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RecA Protein Filaments Disassemble in the 5′ to 3′ Direction on Single-stranded DNA*

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RecA protein forms filaments on both single- and double-stranded DNA. Several studies confirm that filament extension occurs in the 5′ to 3′ direction on single-stranded DNA. These filaments also disassemble in an end-dependent fashion, and several indirect observations suggest that the disassembly occurs on the end opposite to that at which assembly occurs. By labeling the 5′ end of single-stranded DNA with a segment of duplex DNA, we demonstrate unambiguously that RecA filaments disassemble uniquely in the 5′ to 3′ direction.

The active form of the bacterial RecA protein is a filament formed on DNA. RecA has no known activities when it is not part of such a filamentous complex. Further, the action of several other bacterial proteins appears to be directed, at least in part, to the modulation of RecA filament assembly and disassembly on DNA (1–5). An understanding of the formation and disassembly of these filaments is thus critical to a broader understanding of RecA function in recombination processes and to an understanding of the activities of many other recombination proteins.

Filament formation generally includes a distinct and rate-limiting nucleation step followed by rapid filament extension. RecA filaments are formed most readily on ssDNA,[1] where nucleation is followed by extension uniquely in the 5′ to 3′ direction (1, 6). Filament formation on dsDNA is very slow above pH 7 due to slow nucleation (7, 8). On a gapped DNA substrate, RecA will nucleate within the single-stranded gapped region, and the subsequent extension will encompass any adjacent duplex DNA on the 3′-proximal end of the gap (1, 6, 9–11). The RecA protein forms a right-handed helical filament on DNA with visible striations seen using EM (12, 13). Within the filament, bound DNA is highly extended and underwound (12, 14–18).

The bacterial SSB protein modulates many aspects of RecA filament dynamics. Prebound SSB inhibits the nucleation step in RecA filament assembly on ssDNA (19). However, once RecA filament formation is initiated, SSB facilitates the subsequent filament extension by removing secondary structure in the DNA (20). Even though filament nucleation is inhibited by SSB, the same SSB is readily displaced during the extension phase of filament formation. When SSB is overproduced within the *Escherichia coli* cell, recombination of UV-irradiated bacteriophage DNA is specifically inhibited (21).

A net end-dependent disassembly of RecA protein filaments has been observed on dsDNA (11, 22) and ssDNA (1). Net filament disassembly from dsDNA and ssDNA is observed at neutral and higher pH values. On ssDNA, observation of a net disassembly is dependent upon the presence of SSB and ATP (1) and proceeds at a rate of about 60–70 monomers min^{-1} at 37 °C. In the EM, filament disassembly on linear single-stranded DNA appears to occur only on one end (1). Because filament extension occurs unambiguously in the 5′ to 3′ direction, and because extension followed by recession at the same end seemed unlikely, we previously argued that filament disassembly must be occurring at the 5′-proximal end (the end opposite to that at which extension occurred) (1). However, this supposition was not tested directly. In the present experiments, we were able to label the 5′ ends of a linear ssDNA by adding a duplex region to that end. The results provide a direct demonstration that the RecA filaments disassemble from ssDNA uniquely in the 5′ to 3′ direction.

EXPERIMENTAL PROCEDURES

Enzymes and Biochemicals—The *E. coli* RecA protein was purified by a procedure developed for the RecA K72R mutant protein (23). *E. coli* SSB protein was purified as described (1, 24). The concentration of each protein was determined by absorbance at 280 nm using respective extinction coefficients: $\epsilon_{280} = 2.23 \times 10^4\ M^{-1}\ cm^{-1}$ for RecA (25) and $\epsilon_{280} = 2.83 \times 10^4\ M^{-1}\ cm^{-1}$ for SSB (26).

DNA—The supercoiled circular duplex DNA and circular ssDNA from bacteriophage M13mp8 and M13mp8.1037 were prepared as described previously (27–29). Bacteriophage M13mp8.1037 DNA (8266 bp) is M13mp8 (7229 bp) with a 1037-bp insert (*Eco*RV fragment from *E. coli gal*T gene) at the *Sma*I site (30). The concentrations of dsDNA, ssDNA, and gapped DNA stock solutions were measured by absorbance at 260 nm, using 50 and 36 $\mu g\ ml^{-1}\ A_{260}^{-1}$, respectively, as conversion factors for dsDNA and ssDNA, respectively. The conversion factors used for gapped DNA were based on the fractions of duplex and single-stranded DNA in the particular molecule. DNA concentrations are expressed in terms of total nucleotides.

Linear gapped DNA molecules with a precisely defined gap length were prepared by restriction digestion of the products of a large scale RecA reaction. The linear dsDNA fragment (M13mp8 × *Pst*I × *Bsr*GI) substrate for the RecA reaction was prepared as follows. Complete digestion of supercoiled M13mp8 with *Pst*I and *Bsr*GI resulted in two linear dsDNA fragments of 5236 and 1993 bp. After digestion, residual protein was removed by sequential 1:1 extractions with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) followed by ethanol precipitation. The 1993-bp linear dsDNA fragment was purified by separation in 1.0% SeaPlaque (FMC) low melting agarose and extracted from the gel with the QIAquick Gel Extraction kit (Qiagen) as recommended by the supplier.

The M13mp8 × *Pst*I × *Bsr*GI linear dsDNA (1993 bp) and

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ATPγS, adenosine 5′-*O*-(thiotriphosphate); OAc, acetate ion; bp, base pair(s); BSA, bovine serum albumin; EM, electron microscopy; SSB, the single-stranded DNA-binding protein of *E. coli*; PBS, phosphate-buffered saline; BAP, bacterial alkaline phosphatase.

M13mp8.1037 circular ssDNA (8266 bases) were used in a large scale RecA-mediated three-strand exchange reaction as previously described (31). Reactions contained 7 μM RecA, 2 μM SSB, 3 mM ATP, 20 μM M13mp8.1037 circular ssDNA, and 20 μM M13mp8 × *Pst*I × *BsrG*I linear dsDNA. An ATP regeneration system was included at 10 units ml^{-1} pyruvate kinase and 5 mM phosphoenolpyruvate. RecA was allowed to prebind to the ssDNA before the addition of dsDNA. Reactions were initiated by the addition of ATP and SSB. After 135 min at 37 °C, the reaction was halted by the addition of EDTA to 12 mM, SDS to 1%, and Proteinase K to 1 mg ml^{-1} with digestion at 37 °C for an additional 30 min. The DNA was extracted and concentrated using a Microcon-100 Ultrafilter (Amicon). The entire reaction mixture was then digested with *Pvu*II for 60 min at 37 °C to generate a linear gapped DNA molecule with 1894 bp of dsDNA at the 5′ end of the long linear ssDNA region. The linear gapped DNA was extracted, and ethanol was precipitated. The DNA preparations were checked by spreading the sample using a modified cytochrome method (32) and counting the different types of molecules using EM. In the sample used for these trials, 44% of 102 molecules selected at random in the EM field were the linear gapped DNA of interest. Of the remainder, 14% were uncut circles, and the rest were broken linear molecules of various types (some of which may have been broken during spreading).

Preparation of Denatured BSA-coated Carbon Film for Electron Microscopy—A solution containing BSA (0.1%) and 0.5% Sarkosyl was heated in a boiling water bath for 10 min and then stored at 4 °C. Carbon-coated electron microscopy grids were then floated (carbon side down) on the denatured BSA solution for 5 min. Finally, the grids were picked up with clean forceps and immersed in three consecutive beakers containing 100 ml of water for 10 s each, touched to filter paper to absorb most of the liquid from the grid, dried under a heat lamp, and stored in a desiccator.

Electron Microscopy—Reaction mixtures contained 25 mM Tris acetate (80% cation, pH 7.5) or 25 mM HEPES, pH 7.5, 10 mM $Mg(OAc)_2$, 5% (w/v) glycerol, and 3 mM ATP. An ATP regeneration system of 10 mM phosphoenolpyruvate, 20 units ml^{-1} pyruvate kinase, and 3 mM potassium glutamate (PEP/PK) or 12 mM phosphocreatine and 10 units ml^{-1} creatine phosphokinase (PC/CPK) was included. The reactions also included 2 μM RecA protein, 3 μM linear gapped DNA (2.4 μM nucleotides ssDNA), and 0.24 μM SSB. The RecA protein was preincubated with the linear gapped DNA for 10 min at 37 °C in the presence of all reaction components except ATP and SSB. The reaction was then initiated by the addition of ATP and SSB. Reactions were stopped by adding ATPγS to 1 mM and incubating at 37 °C for 5 min. Reactions were spread by a modified adsorption procedure onto carbon films that were activated by either Alcian blue (33) or denatured BSA (as described above), with the following changes. The samples were diluted into 200 mM ammonium acetate, 10 mM HEPES, pH 8.0, and 10% (w/v) glycerol and were adsorbed to carbon films on an EM grid for 3 min. Grids were washed by quickly touching the carbon surface to a drop containing 50 mM ammonium acetate containing 10 mM HEPES pH 8.0 and 10% glycerol. This was followed by floating the grid on a drop of the same solution for 30 s. Glycerol (5% w/v) was included in the 5.0% uranyl acetate staining solution and subsequent water washes. The grids were dried under a heat lamp for 10 min before circularly shadowing with platinum. This protocol is designed for visualization of complete reaction mixtures; however, it has the disadvantage of a higher than normal background because of unreacted protein. Full-length linear molecules were scored in the analysis of filament disassembly. Circular or broken molecules were ignored.

RecA filament lengths were estimated by a procedure described previously (1). In the present experiments, the judgments were again checked for accuracy and bias by comparison with direct measurements from electron micrographs (on average 5 filaments/experiment).

RESULTS

Experimental Design—To demonstrate by EM that RecA protein filaments disassembled in the 5′ to 3′ direction, we needed a method to label one end of the ssDNA. This was accomplished by making a gapped circular DNA with a duplex region extending for 1993 bp and linearizing the molecule so that 1894 bp of the duplex DNA appeared at the 5′ end (Fig. 1). Because RecA filaments have been shown to assemble in the 5′ to 3′ direction, the long duplex DNA in this molecule generally would not be bound by RecA protein.

RecA Protein Filaments Disassemble in the 5′ to 3′ Direction on ssDNA—Filaments were assembled on this linear DNA and

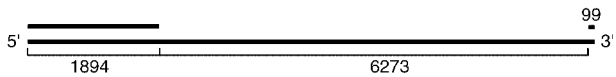

FIG. 1. **DNA substrate used in the experiments.** *Numbers* indicate DNA length in terms of base pairs or nucleotides for dsDNA and ssDNA, respectively.

then examined 5 and 30 min later. At the 5-min time point, most of the DNA molecules had long RecA filaments bound to them (Fig. 2, *A* and *B*). Some of the molecules (7 of 113 examined) had no SSB-bound regions, but a DNA tail was apparent at one end (Fig. 2*A*). Very short vacated single-stranded segments found in most molecules (106 of 113 examined) were coated with SSB, giving the DNA a characteristic condensed beads-on-a-string appearance. Fig. 2*B* shows an example containing a typical short SSB-coated segment. In virtually all cases the SSB-bound region was at one end of the RecA filament, and the duplex tail (without SSB) extended beyond the SSB at the same end of the molecule. In the above count we did not observe any filaments with SSB-coated DNA at both ends. In a broader survey of these grids intended in part to locate such molecules (in which no attempt was made to count and characterize the various types) we were able to find several filaments that may have had SSB-coated DNA at both ends. However, these were both rare and ambiguous. In every instance the possible SSB segments at the end distal from the duplex tail were too short to be reliably distinguished from background.

Samples spread from the 30-min time point were similar, except that the RecA-coated regions were shorter, and the SSB-bound regions were longer (Fig. 2, *C–E*). In this sample as in the 5-min sample, the SSB-coated region was contiguous with the RecA filament. This SSB segment was always at the end nearest the duplex DNA, and the duplex remained protein-free. These results revealed that disassembly occurred uniquely from the end nearest the duplex DNA.

Precise measurements of the growth of the SSB-coated segment were not possible because bound SSB condenses the DNA. The lengths of the RecA filaments could be measured, and the lengths at 5 *versus* 30 min are summarized in Fig. 3. The distribution of RecA filaments is clearly centered on shorter lengths at the 30-min time point. In addition, the molecules that had no bound RecA protein increased from 0 to 2.5% in the 25 min separating the two time points, and the number with full-length filaments declined from 6% to less than 1%. Note that the presence of some long RecA filaments even at late time points is likely to reflect a low but readily detectable degree of rebinding of RecA protein to vacated DNA (34).

DISCUSSION

We conclude that RecA filament disassembly proceeds from the end nearest the 5′ end or the end opposite to that at which filament assembly occurs. The results of this study are summarized in Fig. 4. Filaments are formed on the DNA early in the reaction, coating the single-stranded region and extending to include the 3′ end of the single strand. The short 90-bp duplex at the distal end is encompassed by the filament and is not relevant to the analysis. The long 1894-bp duplex at the 5′-proximal end of the single strand is not bound by RecA protein to any significant degree in these experiments, because filaments formed at neutral pH nucleate in the ssDNA and are extended in the direction away from this duplex region. Once filaments are formed, they begin to disassemble from the 5′-proximal end. The vacated ssDNA is bound by SSB. Limited renucleation at random sites within the SSB-bound segments occurs despite the SSB inhibition, so that some long filaments

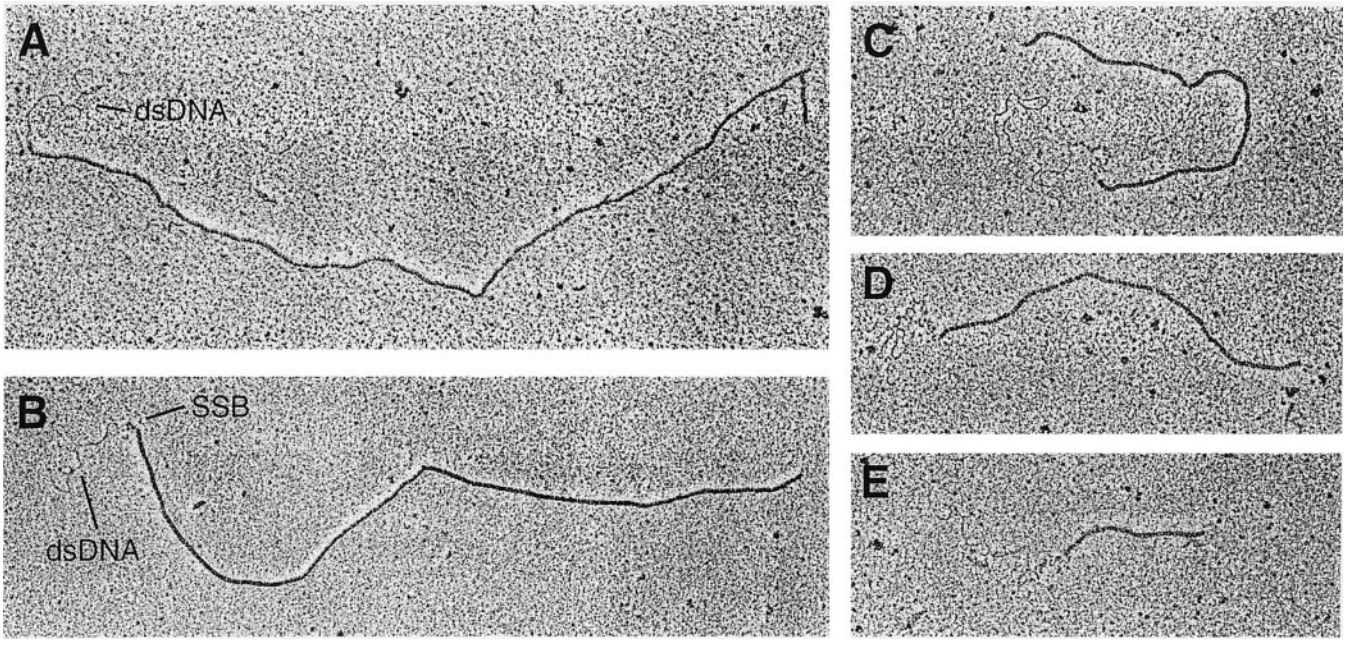

FIG. 2. **EM analysis of RecA filaments undergoing disassembly.** *Panels* show typical molecules found at the 5-min (*A* and *B*) and 30-min (*C–E*) time points. The duplex DNA ends and the SSB-coated regions are indicated for most of the molecules.

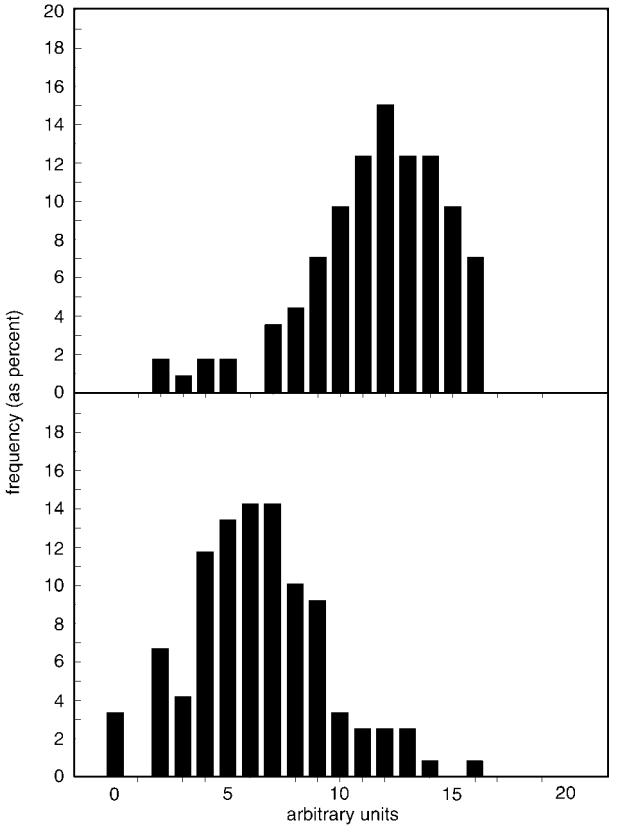

FIG. 3. **Quantitation of the lengths of RecA filaments formed on the linear gapped DNA at 5 and 30 min after reaction initiation.** The number of molecules examined to generate these plots was 57 for the *top panel* and 59 for the *bottom panel*. The arbitrary units represent the 20 segment lengths into which the molecules were divided. A length of "20" would represent a RecA filament that encompassed all of the DNA (ssDNA + dsDNA) in the molecule in Fig. 1.

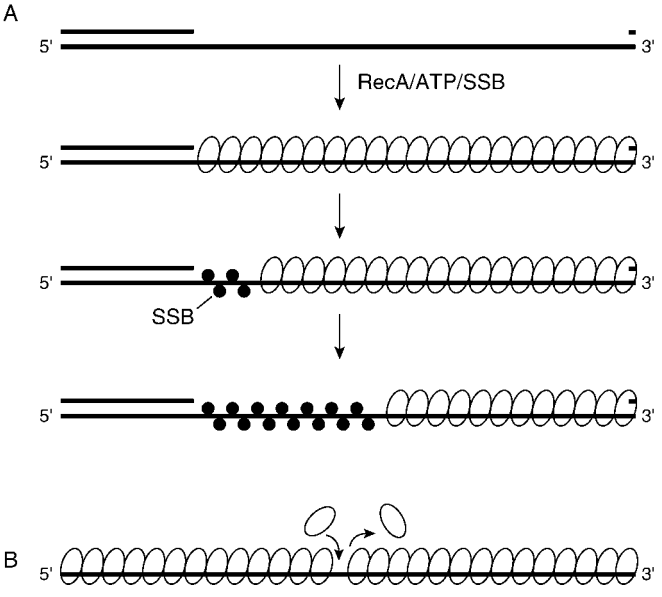

FIG. 4. **Two modes of RecA protein dissociation from filaments.** *A*, model for RecA protein end-dependent disassembly. RecA filaments first form in the single strand gap and are extended 5′ to 3′ to the end of the filament. Disassembly occurs from the 5′-proximal end, and the vacated DNA is replaced with SSB. *B*, a proposed mechanism for RecA monomer exchange in a filament interior. RecA monomers dissociating from a disassembling end at a filament discontinuity are replaced by monomers binding to the assembling end nearby.

can be seen even at late times in the reaction. Disassembly is coupled to ATP hydrolysis, rendering it effectively irreversible. Kinetic analysis has indicated that there is little direct rebinding of RecA protein to the disassembling end of a filament (34).

As we have posited in previous work, the direction of filament assembly and disassembly is the same, and RecA monomers are added largely at one end and subtracted from the other. In the RecA protein-promoted formation of D-loops with linear single-stranded DNA, there is a marked 3′ end-bias noted in many studies (35–37). Such an end-bias is potentially useful, because the 3′ end used in the strand invasion can be subsequently used as a replication primer. The end-bias simply reflects the presence of RecA protein at a particular end (37, 38). The directions of RecA filament assembly and disassembly both contribute to a situation in which, in the absence of other factors, RecA protein will always be more abundant at the 3′

rather than the 5′ end of linear single strands.

We have suggested that ATP hydrolysis results in dissociation only at the disassembling end, because the monomers there are joined to the filament only by one protein interface, although each of the monomers in the filament interior is linked by two interfaces. However, an exchange of RecA monomers between free and bound forms can be detected under some conditions within the interior of RecA protein filaments (39, 40). This may be caused by limited dissociation of RecA monomers (or larger units) within a filament followed by a rapid replacement by other RecA protomers. Alternatively, it may reflect the presence of short gaps in the filaments at which end-dependent disassembly and assembly occur simultaneously (Fig. 4*B*). The significance of these exchanges for RecA activities remains to be elucidated.

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