reaction may occur at these residues. In fact, when a cysteine derivative RecA protein was directly labelled by the fluorescent reagent, the lengths of the RecA filaments that formed with M13mp18 ssDNA were shorter than that expected. This is presumably due to undesirable labelling reaction at cys116, which is located at a protein–protein interface of a RecA polymer in the crystal structure (*21*), and it may prevent the cooperative polymerization reaction of RecA protein. The sulphydryl groups of other two cysteine residues, cys90 and cys129, are fully buried in the protein's hydrophobic core, and seemed to be hard to react with a labelling reagent. Attempts to replace the cys116 residue by other amino acids failed because the mutated gene product adversely affected the bacteria growth. This problem was circumvented by labelling a cysteine derivative RecA protein after the filament formation: namely, the cysteine derivative RecA protein was first complexed with DNA, and the fluorescent labelling reagent was then reacted with the RecA filament. Figure 4 shows the fluorescently labelled RecA filaments prepared by this 'label after filament formation' method. Any excess fluorescent labelling reagent had been removed by gel filtration. The length and shape of the filament was equivalent to that of the wild-type RecA filament visualized by immunostaining. The RecA filaments formed with the wild-type RecA protein were only slightly labelled by this method, and no filament was detected in the viewing field of the fluorescence microscope. The strand exchange activity and DNA-dependent ATPase activity of the

fluorescently labelled RecA protein were measured and the results were shown in Figs 5 and 6. Although the efficiency of strand exchange was a little lower, the fluorescently labelled RecA protein exhibited comparable activities to the wild-type and 353C RecA.

Double Immunofluorescence Staining of RecA Filament and Naked DNA—Not only the RecA filament, but also the naked DNA region, which had not been covered with the RecA protein during the polymerization process, was separately visualized by immunofluorescence staining using the anti-DNA monoclonal antibody. The double-stranded lambda DNA (48,502 bp) was allowed to react with the RecA protein in the presence of ATPγS at 37°C. After 2 min, the filament formation was interrupted, and the reaction solution was infused into the observation chamber, which had been treated with the anti-RecA antibody. The RecA filaments immobilized onto the surface of the coverslips were immediately immunostained with the anti-DNA monoclonal antibody and anti-RecA monoclonal antibody, and observed under a fluorescence microscope (Fig. 7). The region covered with the RecA protein was clearly distinct from that of the naked DNA, consistent with the previous observation that the binding affinity of the RecA protein to the duplex DNA is relatively slow, but once it binds, it polymerizes onto the duplex DNA in a highly cooperative manner (*35–37*).

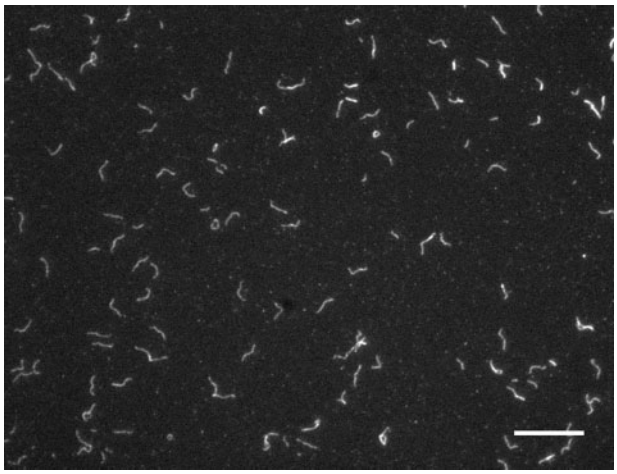

Fig. 4. **Direct fluorescent labelling of the cysteine derivative RecA filaments.** The 0.72 μM M13mp18 linear ssDNA was reacted with 1.8 μM 353C RecA protein in 20 mM Tris-acetate, pH 7.5, 1 mM magnesium acetate and 2 mM dATP at 37°C for 20 min, and the RecA filament was labelled with IC3-PE-maleimido. Scale bar: 10 μm.

Visualization of Filament Dissociation—As well as the duplex DNA, ssDNA was able to be immunostained with the anti-DNA monoclonal antibody, and the location of ssDNA along the RecA filament was also identified by visualization under a fluorescence microscope. The 353C RecA filament was formed with the linear ssDNA, to which a biotin molecule had been attached at the 3′ or 5′ terminus via the PEG linker, and labelled with the fluorescent reagent. The sample was infused into the flow chamber, which had been treated with streptavidin, and allowed to become immobilized onto the glass plate by the biotin–streptavidin binding. After washing with buffer to remove the unbound RecA protein from the chamber solution, the chamber was incubated for 10–15 min at room temperature. The reaction was terminated by the injection of ATPγS buffer, and the ssDNA tail exposed from the filament was located by immunostaining using the anti-DNA monoclonal antibody. The polarity of the filament that was immobilized on the glass surface at either end was determined by injecting the ATPγS buffer into the flow chamber at a constant rate. As shown in Fig. 8, the ssDNA region was located at the 5′-end in both cases of the 3′-end attached filament and 5′-end attached filament, indicating that the RecA filament disassembled from the 5′-side of the ssDNA (Fig. 8 and Supplementary Movies S1 and S2). The staining images before the filament dissociation (0 min) and after the dissociation (50 min) are available as supplementary data (Supplementary Fig. 1).

DISCUSSION

In this report, we describe the fluorescence labelling methods to visualize the RecA filament and its reaction. They include: (i) immunofluorescence staining of the

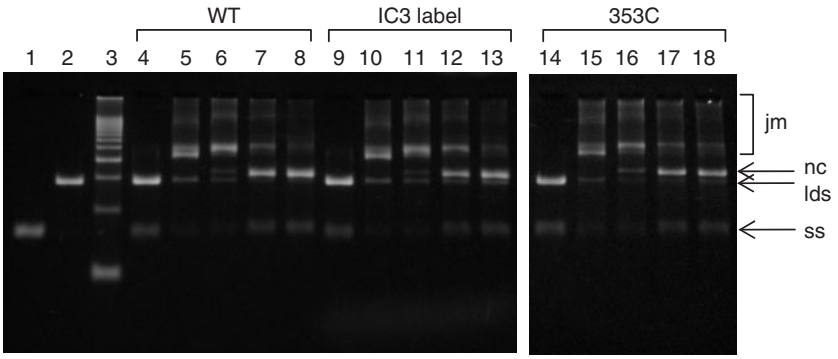

Fig. 5. **The strand exchange activity of fluorescently-labelled RecA protein.** Lane 1, ssDNA, lane 2, dsDNA, lane 3, marker (2.5 kbp ladder); lanes 4–8, reaction with wild-type RecA; lanes 9–13, reaction with IC3-labelled 353C RecA; lanes 14–18, reaction with 353C RecA (non label). Time points for each reaction correspond to 0, 10, 20, 30 and 45 min (left to right). ss, circular and linear single-stranded DNA (substrate and products); lds, linear double-stranded DNA (substrate); nc, nicked circular double-stranded DNA (products); jm, joint molecules (intermediates).

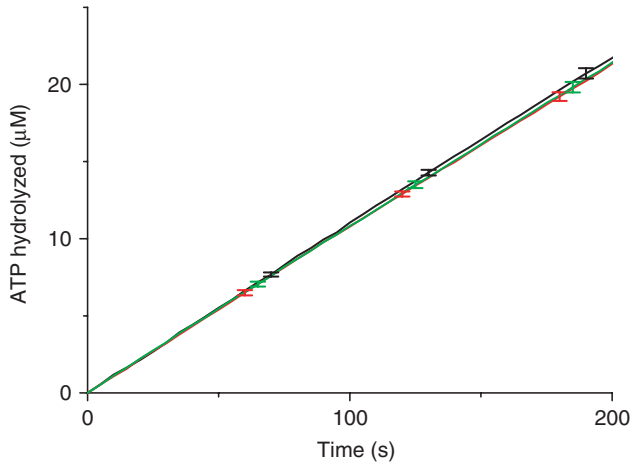

Fig. 6. **The ssDNA-dependent ATPase activity of fluorescently-labelled RecA protein.** Black: wild type. Red: 353C RecA (non label). Green: fluorescently-labelled 353C RecA. For each data, the values of three or four experiments were averaged. Error bars indicate SD.

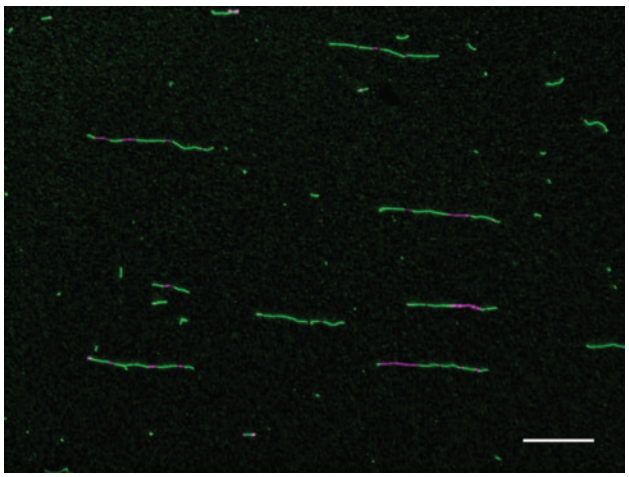

Fig. 7. **Double immunofluorescence staining of the RecA filaments (green) and the naked DNA (purple).** The 1.6 μg ml^{-1} double-stranded lambda DNA was reacted with 2.6 μM RecA protein in 20 mM Tris-acetate, pH 7.5, 1 mM magnesium acetate and 1 mM ATPγS at 37°C for 2 min. Scale bar: 10 μm.

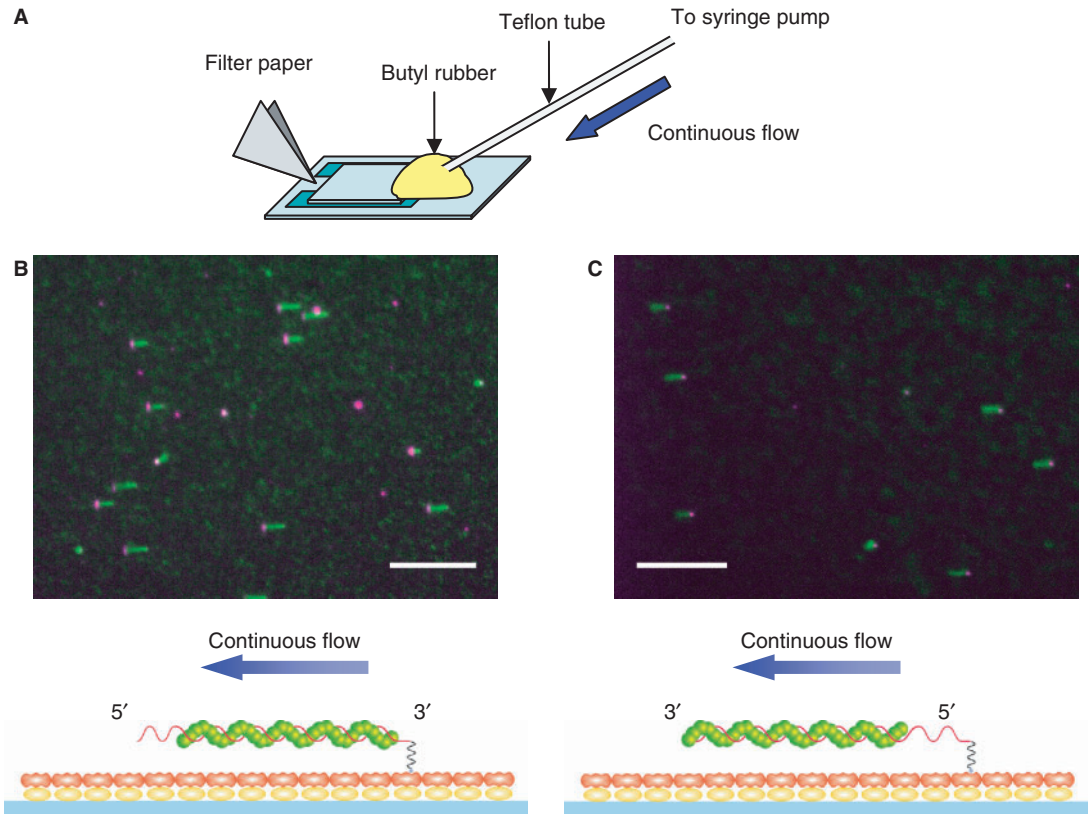

Fig. 8. **The RecA protein dissociates from the 5′-end of ssDNA.** (A) Illustration of the experimental setup. A slantwise-cut Teflon tube was placed at the entrance of the flow cell and sealed with butyl rubber. The Teflon tube was connected to a syringe pump, and a sample buffer was injected at a constant rate. The drained water was removed by a filter paper. (B,C) Fluorescently-labelled RecA filaments were immobilized onto the surface of a glass chamber via biotin–avidin binding at 3′-end (B) or 5′-end (C). After the chamber solution was exchanged by dATP buffer, the sample was incubated for 10 min (B) or 15 min (C). DNA strands were then immunostained by the anti-DNA monoclonal antibody. Solution buffer including ATPγS was injected from the right to left side of the flow chamber at a constant rate. Scale bars: 10 μm. (Up) Green: RecA filaments, Purple: ssDNA. (Down) Red wave line: M13mp18 linear ssDNA. Green: fluorescently-labelled RecA protein. Orange: streptavidin. Yellow: biotin-BSA. Blue: a coverslip. Black wave line: a PEG linker with biotin tag. Movie files for Fig. 8B and 8C, and other information are available as supplementary data (Supplementary Fig. 1 and Movies S1 and S2).

RecA filament using the anti-RecA monoclonal antibody, (ii) immunofluorescence staining of the DNA strands using the anti-DNA monoclonal antibody, and (iii) direct fluorescence labelling of the RecA filament using the cysteine derivative RecA protein. Combined with these methods, we fluorescently labelled both the RecA filaments and naked DNA on the same specimen, and the process of filament dissociation from the ssDNA strand was visualized.

Immunofluorescence staining of the RecA filament is a rapid and simple method of microscope observation. Due to the high labelling efficiency of the antibodies, this method yields clear images with a higher contrast than that obtained by direct fluorescent labelling. Furthermore, since more than 100 filaments can be observed in the field of a microscope, this method has the advantage of a quick evaluation of the experimental results. As we have shown in this report that the location of both the RecA filament and the DNA strands were identified by double immunostaining (Fig. 7), the higher-order nucleoprotein complex formed with other protein components can be visualized by the multiple immunofluorescence staining technique. The anti-RecA antibody ARM191, which we used for the immunostaining, was shown to bind to the outside surface of the RecA filament, and the binding did not inhibit the ssDNA-dependent ATPase activity (*29,30,38*). This suggests that the binding of the ARM191 antibody would cause little change to the whole structure of RecA filaments and its contour lengths. On the other hand, detailed filament structures below a submicron scale cannot be detected using a fluorescence microscope due to the optical resolution limitation. Although it was experimentally shown that the RecA filament has about a 0.6–1 μm persistent length (*8,39,40*), the filaments placed on the slide glass may have an irregularly kinked structure. The values of the filament length measured from the fluorescence microscopic images are therefore underestimated. Nonetheless, our method allows the analysis from a large number of samples ($n \approx 1000$), and gives reliable statistics for the characteristics of the RecA filaments under a defined condition.

In order to characterize the dynamic property of the RecA filaments by a single molecule observation, we prepared the cysteine derivative RecA protein and directly labelled the RecA filament with a fluorescent reagent. We obtained the fluorescently-labelled RecA filament, whose microscopic images were comparable with those obtained by immunofluorescence staining (Fig. 4). While the immunostaining technique results in a loss of activity due to the tight binding of antibodies to the RecA protein (*29,30*), direct labelling of the RecA filament is expected to maintain the biochemical activity. In fact, we were able to obtain the fluorescently-labelled RecA filaments that have comparable ATPase and strand exchange activities to the wild type by labelling cysteine derivative RecA protein with a fluorescence reagent after the filament formation with ssDNA (Figs 5 and 6). Furthermore, the fluorescently-labelled RecA filaments that are immobilized onto a glass surface via the biotin–avidin interaction exhibited an activity that is expected from the results of the prior biochemical experiments. The exposed DNA molecule, which was dissociated from the RecA filament, was located at the 5′-end, consistent with the results from previous studies that the RecA filament dissociates from the 5′-end to the 3′-end of ssDNA (Fig. 8 and Supplementary Movies S1 and S2) (*13,14*). In the future, we plan to investigate the recombination reactions of the RecA protein using this direct labelling technique.

Supplementary data are available at *JB* Online.

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