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RecA Protein Filaments: End-dependent Dissociation from ssDNA and Stabilization by RecO and RecR Proteins

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RecA protein filaments formed on circular (ssDNA) in the presence of ssDNA binding protein (SSB) are generally stable as long as ATP is regenerated. On linear ssDNA, stable RecA filaments are believed to be formed by nucleation at random sites on the DNA followed by filament extension in the 5′ to 3′ direction. This view must now be enlarged as we demonstrate that RecA filaments formed on linear ssDNA are subject to a previously undetected end-dependent disassembly process. RecA protein slowly dissociates from one filament end and is replaced by SSB. The results are most consistent with disassembly from the filament end nearest the 5′ end of the DNA. The bound SSB prevents re-formation of the RecA filaments, rendering the dissociation largely irreversible. The dissociation requires ATP hydrolysis. Disassembly is not observed when the pH is lowered to 6.3 or when dATP replaces ATP. Disassembly is not observed even with ATP when both the RecO and RecR proteins are present in the initial reaction mixture. When the RecO and RecR proteins are added after most of the RecA protein has already dissociated, RecA protein filaments re-form after a short lag. The newly formed filaments contain an amount of RecA protein and exhibit an ATP hydrolysis rate comparable to that observed when the RecO and RecR proteins are included in the initial reaction mixture. The RecO and RecR proteins thereby stabilize RecA filaments even at the 5′ ends of ssDNA, a fact which should affect the recombination potential of 5′ ends relative to 3′ ends. The location and length of RecA filaments involved in recombinational DNA repair is dictated by both the assembly and disassembly processes, as well as by the presence or absence of a variety of other proteins that can modulate either process.

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Keywords: RecA; recombination; repair; DNA; filament

Introduction

RecA is an ancient protein present in all bacteria, with structural and functional homologues in eukaryotes ranging from yeast to human (Ogawa *et al.*, 1993; Roca & Cox, 1990, 1996; Sung, 1994). *In vivo*, RecA protein plays an important role in the processes of recombinational DNA repair, homologous DNA recombination, induction of the SOS response to DNA damage, and the partitioning of chromosomes at cell division (Clark & Sandler, 1994; Cox, 1994, 1995; Kowalczykowski *et al.*, 1994; Kowalczykowski & Eggleston, 1994; Roca & Cox, 1990; West, 1992; Zyskind *et al.*, 1992).

In vitro, RecA protein promotes a set of DNA strand exchange reactions that mimics its presumed role in homologous recombination and recombinational repair (Figure 1). The most frequently used DNA substrates are circular ssDNA and its homologous linear dsDNA. RecA protein first forms a nucleoprotein filament on the circular single-stranded DNA. A homologous linear duplex DNA is then paired with the single strand

Abbreviations used: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; RecA, the wild-type RecA protein; OAc, acetate ion; ATPγS, adenosine 5′-*O*-(3-thio)triphosphate; SSB, the single-stranded DNA binding protein of *E. coli*; LDH, L-lactic dehydrogenase; IPTG, isopropyl β-D-thiogalactopyranoside; Mes, 2-(*N*-morpholino) ethane sulfonic acid; EM, electron microscopy.

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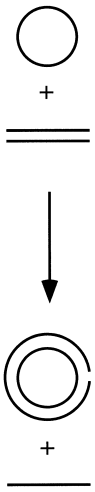


Figure 1. RecA protein-mediated DNA strand exchange reaction. Typical reaction involving three DNA strands is shown.

in the nucleoprotein filament. A strand switch occurs within the filament producing a nascent region of hybrid DNA, which is extended to generate the products, as shown in Figure 1. In a normal reaction, the extension of the hybrid DNA proceeds unidirectionally, 5′ to 3′ relative to the single-stranded DNA to which the RecA protein first binds. RecA protein is a DNA-dependent ATPase, with a monomer k_{cat} value of 30 min^{-1} when bound to ssDNA. ATP is hydrolyzed uniformly throughout the nucleoprotein filament (Brenner *et al*., 1987). By a mechanism not yet established, the ATP hydrolysis is coupled to the unidirectional extension of the hybrid DNA during DNA strand exchange (Bedale & Cox, 1996; Cox, 1994; Shan *et al*., 1996).

The RecA mutant protein RecA K72R, which binds but does not hydrolyze ATP, has proven useful in exploring the role of ATP hydrolysis in RecA reactions (Rehrauer & Kowalczykowski, 1993; Shan & Cox, 1996; Shan *et al*., 1996). This mutant protein forms mixed filaments with wild-type RecA protein under a variety of conditions, and the resulting effects on ATP hydrolysis and DNA binding have been characterized (Shan & Cox, 1996).

Proposed mechanisms for RecA protein-mediated DNA strand exchange can be categorized in part by whether the active RecA filament envisioned is stable throughout the reaction or is subject to RecA monomer dissociation and/or redistribution as part of the strand exchange mechanism (Cox, 1994; Kowalczykowski *et al*., 1994; Kowalczykowski & Eggleston, 1994; Shan & Cox, 1996; Ullsperger & Cox, 1995). The pathways by which RecA filaments assemble and disassemble therefore represent a key element in any overall understanding of DNA strand exchange mechanisms. Here we document a new filament disassembly pathway, as well as the effects of nucleotide cofactors and other proteins on it. In addition to summarizing current information about filament assembly and disassembly, the relevant proteins and their properties are also introduced below.

RecA protein filaments assemble on ssDNA in the 5′ to 3′ direction (Register & Griffith, 1985), generating a contiguous filament encompassing a circular ssDNA molecule. Filament assembly is rapid, with filaments containing over 2000 monomers completed in one to two minutes. Each RecA monomer binds to three nucleotides of ssDNA (Roca & Cox, 1990). When visualized by electron microscopy, RecA filaments on ssDNA exhibit a repeating structure with helical striations and can be easily distinguished from the nucleosome-like SSB-ssDNA complexes (Register & Griffith, 1985). The ssDNA is extended when bound by RecA (by a factor of 1.5 relative to a duplex DNA with an equivalent length in base-pairs), whereas ssDNA is collapsed and compact when bound by SSB.

In contrast, RecA protein binding to dsDNA is limited kinetically by a very slow nucleation step at neutral pH (Pugh & Cox, 1987, 1988). If a single-strand gap is present to provide a rapid nucleation site, filaments will assemble 5′ to 3′ relative to the strand in the gap and rapidly incorporate any adjacent duplex DNA (Lindsley & Cox, 1989, 1990a; Shaner & Radding, 1987). Faster binding to duplex DNA also occurs at pH values around 6.0 (Roca & Cox, 1990). When dsDNA is bound in the filament, one of the two strands occupies the site to which ssDNA would normally be bound, and it is this strand which determines the polarity of the filament itself (Chow *et al*., 1988, Lindsley & Cox, 1989; Stasiak *et al*., 1988). In the continuing discussion, this strand is called the initiating strand. We also define filament assembly or disassembly in terms of RecA monomers, although some larger unit may represent the actual species that binds or dissociates.

RecA filaments will disassemble from any DNA if the ADP/ATP ratio exceeds 1.0 (Cox *et al*., 1983; Lee & Cox, 1990a,b). If ATP is regenerated as it must be *in vivo*, a net disassembly from DNA is rarely observed. Net disassembly or even an equilibrium exchange between free and bound RecA monomers has been documented only in the case of filaments formed on dsDNA. Two such processes have been described and characterized. On linear dsDNA, an end-dependent disassembly occurs in which RecA monomers leave progressively from the filament end opposite to that at which monomer addition occurs during assembly (Lindsley & Cox, 1989, 1990a). The process is pH-dependent, being absent at pH 6.0 and occurring at maximal rates (about three monomers s^{-1}) above pH 8.0 (Lindsley & Cox, 1989). More recently, an exchange of RecA monomers between free and bound forms has been documented that occurs in the interior of filaments bound to duplex DNA (Shan & Cox, 1996). Both processes require

ATP hydrolysis. However, in both cases the observed dissociation or exchange accounts for only a small fraction of the ATP hydrolyzed, so that an ATP hydrolytic event does not necessarily lead to dissociation of the RecA monomer in which it occurs.

Disassembly of filaments from circular ssDNA is generally not observed as long as ATP is regenerated (Neuendorf & Cox, 1986; Shan & Cox, 1996). There is no direct coupling of ATP hydrolysis and dissociation of RecA monomers from the filaments, and little measurable transfer between filaments and a free RecA pool, although some direct transfer between filaments can be observed (Neuendorf & Cox, 1986; Shan & Cox, 1996). Direct transfer between two oligonucleotides *via* a ternary intermediate has also been described (Menetski & Kowalczykowski, 1987a,b).

The nucleotide cofactor used affects RecA filament formation and stability. While RecA-mediated DNA strand exchange proceeds well in the presence of ATP, the reaction is nevertheless enhanced when dATP is used instead (Menetski & Kowalczykowski, 1989). The dATP is hydrolyzed about 20% faster than ATP, and strand exchange also proceeds at an increased rate with dATP. The RecA K72R mutant protein is proficient in many DNA binding and pairing activities, but only when dATP rather than ATP is used (Rehrauer & Kowalczykowski, 1993; Shan & Cox, 1996; Shan *et al*., 1996). The use of dATP instead of ATP also suppresses a very low level exchange of RecA monomers between free and bound forms that can be observed with filaments formed on circular ssDNA (Shan & Cox, 1996).

A number of other proteins affect the assembly of RecA filaments on ssDNA. The single-strand DNA binding protein (SSB) is the best studied. Formation of RecA-ssDNA filaments is greatly facilitated by SSB, which removes the secondary structure in ssDNA that normally impedes formation of a RecA filament (Cox & Lehman, 1982; Kowalczykowski & Krupp, 1987; Morrical *et al*., 1986; Muniyappa *et al*., 1990). SSB inhibits the nucleation but not the extension of RecA filaments, and is therefore most effective when added after rather than before RecA protein in a reaction mixture. SSB remains loosely associated with the filaments (Morrical *et al*., 1986; Muniyappa *et al*., 1990; M.M. Cox, unpublished results).

The inhibition of RecA filament nucleation by prebound SSB can be overcome by the addition of the RecO and RecR proteins (Umezu & Kolodner, 1994). RecO and RecR are considered components of the RecF pathway of homologous genetic recombination (Clark & Sandler, 1994; Smith, 1989). The *recO* gene was identified in 1985 by Kolodner *et al*. (1985) and encodes a protein with 242 amino acids (26,000 Da). The *recR* gene was identified by Mahdi & Lloyd (1989a,b) and encodes a 201 amino acid protein of a predicted molecular weight of 21,965. The RecO and RecR proteins, acting together, stimulate RecA protein binding to ssDNA coated with SSB (Umezu & Kolodner, 1994). The resulting filament can still promote DNA strand exchange. Significant amounts of RecO, RecR, and SSB remained associated with RecA nucleoprotein filaments formed on ssDNA (Umezu & Kolodner, 1994). Neither the RecO nor the RecR protein possess an intrinsic ATPase activity.

There has been no demonstration of a RecA filament disassembly pathway for filaments formed on ssDNA, and these filaments have generally been assumed to be stable. The 5′ to 3′ polarity of RecA filament assembly on ssDNA (Register & Griffith, 1985) has an implication that has been highlighted in many current models for recombination. On linear ssDNA, RecA protein should nucleate filament formation at random locations and extension should proceed in the 5′ to 3′ direction until the 3′ end is reached. The 3′ end is therefore much more likely to be coated with RecA protein than the 5′ end, leading to models in which 3′ ends exposed by the action of nucleases or helicases are more recombinogenic or invasive than 5′ ends. This effect has been directly demonstrated *in vitro* (Dixon & Kowalczykowski, 1991; Dutreix *et al*., 1991; Konforti & Davis, 1990, 1991, 1992).

In re-examining the issue of RecA protein filament assembly 5′ to 3′ on linear ssDNA, we have found that the filaments also disassemble with the same apparent polarity. While a disassembly of RecA from the DNA nearest the 5′ end would tend to exacerbate the bias toward DNA pairing reactions at the 3′ end, filaments coating the linear single strands in a contiguous manner from the 5′ to 3′ ends can be readily assembled and stabilized by including additional proteins and nucleotides present *in vivo*. This novel disassembly path, and its suppression by pH, dATP and/or the RecOR proteins, are the subject of this paper.

Results

Experimental design

This study was designed to examine the stability of RecA protein filaments on ssDNA. The effects of pH and a variety of proteins and nucleotide cofactors on that stability were also investigated. The stability of RecA filaments was examined by three independent assays.

The first approach used ssDNA-dependent NTPase assays. In general, the observed ATP hydrolysis rate derived from the bound RecA can be compared to the expected rate (based on the intrinsic k_{cat}) if the maximum possible amount of RecA protein were bound to DNA. This provides an estimate of the amount of RecA bound to ssDNA. Although the DNA-dependent ATPase assay is an indirect assay to monitor RecA protein binding to DNA, it offers convenience, high accuracy, reproducibility, and detection of dynamic changes in real time. Previous studies have shown

that this assay generally correlates well with other assays which more directly measure the amount of RecA bound to DNA, and applicable caveats have been defined (Lindsley & Cox, 1990a; Menetski *et al*., 1988; Pugh & Cox, 1988; Shan & Cox, 1996).

The second approach was sucrose gradient sedimentation using ^{35}S-labeled RecA protein. After halting a reaction by addition of ATPγS, free and bound RecA protein migrate as separate peaks in the gradients. The amount of radiolabeled RecA bound to DNA can be quantitatively followed by scintillation counting. Any additional proteins in the DNA bound fractions can be identified by either SDS-PAGE or by Western blot. Sucrose gradient sedimentation analysis is a relatively direct measure of RecA binding to DNA. However, it is sufficiently tedious that the number of assays that can be performed is limited. It is also not as reproducible and accurate as the ATPase assay. We primarily used this assay to confirm key results from ATPase assays.

The third approach was electron microscopy (EM), which allows direct visualization of filaments, again after halting reactions with ATPγS. This assay is one of the most direct approaches to study RecA-DNA interactions. The assay provides information about the physical properties of RecA filaments formed under different conditions, the dissociation of RecA protein from RecA-linear ssDNA filaments, and the binding of SSB as RecA protein dissociates. However, information gained from EM is at the individual filament level and heterogeneous populations of RecA filaments exist in most of the samples. A large number of filaments need to be surveyed in order to describe what happens in a particular reaction.

The second and third approaches rely on ATPγS addition to halt disassembly and stabilize whatever filaments remain. Hence, controls are needed to define effects of ATPγS addition on the results. Nevertheless, when combined, these approaches complement each other and provide a reasonably complete picture of the status of the RecA filaments under most conditions.

RecA protein filaments dissociate from linear ssDNA in an end-dependent manner

The stability of RecA filaments formed on either linear or circular ssDNA was first monitored with the ATPase assay. Typical time courses of these reactions are shown in Figure 2. The circular ssDNA-dependent ATP hydrolysis rate (29.7 μM min^{-1}) was constant during the 90 minute reaction. Since a maximum of 1 μM RecA protein can be bound to the DNA in this experiment, the apparent k_{cat} (29.7 min^{-1}) compares well with the inherent k_{cat} of 30 min^{-1} and indicates that the DNA is saturated with RecA protein. However, the rate of ATP hydrolysis gradually declined when linear ssDNA was used. Initially, filaments formed that generated a rate of ATP hydrolysis comparable to

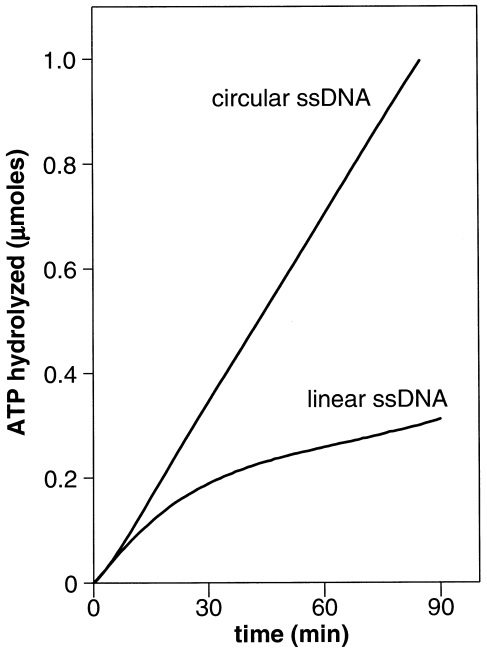
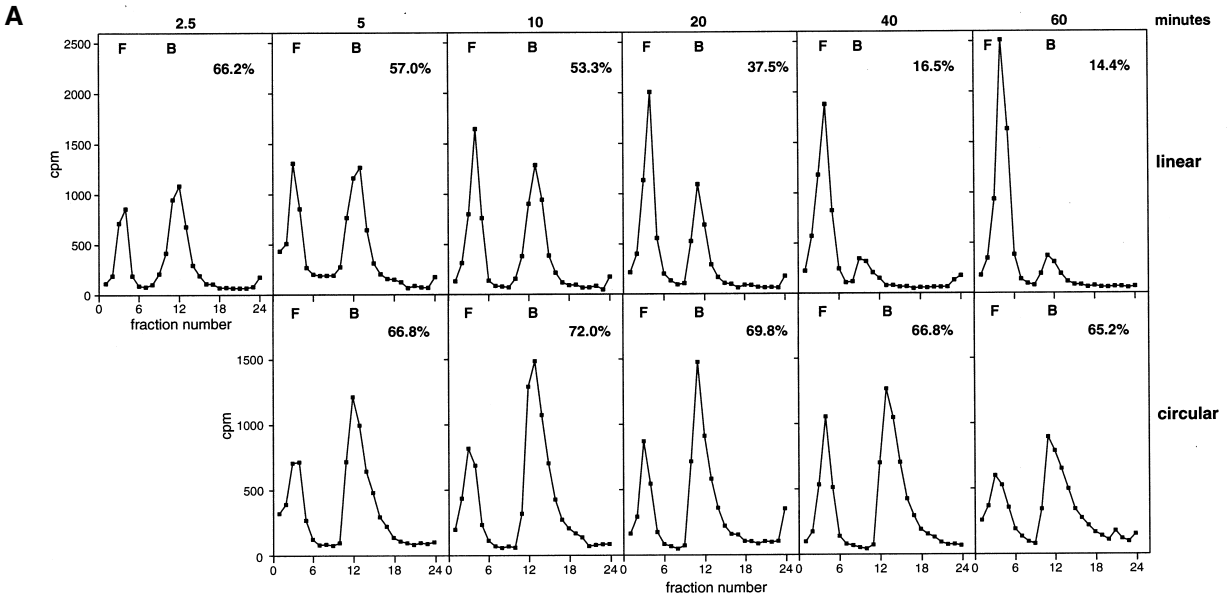


Figure 2. Comparing rates of RecA-mediated ATP hydrolysis in filaments formed on linear *versus* circular ssDNA. Reactions were carried out as described in Materials and Methods, containing 3 μM M13mp8.1037 ssDNA (circular or linearized as noted), 5 μM RecA protein, 0.3 μM SSB protein, and 3 mM ATP. Timepoints on which the lines are based were taken every 60 seconds.

that in the circular ssDNA reaction. The ATP hydrolysis rate gradually decayed to a much lower steady-state rate (4.2 μM min^{-1}) over a period of about 40 minutes. The decline in the ATP hydrolysis rate suggests that RecA protein dissociates from linear ssDNA. A final state is reached where only about 10% to 15% of the linear ssDNA is bound by RecA protein as measured in this indirect assay.

Dissociation of RecA protein from linear ssDNA was confirmed by sucrose gradient sedimentation assays (Figure 3). The 12 μM ssDNA used in these assays is enough to bind 80% of the 5 μM labeled RecA protein. The upper panel in Figure 3A shows RecA binding to linear ssDNA. In the 2.5 minute reaction, 66.2% of RecA was found in the DNA bound peak; the amount was decreased to 16.5% in the 40 minute reaction. Note that if 16% of the total RecA protein is bound, 20% of the total ssDNA is coated with RecA protein. In comparison, the gradient profiles in the bottom panel of Figure 3A show that the same amount of RecA was bound to circular ssDNA after either 5 or 40 minutes. Results from these and other timepoints are summarized in Figure 3B. The results show that the amount of RecA protein in RecA-linear ssDNA filaments is initially about the same as that in RecA-circular ssDNA filaments. RecA protein gradually dissociates from linear ssDNA, but stays bound to circular ssDNA. The dissociation rate of



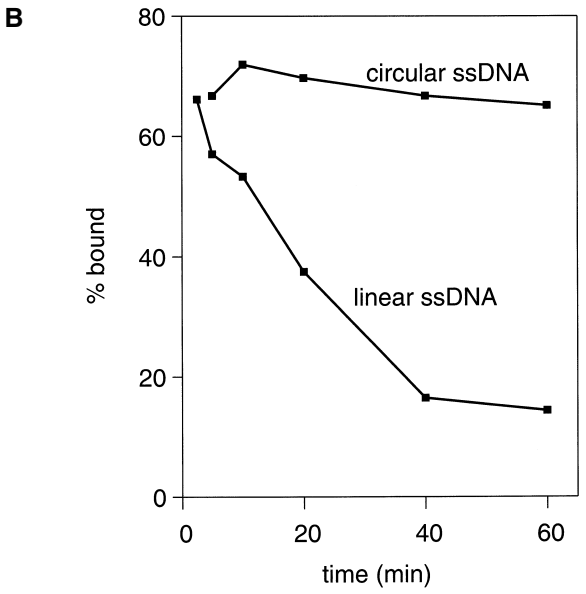


Figure 3. Sedimentation analysis of the relative stability of RecA filaments formed on linear *versus* circular ssDNA. A, Sucrose gradient sedimentation experiments. Reactions were carried out as described in Materials and Methods, using 12 μM M13mp8.1037 ssDNA, 5 μM ^{35}S-labeled RecA protein, 1.2 μM SSB protein, and 3 mM ATP. The amount of ssDNA is enough to bind 80% of the labeled RecA. In these gradients and those subsequent Figures, the top of the gradient is at the left and the first peak is the unbound (free) RecA protein. The labels F and B identify peaks representing free and DNA-bound RecA protein, respectively. Gradients in the upper row represent RecA-linear ssDNA binding reactions. The reaction times are 2.5, 5, 10, 20, 40, and 60 minutes from left to right, respectively. Gradients in the bottom row represent RecA-circular ssDNA binding reactions. The reaction times are 5, 10, 20, 40, and 60 minutes from left to right, respectively. The number in each gradient profile represents the amount of labeled RecA bound to ssDNA, calculated as described in Materials and Methods. B, Summary of sucrose gradient results from A, showing the percentage RecA protein bound to linear and circular ssDNA.

RecA protein from linear ssDNA can also be estimated from Figure 3B. If the decrease in binding from 5 to 40 minutes is averaged over all of the filaments present, the dissociation rate was about 55 RecA monomers min^{-1} filament^{-1}.

In the sucrose gradient experiments, ATPγS is added to stabilize the filaments prior to loading the gradients. Controls to determine if the added ATPγS causes any re-binding of released RecA protein to DNA are described below after the discussion of Figure 4. In the course of loading and running the gradients, the ATPγS is diluted 50-fold (to 20 μM). The early timepoints, where RecA protein binding to DNA is nearly complete, demonstrate that the protocol is sufficient to retain any RecA present on the DNA in a bound state. In addition to the added ATPγS, we assume that the high sucrose concentration and the low temperature conditions under which the gradients are run contribute to filament stability.

RecA could in principle dissociate from one end, both ends, or within a RecA-linear ssDNA filament. We attempted to distinguish between these possibilities by examining a time course of a RecA-linear ssDNA binding reaction using EM. Representative molecules are shown in Figure 4. At five minutes, the predominant species were RecA-coated filaments with short tails coated with SSB protein (Figure 4A), and 12 % of the molecules contained no RecA but appeared to be fully SSB-coated (Figure 4B). As time progressed, the length of the RecA filaments decreased as seen in Figure 4C and D. In many of these it appeared that there was a reciprocal relationship between RecA filament length at one end and the amount

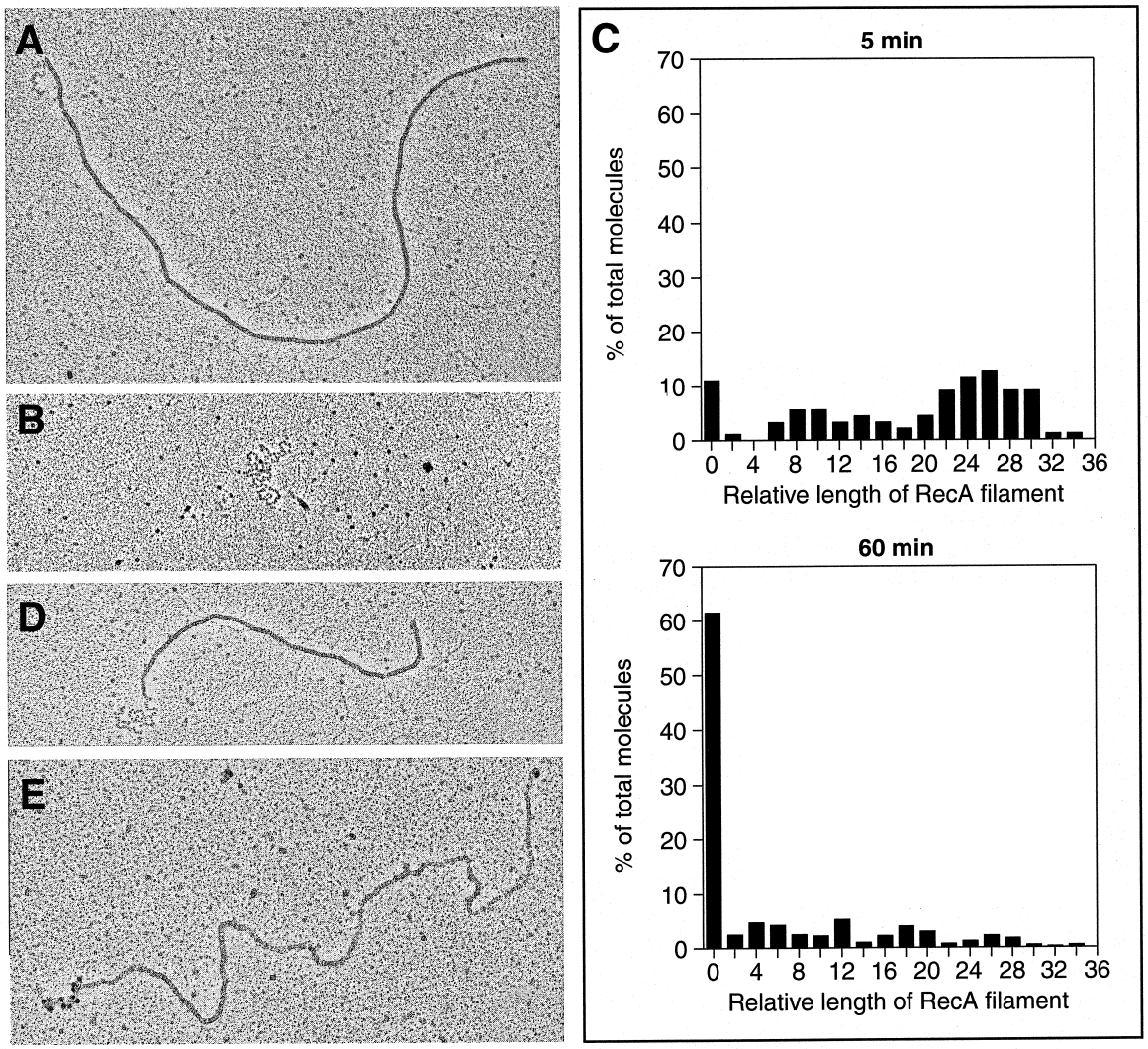


Figure 4. Visualization of RecA filaments on ssDNA molecules. Reactions containing 12 μM M13mp8.1037 linear ssDNA, 5 μM RecA, 1.2 μM SSB, and 3 mM ATP were carried out at 37°C, stopped by the addition of ATPγS to 1 mM, diluted 60-fold and spread as described in Materials and Methods. The molecules shown in the micrographs are representatives of the respective samples. A, RecA filament on linear ssDNA with a short SSB-coated end typically found after short incubations. B, SSB protein bound to linear ssDNA. C, Histograms showing the distribution of RecA filament lengths on linear ssDNA after five minutes and 60 minutes of incubation, judged as described in Materials and Methods. D, A shorter RecA filament on linear ssDNA with a longer SSB-coated end typically found after long incubations. E, An immunoaffinity gold labeling procedure confirming the presence of SSB protein on one end of the linear ssDNA (dark spheres clustered at the left end).

of SSB-coated ssDNA at the other end, although we cannot quantify this conclusion because the compact nature of the SSB-coated ssDNA makes accurate length measurements of the SSB-DNA complexes very difficult. The decrease in the length of the RecA filaments was not due to contaminating nucleases or physical breakage of the DNA molecules (data not shown). Figure 4C shows histograms of judged RecA filament lengths in these experiments. The filaments with relative lengths of between 20 and 30, which represent the major portion of those in the top panel of Figure 4C, are typified by the molecule shown in Figure 4A. The molecule shown in Figure 4D has a relative length of about 11 on the scale of the bottom panel of Figure 4C. The types shown with zero filament length correspond to SSB-coated ssDNA as shown in Figure 4B. After 60 minutes, SSB-coated molecules were the predominant species (63%). Few partial RecA-coated filaments had SSB-coated regions at both ends (less than 3% in all samples examined). The presence of SSB protein on one end of the linear ssDNA was confirmed by an SSB antibody-specific gold labeling procedure, with a representative molecule shown in Figure 4E. The net dissociation of RecA filaments is again not observed on circular ssDNA. After 30 minutes of reaction, the majority of RecA filaments on circular ssDNA were completely intact, while 10% were imperfect with short regions coated with SSB and 7% were fully coated with SSB (data not shown).

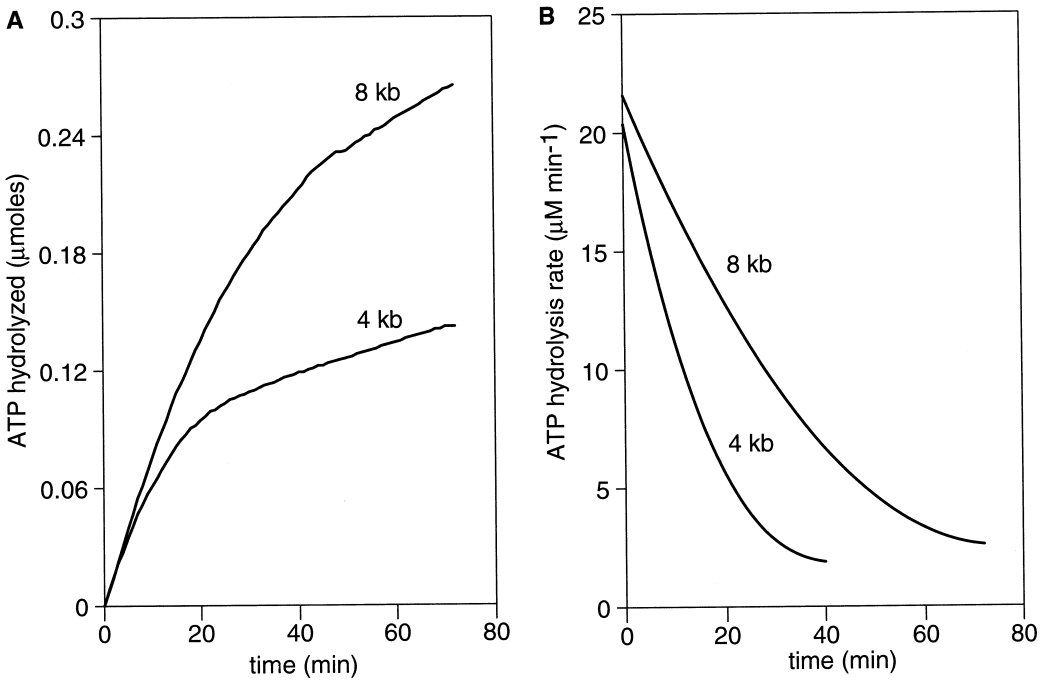


Figure 5. Dependence of RecA protein dissociation rate on the number of DNA ends. Reactions were carried out as described in Materials and Methods, using 3 μM M13mp8 linear ssDNA, 5 μM RecA protein, 0.3 μM SSB protein, and 3 mM ATP. One reaction (8 kb) contained full length M13mp8.1037 linear ssDNA (linearized by *Eco*RI, 8266 bases); the other (4 kb) contained two linear ssDNA fragments also derived from M13mp8.1037 (linearized by *Eco*RI and *Alw*NI, 4044 bases and 4222 bases), and contained twice as many ends (for the same concentration of DNA in nucleotides) as the full length DNA substrate. A, Time courses of ATP hydrolysis of RecA protein. Timepoints on which the lines are based were taken every 60 seconds. B, ATP hydrolytic rates of RecA protein derived from the data in A. Data in A were first fitted by polynomial regression and the first-order derivatives were calculated and plotted in B. Timepoints on which the lines are based were taken every 60 seconds.

RecA protein is known to bind to SSB-coated ssDNA in the presence of ATPγS, producing filaments promoting optimal DNA pairing reactions within ten minutes (Honigberg *et al.*, 1985; Menetski *et al.*, 1990). Since we are using ATPγS to stabilize filaments prior to sucrose gradient sedimentation or spreading for EM, any RecA protein re-binding occurring in the three minutes after ATPγS addition would lead to an over-estimation of total RecA binding at any time point. The amount of re-binding under our experimental conditions was estimated in several ways. First, the late timepoints serve as internal controls. Both the EM and sucrose density gradient experiments indicate that less than 20% of the ssDNA is bound with RecA protein after 40 to 60 minutes. These results compare well to the 10% to 15% binding estimated at late times from the ATPase assays (which does not require the addition of ATPγS). This indicates that re-binding, to the extent it occurs, is minimal under our experimental conditions. To place an upper limit to the extent of re-binding, RecA protein and ATPγS were added to SSB-coated linear ssDNA under the conditions of the EM experiments (without ATP), and the samples were spread three minutes later. In these trials, most DNA molecules had little or no RecA protein bound, but judgments made on those DNA molecules with RecA indicated that 20% of the total available ssDNA was coated with RecA protein. This represents an overestimate of the amount of re-binding in a typical experiment, since there is less RecA and SSB-coated ssDNA available in disassembly reactions which have only gone partially to completion. We have noted that ATPγS-stimulated re-binding is greatly reduced in samples in which RecA protein has previously been bound to ssDNA and hydrolyzing ATP (relative to reactions in which RecA filaments are assembled on ssDNA *de novo* in the presence of ATPγS without prior exposure to ATP). We have also checked the possible effects of ATPγS on the results shown in Figure 4 by simply omitting the ATPγS at the end of the reaction. Again the results confirm the large time-dependent decrease in the length of the RecA filaments. However, a quantitative comparison cannot be made because the filaments spread in the absence of ATPγS exhibit a substantial general reduction in RecA filament length (we tentatively attribute this to filament collapse as ATP is removed during spreading). We conclude that the rates of disassembly derived from the results of experiments like those in Figures 3 and 4 may be slightly underestimated. However, they are generally consistent with experiments in which filaments were not stabilized with ATPγS, and the degree of any underestimation is unlikely to be more than 10%

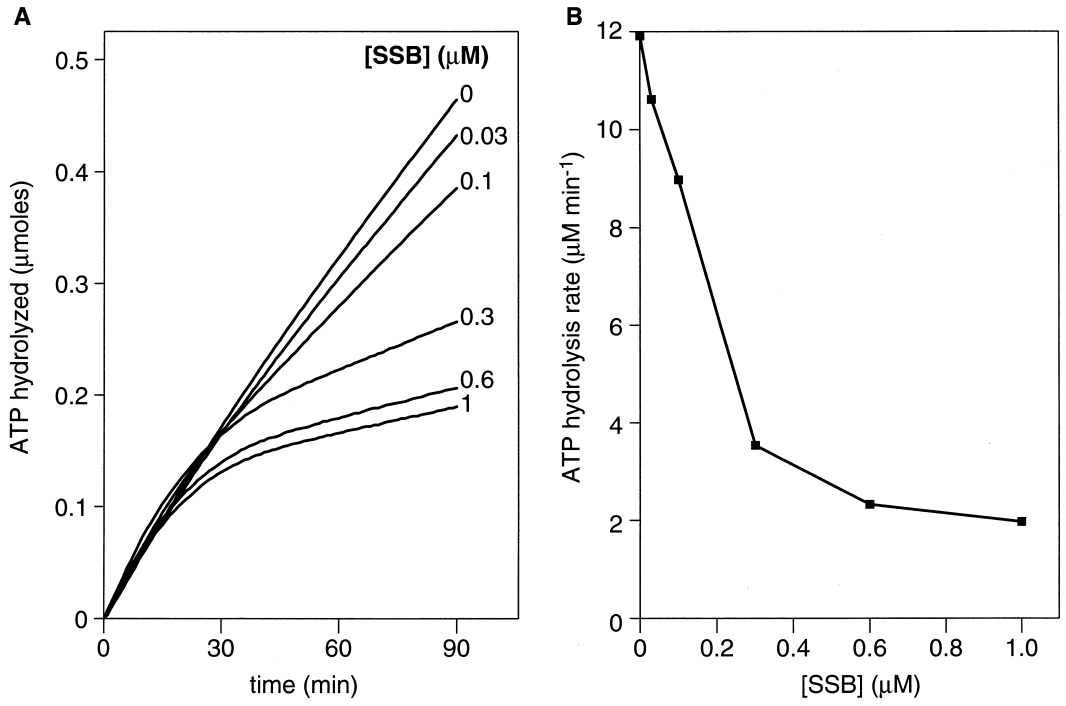


Figure 6. Effects of SSB concentrations on the stability of RecA-linear ssDNA filaments. Reactions were carried out as described in Materials and Methods, containing 3 μM M13mp8.1037 linear ssDNA, 5 μM RecA protein, and 3 mM ATP. The SSB protein concentration was varied from 0 to 1 μM. A, Time courses of ATP hydrolysis of RecA protein. SSB concentrations were 0, 0.03, 0.1, 0.3, 0.6, and 1 μM, from top to bottom, respectively. B, ATP hydrolysis rates at different SSB concentrations, calculated in the time interval from 60 to 80 minutes.

to 20%. As noted below, the rates of disassembly derived from the sucrose density gradient experiments are actually somewhat greater than those derived from the observed decline in ATPase activity.

The EM results reveal that RecA dissociates from only one end of RecA-linear ssDNA filaments in concert with SSB protein binding to the DNA binding sites vacated by the dissociated RecA protein. Dissociation from both ends would produce free or SSB-bound DNA at both ends, which is not observed at significant levels. The results additionally indicate that the RecA dissociation process is not perfectly synchronized on all filaments, although the vast majority undergo a steady decline in bound RecA protein.

Another way to demonstrate that the dissociation is end-dependent is to determine how the rate of dissociation is affected by the number of ssDNA ends. We tested this notion by comparing RecA dissociation rates in two RecA filament disassembly reactions in which the length of the ssDNAs, and consequently the number of DNA ends, was varied with the overall DNA concentration held constant in total nucleotides. In one reaction, the ssDNA was full length M13mp8.1037 (8266 bases); in the other reaction, the linear ssDNA substrate was reduced to two DNA fragments of half the size (4044 and 4222 bases). The time course for the ATPase activity of RecA filaments formed on these DNAs is shown in Figure 5. The RecA dissociation rates were estimated from these data in the following way. The time-dependent ATP hydrolysis curves in Figure 5A were first fitted by polynomial regression; the first-order derivatives of these equations, reflecting ATP hydrolysis rate at any given time, were plotted in Figure 5B. The slopes from the first ten minutes in Figure 5B, reflecting the time-dependent drop in ATPase rates, were calculated by least-squares linear regression. The ATP hydrolysis rate drop was doubled when the concentration of ends was doubled (4 kb fragments, 0.77 μM min^{-2}; compared to 8 kb fragments, 0.41 μM min^{-2}). The estimated dissociation rates are 36 monomers min^{-1} $filament^{-1}$ in the 4 kb reaction, and 38 monomers min^{-1} $filament^{-1}$ in the 8 kb reaction. These dissociation rates agree reasonably well with the 55 RecA monomers min^{-1} $filament^{-1}$ estimated from the sucrose gradient experiments, especially when the reduced accuracy of the sucrose gradient approach is factored in. When combined with other data, the results indicate that RecA protein dissociates progressively from one end of filaments bound to linear ssDNA.

We further assessed the role of SSB protein in this process by examining the concentration dependence of SSB on the stability of RecA linear-ssDNA filaments (Figure 6). Reactions contained 3 μM linear ssDNA, 5 μM RecA, and 3 mM ATP. SSB protein concentrations were varied from 0 to 1 μM, and reactions were initiated by addition of ATP. The time courses of ATP hydrolysis are shown in Figure 6A, and final rates calculated

from the steady state regions (from 60 minutes to 80 minutes) were plotted against SSB concentrations as shown in Figure 6B. In the absence of SSB protein, the ATP hydrolysis rate was constant, albeit slow (11.9 μM min^{-1}). This indicates that in the absence of SSB, complete binding of RecA to the ssDNA is precluded by secondary structure in the DNA, although the binding that does occur is maintained at a constant steady state. In the presence of SSB protein, ATP hydrolytic activity of RecA was stimulated at early times, and then declined to a much slower steady state rate. The optimal SSB concentration to stimulate RecA's ATP hydrolysis was 0.3 μM (one SSB monomer per ten nucleotides of ssDNA). The final steady-state ATP hydrolytic rates decreased with increasing SSB concentration, reaching a minimum as the SSB concentration exceeded 0.3 μM.

As noted in the Introduction, SSB inhibits the nucleation of filament formation but not filament extension. In the experiments described above, RecA protein is added to the linear ssDNA before the SSB, and the results can be interpreted in terms of two different effects of SSB. When RecA-linear ssDNA filaments are formed, SSB facilitates RecA filament extension by removing secondary structure in the ssDNA, leading to the transient stimulation of ATP hydrolysis. However, SSB will replace any RecA dissociating from a filament end and inhibit re-nucleation of filament formation. The result is a net disassembly of the RecA filaments.

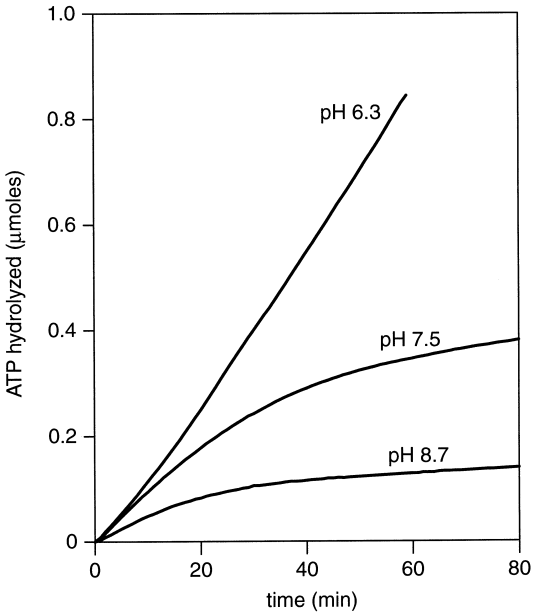


Figure 7. Effects of pH on the stability of RecA-linear ssDNA filaments. Reactions were carried out as described in Materials and Methods in a 25 mM buffer containing Mes-NaOH (56% −, pH 6.3), Tris-acetate (80% +, pH 7.5), or Tris-acetate (30% +, pH 8.7). Reactions also contained 3 μM M13mp8.1037 linear ssDNA, 5 μM RecA, 0.3 μM SSB, and 3 mM ATP. ATP hydrolysis of RecA protein was monitored by the coupled spectrophotometric assay. Timepoints on which the lines are based were taken every 60 seconds.

End-dependent RecA filament disassembly is pH-dependent

We were interested in the relationship of the disassembly reactions observed on ssDNA and dsDNA. Since the rate of end-dependent RecA filament disassembly from linear dsDNA increases with pH over the range of 6.5 to 8.0 (Lindsley & Cox, 1989), we examined the pH dependence of the disassembly reaction from linear ssDNA. Net disassembly was prevented when the RecA filaments were incubated at pH 6.3, as indicated by a stable rate of ATP hydrolysis. Net disassembly was observed at higher pH values, with maximal rates observed above pH 8. Representative results are presented in Figure 7. The results suggest that rates of end-dependent filament disassembly from linear DNAs are similar whether the DNA is single- or double-stranded.

Filament disassembly occurs from the filament end nearest the 5′ end of the ssDNA

If RecA filaments assemble in the 5′ to 3′ direction on ssDNA as demonstrated by Register & Griffith (1985), the data on end-dependent disassembly in this study is consistent only with a disassembly from the filament end nearest the 5′ end of the bound DNA. Filament formation would nucleate at random locations, leaving some SSB-bound DNA near the 5′ end. Disassembly from the 3′ end or both ends would then generate linear ssDNA molecules with unbound regions at both ends as reaction intermediates, an outcome that is not observed. The polarity of filament formation on ssDNA observed by Register & Griffith (1985) is quite unambiguous, and is consistent with a number of subsequent reports examining the polarity of filament assembly on dsDNA when nucleated on an adjacent single strand tail (Shaner & Radding, 1987; Lindsley & Cox, 1989). The only point of some contention was the failure of Register & Griffith (1985) to observe RecA protein bound to dsDNA adjacent to the ssDNA, under conditions where later work (Shaner & Radding, 1987; Lindsley & Cox, 1989, 1990a; Pugh *et al.*, 1989; Roca & Cox, 1996) indicated it should be present. More recent studies have shown that this reflects a technical problem. EM spreading procedures used in the 1985 study do not preserve RecA complexes formed on dsDNA in the presence of ATP†

The polarity of RecA filament assembly and disassembly processes is a parameter of sufficient im-

†For a discussion of the technical issues, see p. 423 of Roca & Cox (1990). Also see Shaner *et al.* (1987), and Register & Griffith (1988) for details.

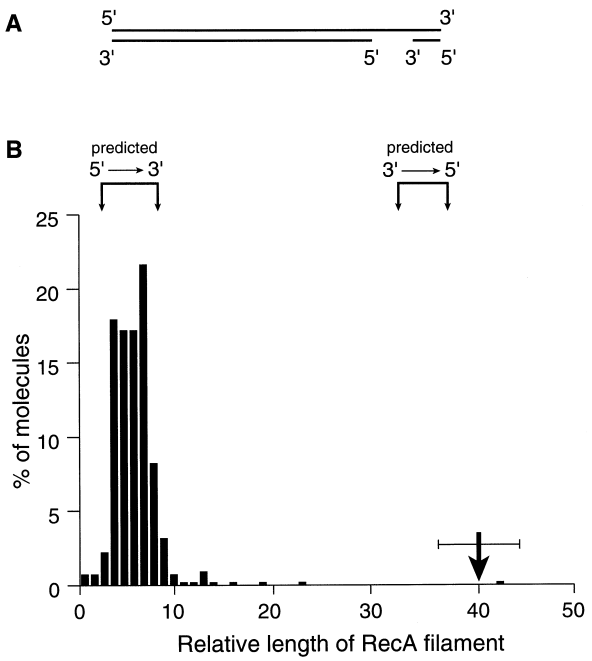


Figure 8. Polarity of RecA protein filament assembly. RecA protein filaments were formed on the DNA molecule illustrated in A, and examined by electron microscopy. Gapped DNA GD1037 was prepared as described (Shan *et al.*, 1996), then linearized by *Bgl*II cleavage. The resulting DNA contains a continuous strand of 8266 nucleotides, with 6556 and 673 bp of duplex DNA on the left and right ends, respectively. The gap is 1037 nucleotides in length. Reactions contained 6 μM of the gapped DNA, 2 μM RecA protein, 0.02 μM SSB, and 3 mM ATP under otherwise standard reaction conditions (pH 7.5, 37°C). Reactions were stopped after ten minutes by addition of ATPγS to 1 mM. The filaments were spread and filament lengths estimated as described in Materials and Methods. The lengths of filaments extending to one DNA end, representing 462 of the 498 linear RecA-DNA complexes examined and judged, are presented in B, with the bound DNA end oriented at the left. For standards, 66 full length filaments formed on circular gapped GD1037 DNA were measured within the same sample, giving a length of 40 ± 4 (arrow and standard deviation bar at the bottom right of the histogram). The predicted lengths of filaments, based on nucleation at any point within the gap and extension in the direction indicated relative to the single strand in the gap, are indicated along the top of the histogram. The observed lengths are consistent with nucleation in the gap, and extension to the right (5′ to 3′) on the DNA molecule as it is oriented in A.

portance that confirmation of the direction of assembly (and thereby the direction of disassembly) seemed warranted. We therefore carried out the experiment presented in Figure 8. The experimental design reflects earlier studies demonstrating that the binding of RecA protein to duplex DNA at pH 7.5 is limited by a very slow nucleation step (Pugh & Cox, 1987, 1988). Nucleation on dsDNA is facilitated by a number of DNA structural features, with by far the most effective nucleation sites being single-strand gaps (Shaner & Radding, 1987; Lindsley & Cox, 1989, 1990a). A linear duplex DNA molecule was constructed with an asymmetrically placed gap. The single-stranded DNA in the gap provides a point where RecA filament formation can be nucleated, followed by extension into adjacent dsDNA. If extension follows the strand in the gap in the 5′ to 3′ direction, the resulting filaments should cover 673 to 1710 nucleotides or base-pairs, corresponding to three to eight length units in the histogram of Figure 8. This assumes that filament nucleation occurs anywhere within the gap. A 3′ to 5′ extension would yield much longer filaments (32 to 37 length units). The experiment uses ATP, along with a short (ten minutes) incubation time and a low SSB concentration to minimize disassembly. Filament lengths were estimated using as standards full-length filaments formed on gapped circular DNA, representing about 12% of the molecules in the sample as a result of incomplete linearization of the gapped DNA by restriction digestion (the circular molecules are not included in the counts below).

The histogram of Figure 8B presents the judged lengths of 462 linear DNA molecules with RecA filaments extending to one DNA end. Of these, 93% fall within the range predicted for a 5′ to 3′ polarity of filament extension. There is a sharp cut-off at eight to nine length units. In addition to the molecules plotted, there were 36 molecules found in the overall sample of 498 linear complexes that were anomalous. Of these 13 had either two filamented regions or exhibited some uncoated DNA at both ends, and 23 had a RecA filament extending over the entire length of the DNA. We suspect, but cannot prove, that the latter class reflect coated circular DNA molecules that were broken during the preparation for electron microscopy.

The results in Figure 8 are consistent with a filament extension proceeding 5′ to 3′ relative to the single strand in the gap, as expected from the work of Register & Griffith (1985). The results in Figure 4 are consistent with a filament disassembly process that occurs on the end opposite to that at which filament assembly occurs, such that the disassembly must occur on the filament end nearest to the 5′ end of the ssDNA. This result provides another link to the disassembly process previously characterized on dsDNA (Lindsley & Cox, 1990a).

End-dependent RecA filament disassembly is prevented by dATP

The stability of RecA-linear ssDNA filaments in the presence of ATP or dATP was monitored by the ATPase assay. As shown in Figure 9, ATP hydrolysis gradually declined over time as previously shown in Figure 2. However, the dATP hydrolysis rate (34.6 μM min^{-1}) was constant

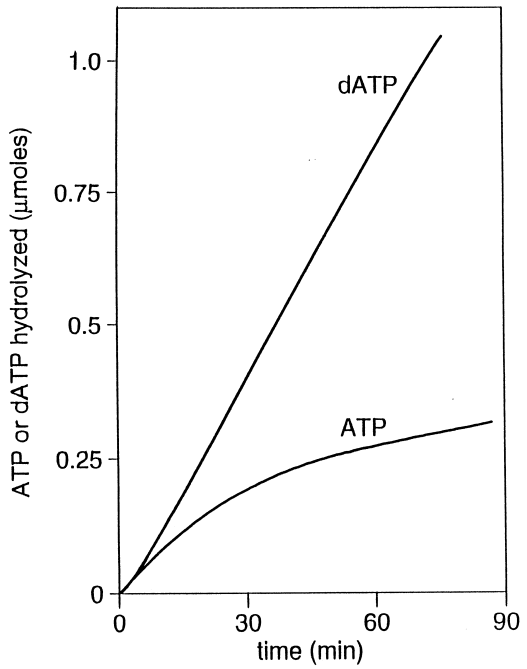


Figure 9. Effects of nucleotide cofactors on the stability of RecA-linear ssDNA filaments. Reactions were carried out as described in Materials and Methods, containing 3 μM M13mp8.1037 linear ssDNA, 5 μM RecA protein, 0.3 μM SSB, and 3 mM ATP or dATP. ATP or dATP hydrolysis was monitored by the coupled spectrophotometric assay. The dATP hydrolysis was constant during the entire time course, and the dATP hydrolysis rate was 34.6 μM min^{-1}.

during the entire 90 minute time course, indicating an absence of net disassembly.

A time course of RecA-linear ssDNA binding reaction in the presence of dATP was also examined by electron microscopy (data not shown). There is no detectable difference in physical appearance between filaments formed in the presence of ATP or dATP, except for the lengths of RecA coated regions. The predominant species present throughout the 40 minute time course was linear ssDNA that was fully RecA-coated. A minority of the filaments (18% and 31% of 198 molecules counted in each of two experiments at five minutes, and 53% of 75 molecules counted at 40 minutes) had very short ssDNA tails. In virtually every case, the ssDNA tails were so short that they were nearly at the resolution limit of these samples, and they did not lengthen with time. The single-strand tails were clearly very much shorter than those observed with ATP, and indicate that RecA protein filaments do not undergo end-dependent dissociation from linear ssDNA in the presence of dATP.

We also examined the disassembly reaction in the presence of a more physiological mixture of ATP and dATP (3 mM and 175 μM, respectively, as reported by Bochner & Ames (1982)). Under these conditions, dATP did not prevent disassembly (data not shown) as it does when ATP is not present.

End-dependent RecA filament disassembly is prevented by the RecO and RecR proteins

The linear ssDNA-dependent ATP hydrolysis of RecA protein in the absence or presence of the RecO and RecR proteins is shown in Figure 10A. Reactions were otherwise identical to those in Figure 2. The RecO and RecR proteins were each present at levels equivalent to one monomer per 40 and 16.7 nucleotides of ssDNA, respectively, and were added to the reaction as a mixture with ATP and SSB. The ATP hydrolysis rate in the presence of the RecO and RecR proteins (29.1 μM min^{-1}) was constant over the 90 minute time course, and was the same as the rate when circular ssDNA was used (29.7 μM min^{-1}, from Figure 2). These results suggest that RecA protein forms stable filaments on linear ssDNA in the presence of the RecO and RecR proteins.

The conclusion drawn from the ATPase assays was further supported by the sucrose gradient sedimentation assays (Figure 10B). The 12 μM ssDNA used in these assays was again enough to bind 80% of the 5 μM labeled RecA protein. In the presence of the RecO and RecR proteins, the linear ssDNA was more stably and completely bound than under any other set of conditions used in this study. Over a 40 minute time course, about 85% of the labeled RecA protein was consistently found associated with the "bound" peak in the gradients. The sucrose gradient fractions at the ten minute time point were also analyzed by Western blot using SSB antibody or RecR antibody (data not shown). Both SSB protein and RecR protein were detected in the ssDNA bound fractions. The peaks of RecR and SSB proteins agreed with the bound RecA protein peak shown in Figure 10B. The results suggest that the resulting RecA filaments also include SSB, and RecR proteins, in agreement with the results of Umezu & Kolodner (1994). As reported by Umezu & Kolodner (1994), the complexes also probably contain RecO protein. We did not directly test for the presence of RecO protein since RecO antibodies were not available.

When RecA-linear ssDNA filaments formed in the presence of the RecO and RecR proteins were examined by EM, virtually all the ssDNA molecules were fully coated by RecA protein, as illustrated by the representative filament in Figure 9C. Less than 4% of the molecules surveyed had detectable SSB-coated ends. There is no discernible difference in physical appearance between filaments formed in the presence or the absence of the RecO and RecR proteins, except for the absence of SSB-coated ssDNA at an end. We can therefore not detect the RecO and RecR proteins simply by visualization in these experiments. About 15% of the examined filaments were broken, as indicated by a shorter length of these fila-

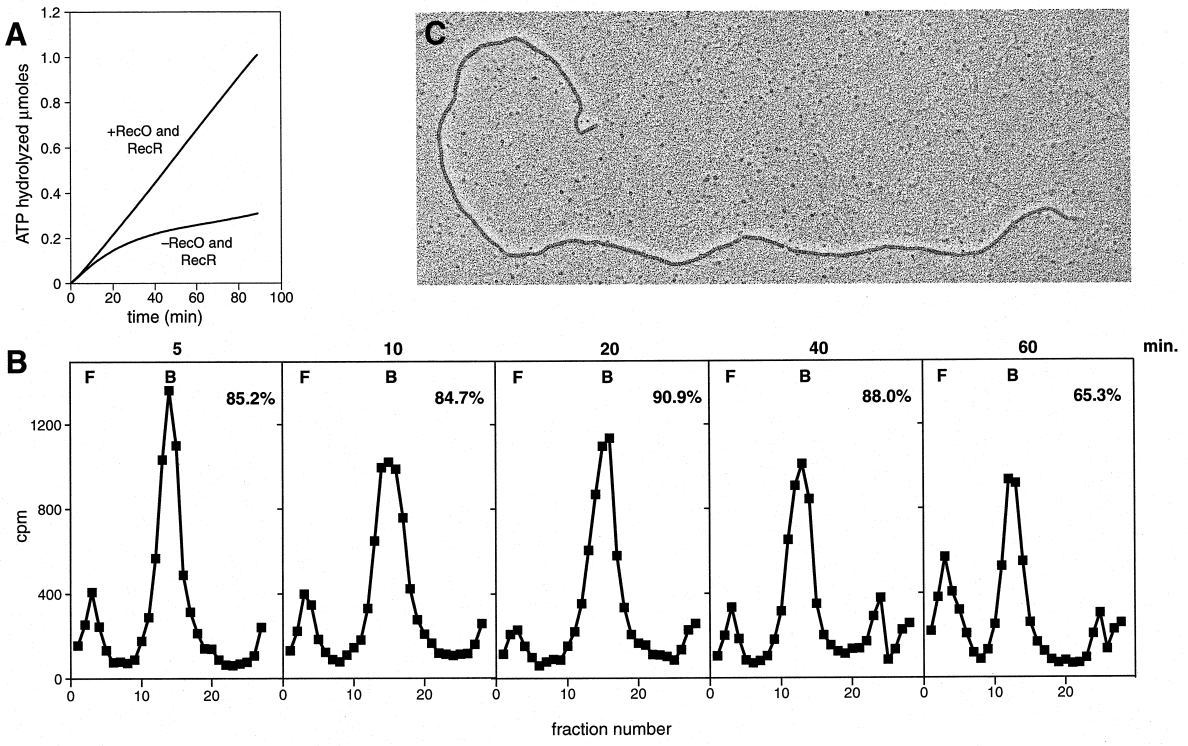


Figure 10. Facilitation of RecA protein filament formation on linear ssDNA by the RecO and RecR proteins. A, ssDNA-dependent ATPase assays. Reactions were carried out as described in Materials and Methods, containing 3 µM M13mp8.1037 linear ssDNA, 5 µM RecA protein, 0.3 µM SSB protein, 75 nM RecO protein, 180 nM RecR protein, and 3 mM ATP. Timepoints on which the lines are based were taken every 60 seconds. B, Sucrose gradient sedimentation assays. Reactions were carried out as described in Materials and Methods, using 12 µM M13mp8.1037 ssDNA, 5 µM ^{35}S-labeled RecA protein, 1.2 µM SSB protein, 0.3 µM RecO protein, 0.72 µM RecR protein, and 3 mM ATP. The ssDNA concentration is enough to bind 80% of labeled RecA. Gradient profiles represent RecA-linear ssDNA binding reactions incubated for 5, 10, 20, 40, and 60 minutes, from left to right, respectively. The number in each gradient profile represents the amount of labeled RecA bound to ssDNA, calculated as described in Materials and Methods. C, Electron micrograph showing a RecA filament stabilized on linear ssDNA by the presence of the RecO and RecR proteins. The reaction contained 12 µM M13mp8.1037 linear ssDNA, 5 µM RecA, 1.2 µM SSB, 3 mM ATP, 300 nM RecO, and 720 nM RecR, was stopped by the addition of ATPγS to 1 mM, diluted 60-fold and spread as described in Materials and Methods.

ments. This shortened length was not due to nuclease contamination, DNA-independent filament formation, or folding back of part of the DNA within the filaments (data not shown). It is possible that the RecA-ssDNA filaments become more rigid in the presence of the RecO and RecR proteins so that the filaments become more susceptible to mechanical breakage during spreading for EM.

We next investigated whether the stabilizing effects of RecO protein and RecR protein are synergistic or additive. RecA-linear ssDNA binding reactions (similar to that shown in Figure 2) were carried out in the presence of either the RecO or RecR proteins individually. Formation of RecA-linear ssDNA filaments was monitored by the ATPase assay, as shown in Figure 11A. The decline in the rate of ATP hydrolysis was not relieved by either RecO protein or RecR protein alone. Similar experiments containing only RecO or RecR protein were examined by EM after 30 minutes of reaction, and less than 5% of the molecules had full-length RecA filaments (data not shown), similar to results obtained with RecA alone. In comparison to the control reaction without RecO and RecR protein, ATP hydrolysis was not affected by RecR protein, whereas it declined somewhat faster when RecO protein was present without RecR. However, when the other protein was added at a time point after which a majority of the RecA protein had dissociated, the rate of ATP hydrolysis quickly recovered to its maximum after a short lag. The same result was also obtained when a mixture of RecO and RecR was added together to a reaction after most of RecA was dissociated, as shown in Figure 11B. The results indicate that both the RecO and RecR proteins are required for the stabilizing effects observed with RecA filaments. The order in which RecO and RecR are added to the reaction does not affect the result. As with most experiments described above, the conclusions derived from the results of Figure 11 were fully confirmed with the sucrose gradient assay and with electron microscopy (data not shown).

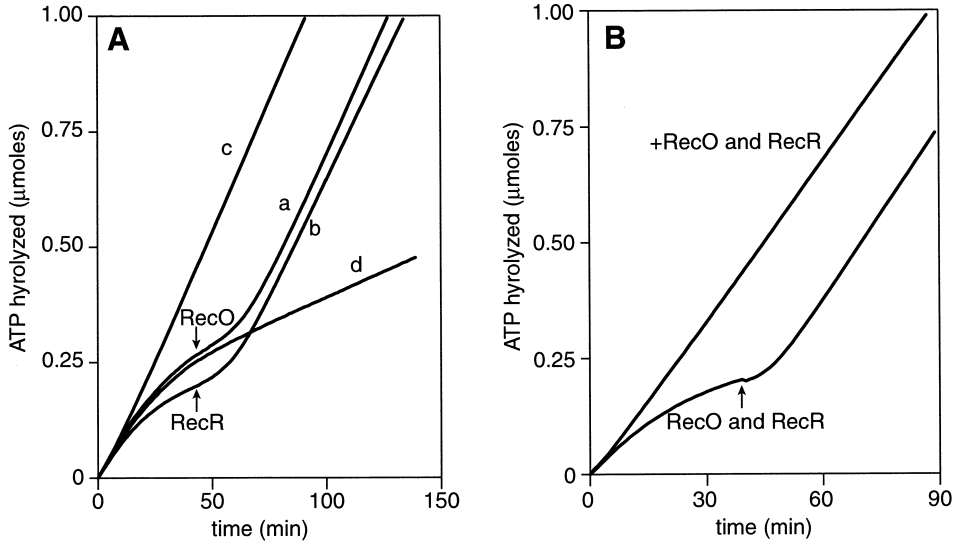


Figure 11. Effects of RecO protein or RecR protein on the stability of RecA-linear ssDNA filaments. A, Linear ssDNA-dependent ATPase assay. Reactions were carried out as described in Materials and Methods, containing 3 μM M13mp8.1037 linear ssDNA, 5 μM RecA protein, 0.3 μM SSB protein, and 3 mM ATP. Reactions also contained 75 nM RecO protein and/or 180 nM RecR protein as indicated. The ATP hydrolysis of RecA protein was followed spectrophotometrically. Timepoints on which the lines are based were taken every 60 seconds. The reactions labeled a and b were initiated only with RecR or RecO protein, respectively. ATP hydrolytic activities of RecA protein were attenuated to a very low level after about 40 minutes. RecO protein (to a) or RecR protein (to b) was then added as indicated by the vertical arrows in the plot. After a short lag, the ATP hydrolysis rates were restored to 27.5 μM min^{-1} and 25.8 μM min^{-1}, for reactions a and b, respectively. In one control reaction (c), the RecO protein and RecR protein were added together in the initial reaction mixture, and ATP hydrolysis rate was 27.9 μM min^{-1}. In a second control reaction (d), neither RecO nor RecR protein was added. B, Linear ssDNA-dependent ATPase assay. A similar reaction as in A was initiated in the absence of the RecO and RecR proteins. The RecO and RecR proteins were added to the reaction as indicated by the arrow. The steady-state ATP hydrolysis rate after RecOR addition was 29.2 μM min^{-1}; whereas the ATPase rate of the control reaction in which the RecO and RecR proteins were included in the initial reaction mixture was 31.3 μM min^{-1}.

The amounts of RecO and RecR proteins required to bring about the stabilizing effect was investigated. The minimal amount of RecO protein required to stabilize RecA-linear ssDNA filaments was determined by titrating RecO protein in the presence of RecR protein. The stability of RecA-linear ssDNA filament was monitored by the ssDNA-dependent ATPase assay (Figure 12). Reactions contained 3 μM linear ssDNA, 5 μM RecA, and 0.3 μM SSB. The RecR protein concentration was kept constant at 180 nM (one RecR monomer per 16.7 nucleotides of ssDNA) whereas the RecO protein concentration was varied from 0 to 75 nM. The time courses of ssDNA-dependent ATPase in the presence of different amounts of RecO protein are shown in Figure 12A. The decline in the rate of ATP hydrolysis was alleviated by the presence of an increasing amount of RecO protein. The final steady-state ATP hydrolysis rates (determined from data gathered after 60 minutes of reaction) were plotted against RecO protein concentration as shown in Figure 12B. The ATP hydrolysis rate increased with an increasing amount of RecO protein, and approached its maximum when RecO was over 30 nM. With 75 nM RecO protein, the ATPase rate was 28.0 μM min^{-1}. The apparent stoichiometry of RecO protein to ssDNA required for the stabilizing effects was about one RecO monomer per 143 nucleotides of ssDNA.

Similar titration experiments were done using different amounts of RecR protein in the presence of RecO protein. In this set of experiments, the RecO protein concentration was held constant at 75 nM, and RecR protein concentration was varied from 0 to 180 nM. The time courses and steady-state rates are summarized in Figure 12C and 12D, respectively. The decline in ATP hydrolysis was also alleviated as the concentration of RecR protein increased. The ATPase rate increased linearly when the RecR concentration was lower than 36 nM, and approached its maximum when RecR exceeded 72 nM. The ATP hydrolysis rate was 28.4 μM min^{-1} in the presence of 180 nM RecR. The stoichiometry between RecR protein and ssDNA derived from Figure 12D was one RecR monomer per 83 nucleotides of ssDNA.

Results from these experiments show that the RecO and RecR proteins act together, in a ratio that is, within experimental error, 1:2. We note that due to differences in the methods used to measure protein concentration (see Materials and Methods), the RecR protein concentration is known to a higher degree of confidence than the RecO protein concentration, and this stoichiometry may change somewhat when an extinction coeffi-

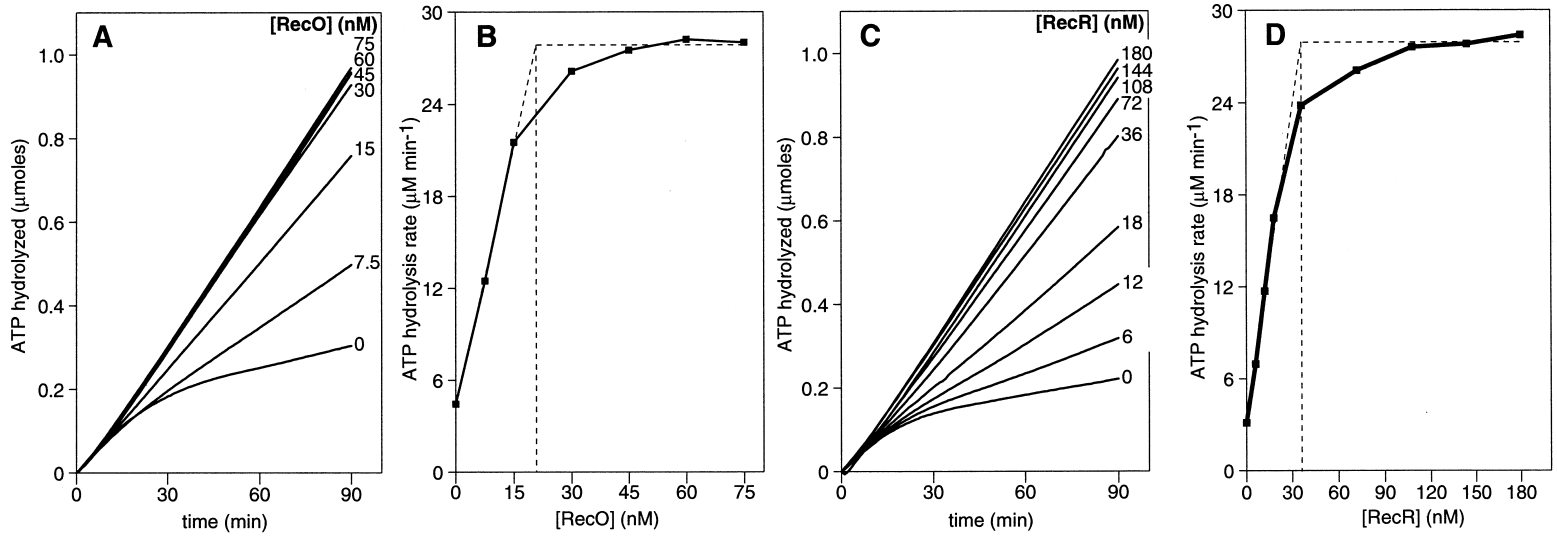


Figure 12. Stoichiometries between RecO, RecR and ssDNA. Reactions contained 3 μM M13mp8.1037 linear ssDNA, 5 μM RecA protein, 0.3 μM SSB protein, and 3 mM ATP. The RecA filament formation on linear ssDNA was followed by monitoring ATP hydrolysis. Timepoints on which the lines are based were taken every 60 seconds. A, RecO protein titration. Reactions contained 180 nM RecR protein, and RecO concentration was varied from 0 to 75 nM. Curves from bottom to top represent reactions containing 0, 7.5, 15, 30, 45, 60, and 75 nM RecO protein, respectively. B, ATP hydrolysis rates as a function of RecO concentration, derived from data in A. C, RecR protein titration. Reactions contained 75 nM RecO protein, and RecR concentration was varied from 0 to 180 nM. Curves from bottom to top represent reactions containing 0, 6, 12, 18, 36, 72, 108, 144, and 180 nM RecR protein, respectively. D, ATP hydrolysis rates as a function of RecR concentration, derived from data in C. Estimates of the stoichiometric requirements for RecO or RecR protein cited in the text are based on the broken lines in B and D.

cient becomes available for RecO. The proteins are required at concentrations well below the concentration of RecA protein. We do not know how the RecO and RecR proteins are associated with, or distributed in, the RecA filaments.

The requirements for each protein could reflect a functional stoichiometry. Alternatively, there might be a critical concentration of each protein required to form an active complex. To address this question for the RecO protein, additional titrations were conducted in which the RecA-ssDNA filament concentration was changed by increasing or decreasing the ssDNA concentration. Other conditions remained the same as those used in the experiments of Figure 12. When the ssDNA concentration was halved or doubled, the requirements for RecO protein were approximately halved or doubled as well (data not shown). RecR protein was maintained at levels that were saturating in these trials. Equivalent experiments were carried out to examine the requirement for RecR protein with similar results (data not shown). We tentatively conclude that the RecO and RecR proteins are required in amounts proportional to the concentration of RecA nucleoprotein filaments. The results of Figure 12 do not reflect a critical concentration for the formation of some active complex involving RecO and R.

Discussion

There are two primary conclusions arising from this study. First, RecA filaments formed on ssDNA will undergo a progressive disassembly from the 5′ end if such an end is available. The rate of disassembly at 37°C is approximately 35 to 40 RecA monomers min^{-1} $filament^{-1}$ at pH 7.6. The disassembly reaction rate increases with pH over the range of 6.3 to 8.7. Second, the end-dependent disassembly can be prevented by substituting dATP for ATP as the nucleotide cofactor, or by including modest amounts of both the RecO and RecR proteins in the reaction. These results are relevant to several divergent topics within the enzymology of recombinational DNA repair.

Disassembly of RecA filaments

We believe that this study completes a catalogue of RecA disassembly pathways. There are three general processes by which RecA monomers can dissociate from a filament bound to DNA. The first is the general disruption of filaments that occurs when ADP levels increase so that the ADP/ATP ratio approaches 1.0 (Cox *et al.*, 1983; Lee & Cox, 1990a,b). It is not clear that this ever occurs *in vivo*, so that the physiological relevance of this disassembly process is questionable.

If ATP is adequately regenerated there are only two disassembly pathways. One is an end-dependent dissociation of RecA monomers or some other protomeric unit. Disassembly is not difficult to detect, and the lack of previous reports on this process might be attributed to the prevalence of circular ssDNA substrates in RecA studies coupled to a requirement for the inclusion of SSB to observe a net disassembly of RecA filaments.

The results indicate that disassembly occurs from the filament end nearest the 5′ end of the DNA. The results shown in Figure 8 reinforce the conclusion of Register & Griffith (1985) that assembly proceeds 5′ to 3′. During disassembly, free DNA is observed only at one end of the filaments, a result that is most consistent with disassembly only on the end opposite to that at which assembly occurs. Removal of RecA monomers from the 5′ end of the nucleoprotein filament, as defined by the bound DNA, would produce a disassembly process with the same 5′ to 3′ polarity as assembly. Disassembly of RecA filaments from both ends would generate complexes with free DNA on both ends as intermediates, and these are not observed. There is only one other possibility one might consider that could generate filament disassembly such that free DNA appeared only at one end. Filament assembly would have to begin precisely at the 5′ end (so as to leave no unbound DNA), and proceed 5′ to 3′ until it reaches a DNA end. Then some abrupt change in the binding equilibrium would have to occur on the assembly end of the filament such that disassembly would then proceed uniquely from that same end (3′ to 5′). At least three considerations make it highly unlikely that our results could reflect a 3′ to 5′ disassembly of RecA filaments from ssDNA. First, ATP is hydrolyzed uniformly throughout the filaments during these experiments (Brenner *et al.*, 1987), and ATP is regenerated in the reaction mixture. There is no change in reaction conditions that might produce the dramatic shift in the RecA binding equilibrium required in this scenario. Second, it has been demonstrated that assembly and disassembly of RecA filaments on dsDNA occurs at opposite filament ends (Lindsley & Cox, 1990a), and it seems reasonable to expect the process on ssDNA to follow suit. Finally, the 3′ to 5′ disassembly scenario would remove RecA protein uniquely from the 3′ end of the DNA. This is inconsistent with the strong bias favoring RecA-mediated DNA pairing at 3′ rather that 5′ ends of linear ssDNA observed in several studies (Dutreix *et al.*, 1991; Konforti & Davis, 1991, 1992).

The second dissociation pathway is the exchange between free and bound RecA monomers in filament interiors as characterized in a recent study (Shan & Cox, 1996). This exchange occurs at very low levels in filaments on ssDNA in the presence of ATP, and not at all in the presence of dATP. The exchange occurs at much higher levels in filaments bound to dsDNA, providing evidence for a substantial change in filament state brought on when a second DNA strand is bound (Shan & Cox, 1996). The mechanism of the internal exchange process is unclear. It may be a simple dissociation and replacement process, or it may reflect a direct transfer of monomers between a

dsDNA bound-RecA filament and an unbound but structurally organized aggregate or filament of RecA protein with which it comes into transient contact. Previous work has demonstrated that direct exchange of RecA monomers between two nucleoprotein filaments in transient contact can be detected even when exchange between free and bound forms cannot be detected (Neuendorf & Cox, 1986).

We do not know the aggregation state of RecA protein that is not bound to DNA under the precise conditions of these experiments, or how it might be affected by RecO and RecR proteins or dATP. This represents another parameter that clearly must be better defined before a complete description of filament assembly/disassembly processes can be obtained. Several studies on the RecA aggregation state and its dependence on solution conditions (Brenner *et al.*, 1988, 1990) have not resolved the question of which species is/are active in assembly/disassembly.

The end-dependent filament assembly and disassembly processes must proceed with some equilibrium not yet measured, and it is logical to assume that some disassembly must occur at the assembly end and *vice versa* under at least some conditions. However, with the methods and conditions used to date, the assembly and disassembly ends of a RecA filament seem to be remarkably well-defined. As pointed out elsewhere, conditions that favor the growth of a filament at one end but disassembly at the other are unlikely or impossible without an input of chemical energy (Wegner, 1976; Lindsley & Cox, 1990a). The disassembly reaction does not occur in the absence of ATP hydrolysis, and must be viewed as one outcome of an ATP hydrolytic event in a RecA monomer at the filament end nearest the 5′ end of the ssDNA. At the same time, ATP hydrolysis is not tightly coupled to dissociation, and dissociation should not be viewed as the only function of ATP hydrolysis. ATP hydrolysis occurs throughout RecA filaments in monomers where no dissociation occurs. In the presence of dATP, which is hydrolyzed somewhat faster than ATP, no filament disassembly or monomer exchange reactions are observed at all in filaments formed on ssDNA (Shan & Cox, 1996; this work). We do not know if this effect of dATP is physiologically significant, since mixtures of ATP and dATP designed to mimic *in vivo* levels of both nucleotides do not prevent disassembly.

The disassembly process in the presence of ATP is clearly pH-dependent. The increase in the rate of filament disassembly from ssDNA with increasing pH at least qualitatively parallels that observed for the disassembly of filaments from linear dsDNA. This suggests that the processes are closely related and that the mechanism of filament disassembly is similar regardless of whether one or two DNA strands are bound within it. A detailed comparison of disassembly from ssDNA and dsDNA will appear elsewhere.

With respect to SSB, the results are also compatible only with a scenario in which SSB inhibits nucleation of RecA filament formation but is readily displaced by RecA filament extension. Observation of a net disassembly of RecA filaments on ssDNA requires SSB. SSB bound near the 5′ end of a linear ssDNA inhibits RecA nucleation, and it cannot be displaced because RecA filament extension does not proceed in the 3′ to 5′ direction. The net effect is to render the dissociation largely irreversible. Some low levels of RecA binding to the ssDNA persist only because the inhibition of RecA nucleation by SSB is not absolute.

The function of the RecO and RecR proteins

It is clear from previous work that the RecO and RecR proteins greatly stimulate the binding of RecA protein to SSB-bound ssDNA (Umezu & Kolodner, 1994). The present work extends these results in demonstrating a continuing function for RecO and RecR proteins after the filaments have been formed. The stabilizing effects of these proteins may reflect a direct interaction between the RecO and RecR proteins and RecA protein that enhances the overall filament stability. Alternatively, the RecO and RecR proteins could simply act as nucleation sites to promote rapid re-binding of any RecA protein that dissociates. In principle, the RecO and RecR proteins could be distributed throughout the filaments, or they could simply bind and stabilize the RecA protein at the 5′ end of the DNA. The titration experiments indicate that about 100 monomers of RecR protein and perhaps half as much RecO protein are required to stabilize each filament formed on our 8266 nucleotide ssDNA substrate. End-bound aggregates of RecO and RecR proteins of that size seem unlikely and are not evident in the electron micrographs, although one could imagine a filamentous structure of RecO and RecR bound near the 5′ end of the ssDNA and contiguous with the RecA filament. At present we feel the data are more consistent with a distribution of the RecO and RecR proteins throughout the RecA filament. It may be appropriate to think of a functionally integrated RecAOR filament. Many implications of this idea remain to be investigated, and the effects of other proteins which might affect this complex (such as the RecF protein) remain to be determined.

Previous results (Umezu & Kolodner, 1994) indicated that RecA filaments formed on SSB-coated circular ssDNA in the presence of the RecO and RecR proteins contain only 70% of the amount of RecA protein normally present at saturation. One implication is that some of the RecA protein is replaced by the RecO and RecR proteins. However, our results refine and alter this picture to some degree. First, rates of RecA-mediated ATP hydrolysis change less than 10% when RecO and RecR proteins are included in the reaction. Sucrose gradient assays indicate that there is about the same amount of RecA in the RecA filaments

formed in the presence of RecO and RecR proteins as in a RecA filament formed on circular ssDNA (Figures 3 and 10). Finally and most importantly, the RecA filaments formed in the presence of the RecO and RecR proteins appear to be fully coated by RecA protein when visualized by electron microscopy, although relatively small amounts of RecO and RecR proteins distributed in the filament might not be readily apparent. The small quantitative discrepancies between our results and the earlier work are probably explained by the many differences in the assays and DNA substrates employed.

The role of 3′ *versus* 5′ single-stranded DNA ends in recombinational processes

Many recent efforts to reconstitute portions of homologous recombination reactions *in vitro* have stressed the importance of prepared regions of single-stranded DNA with free 3′ ends in the initiation of genetic exchanges (Dixon & Kowalczykowski, 1991; Kowalczykowski, 1994; Smith, 1994). In bacteria, the *in vivo* evidence that 3′ ends are more recombinogenic or invasive is limited. The observation that sbcB mutations, which affect the 3′ to 5′ exonuclease I, restore recombination levels in RecBC mutants (Clark, 1971; Clark & Sandler, 1994; Phillips *et al.*, 1988; Smith, 1989) has often been cited as evidence that the generation and protection of 3′ ends is important. However, some mutations or deletions which eliminate exonuclease I function do not restore recombination in *recBC* mutants (Razavy *et al.*, 1996). A key *in vitro* observation relevant to the role of 3′ ends has been the 5′ to 3′ assembly of RecA filaments. A number of studies showed that the 3′ ends of linear ssDNA were used more efficiently in RecA-mediated DNA pairing reactions than 5′ ends (Dutreix *et al.*, 1991; Konforti & Davis, 1991, 1992).

These results have been explained to date by the polarity of RecA filament formation. Based on earlier studies (Register & Griffith, 1985; Thresher *et al.*, 1988), RecA filaments were presumed to nucleate at random along the ssDNA, and extend from there to the 3′ end. For this reason, most molecules of ssDNA would lack RecA over some segment of DNA at the 5′ end, but almost all would have RecA bound at the 3′ end. This would lead to a RecA-based bias in the pairing reactions in favor of the 3′ ends (Kowalczykowski, 1994; Smith, 1994). Previous *in vitro* studies carried out to examine the roles of 3′ or 5′ ends (Dixon & Kowalczykowski, 1991; Dutreix *et al.*, 1991; Konforti & Davis, 1991, 1992) were done without knowledge of the RecA filament disassembly process documented in the present study, which would tend to exacerbate an observed 3′ end bias in studies with RecA protein alone. The same studies were also carried out without the RecO and RecR proteins. We point out that any RecA-based bias favoring 3′ ends may be eliminated in any cell or experiment where the RecO and RecR proteins are present. In general, the location and size of RecA protein filaments during recombination and recombinational DNA repair must now be viewed as a dynamic consequence of the interplay of filament assembly and disassembly processes, both of which are modulated by a variety of other proteins.

Materials and Methods

Enzymes and biochemicals

Escherichia coli wtRecA and RecA K72R mutant proteins were both purified by a procedure developed for the RecA K72R mutant protein (Shan *et al.*, 1996). Labeling of RecA protein with ^{35}S was carried out using a published procedure (Soltis & Lehman, 1983) and purified in the same manner as unlabeled RecA protein. The specific activity of labeled RecA protein was 6.1×10^7 cpm μmole^{-1} RecA. RecA protein (labeled, unlabeled, or mutant) was stored in R buffer (20 mM TrisOAc 80%+, (pH 7.5), 1 mM DTT, 0.1 mM EDTA, 10% (w/v) glycerol). The ssDNA-dependent ATPase and DNA strand exchange activities of the labeled protein were comparable to the unlabeled RecA protein. All RecA protein preparations were more than 95% pure and free of detectable nucleases. The concentrations of all RecA protein preparations were determined by absorbance at 280 nm using an extinction coefficient of $\varepsilon_{280} = 0.59$ A_{280} mg^{-1} ml (Craig & Roberts, 1981). *E. coli* single-stranded DNA binding protein (SSB) was purified as described (Lohman *et al.*, 1986) with the minor modification that a DEAE-Sepharose column was added to ensure removal of single-stranded exonucleases. The concentration of SSB protein was determined by absorbance at 280 nm using an extinction coefficient of $\varepsilon_{280} = 1.5$ A_{280} mg^{-1} ml (Lohman & Overman, 1985). RecR protein was purified as described (Webb *et al.*, 1995). SSB antibody (rabbit) was a generous gift from Dr Tim Lohman (Washington University). RecR antibody (chicken) was a gift from Promega. Restriction endonucleases, phage T4 polynucleotide kinase and β-agarase were purchased from New England Biolabs. Terminal transferase was purchased from Promega. Tris buffer was from Fisher Scientific. ATP, Proteinase K, lactic dehydrogenase, pyruvate kinase, phosphoenolpyruvate, and nicotinamide adenine dinucleotide (reduced form, $NADH^+$) were purchased from Sigma. Ultrapure dATP, DEAE-Sepharose resin, butyl Sepharose 4B resin, MonoQ, and MonoS columns were from Pharmacia Biotech Inc. Hydroxylapatite resin and BioRex 70 cation exchange resin were from Bio-Rad. $H_2[^{35}S]O_4$ was purchased from New England Nuclear.

Determination of the extinction coefficient for RecR protein

The determination of the extinction coefficient for native RecR protein is based on a published procedure (Lohman *et al.*, 1989), modified as described (Marrione & Cox, 1995). The RecR protein has four tyrosine and no tryptophan residues. Although the resulting low extinction coefficient limits the accuracy of this method somewhat, our experience indicates that the error in protein concentration determination by this method is still significantly lower than that of the Bradford

method. The extinction coefficient for RecR protein in 6 M guanidine-HCl (Gdn-HCl), based on the reported extinction coefficient of 1280 $M^{-1}cm^{-1}$ for glycyl-L-tyrosylglycine, is calculated as: $\varepsilon_{280\ (6\ M\ Gdn\text{-}HCl)} = 4\ (1280) = 5.12 \times 10^3\ M^{-1}cm^{-1}$. UV absorbance spectra of native (in 20 mM Tris-HCl (80% cation, pH 7.5), 200 mM NaCl, 15% glycerol (w/v), and 1 mM EDTA) and denatured (in 6 M Gdn-HCl) RecR were scanned at 25°C from 360 to 240 nm at four different dilutions of stock protein. The extinction coefficient of the native RecR protein was determined at 280 nm according to the following expression:

$$\varepsilon_{28\prime,M,native} = [(Abs_{native,28\prime})(\varepsilon_{M,Gdn\text{-}HCl})] - Abs_{Gdn\text{-}HCl,28\prime}:$$

The results of the four determinations were averaged to give an extinction coefficient of native RecR as $\varepsilon_{280,M,native} = 5.60\ \pm 0.18 \times 10^3\ M^{-1}\ cm^{-1}$. The RecR protein concentration obtained using this method is 2 to 2.5 times higher than the concentration estimated using the Bradford method.

Cloning of the *E. coli recO* gene

The *recO* gene was isolated from the *E. coli* genome (strain MG1655, a gift from George Weinstock (Guyer *et al.*, 1981)) by polymerase chain reaction using a Perkin-Elmer DNA thermal cycler. This was accomplished by designing DNA primers which were complementary to the 5′ and 3′ DNA sequences of the *recO* gene. The 5′ DNA primer corresponded to bases 772 to 786 of the published DNA sequence (Morrison *et al.*, 1989), with the sequence 5′-CTAGCC added to the 5′ end to provide an *Nco*I restriction site. The 3′ DNA primer corresponded to bases 1486 to 1500, with the sequence 5′-CCCAAGCTT added to the 5′ end to provide a *Hin*dIII restriction site. These primers were used to amplify a DNA fragment of the appropriate size (726 bp) by the polymerase chain reaction. The *recO* gene fragment was digested with *Nco*I and *Hin*dIII and then ligated to the cloning vector pET21d (Novagen). This plasmid containing the *recO* gene was designated pBLW21. The integrity of the entire *recO* gene in this construct was verified by direct sequencing (Sequenase kit and protocol, U.S. Biochemical Corp.).

Overexpression and purification of the *E. coli* RecO protein

A 40-liter culture of *E. coli* strain, BL21(DE3), with the plasmid pBLW21 was grown with aeration in a New Brunswick MPPF 40 liter fermentor at 37°C in Luria broth (supplemented with 100 mg/ml ampicillin) to an A_{650}=1.0. RecO protein expression was induced by adding IPTG to a final concentration of 0.5 mM. The culture was then incubated at 25°C for two hours. The lower outgrowth temperature greatly increased the amount of soluble RecO protein. The cells were harvested by centrifugation and frozen in liquid nitrogen. The frozen cell pellet from 40 liters of cells (250 g) was thawed and resuspended in lysis buffer (50 mM Tris-HCl (78% cation, pH 7.5), 10% (w/v) sucrose) to a final volume of 500 ml, frozen in liquid nitrogen and stored at −70°C. RecO protein was purified by a published procedure (Luisi-DeLuca & Kolodner, 1994) with the following changes. The lysis supernatant, from 33 g of frozen cells, was applied directly to the BioRex 70 cation exchange column. The pooled fraction from the BioRex 70 column was further purified on an hydroxylapatite column, and a butyl Sepharose column. The RecO protein eluted from the butyl Sepharose column, with a 200 ml linear gradient decreasing from 1.2 M to 0 M ammonium sulfate in buffer A (20 mM Tris-HCl (78% cation, pH 7.5), 10 mM β-mercaptoethanol, 0.1 mM EDTA, 10% (w/v) glycerol), as one peak at 600 mM ammonium sulfate. The peak fractions were loaded onto a 1 ml Mono S column equilibrated in buffer A containing 200 mM NaCl at 0.5 ml/min. The column was washed with 5 ml of buffer A containing 200 mM NaCl, and developed in a 10 ml 200 mM to 1000 mM NaCl linear gradient in buffer A. The RecO protein eluted at 600 mM NaCl, was dialyzed against buffer A containing 200 mM NaCl and 60% (w/v) glycerol, and was stored at −70°C. The final yield from 33 g of frozen cells was 240 μg of at least 95% pure RecO protein, as estimated by SDS-PAGE. The purified RecO protein contained no detectable nuclease activities. The concentration of the RecO protein was measured by Bradford assay using bovine serum albumin as a standard. The quantities of pure RecO protein available are as yet insufficient to provide for an accurate determination of an extinction coefficient.

DNA

Supercoiled circular duplex DNA and circular ssDNA from bacteriophage M13mp8 were prepared as described (Davis *et al.*, 1980; Messing, 1983; Neuendorf & Cox, 1986). Bacteriophage M13mp8.1037 (8266 bp) is bacteriophage M13mp8 with a 1037 bp (*Eco*RV fragment from *E. coli galT* gene) inserted into the *Sma*I site (Lindsley & Cox, 1990b). The concentrations of dsDNA and ssDNA stock solutions were determined by absorbance at 260 nm, using 50 and 36 μg ml^{-1} $A_{260}{}^{-1}$, respectively, as conversion factors. DNA concentrations are expressed in terms of total nucleotides. Linear dsDNA substrates were generated by complete digestion of supercoiled DNA by appropriate restriction endonucleases, using the protocol recommended by the endonuclease supplier. The protein was removed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1, by volume) and chloroform/isoamyl alcohol (24:1 (v/v)) followed by precipitation with ethanol.

To generate linear ssDNA substrates, M13mp8.1037 circular ssDNA was first annealed with an oligonuclestide carrying an unique restriction enzyme site, and then digested by restriction endonuclease. The sequence of the two oligos used are: *Eco*RI oligo, 5′-GGATGG-GAATTCGTAATC-3′, and *Alw*NI oligo, 5′-AAGCG-CAGTCTCTGAATT-3′. After digestion was complete, protein was removed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1, by volume) and chloroform/isoamyl alcohol (24:1 (v/v)) followed by precipitation with ethanol. ssDNA was resuspended in 450 μl of TE buffer (Sambrook *et al.*, 1989). Remaining oligonucleotides were removed from the ssDNA template by heating the sample at 65°C for five minutes and immediately transferring the sample to ice. The oligo was then removed by filtration through a Microcon-30 Ultrafilter (nucleotide cutoff is 300 nt ssDNA, Amicon). The final volume after filtration was about 50 μl. This process was repeated twice. Linear ssDNA appeared to be a single band migrating at the same position as a linear M13mp8.1037 marker on a 1.4% agarose gel. When M13mp8.1037 circular ssDNA was digested by both *Eco*RI and *Alw*NI, more than 90% of M13mp8.1037 circular ssDNA was linearized into two 4 kb fragments (4044 bases, and 4222 bases). Unless

otherwise specified, the ssDNA used in ssDNA-dependent ATP or dATPase assays was derived from M13mp8.1037 DNA.

ATPase and dATPase assays

NTP hydrolysis activities were measured by a coupled enzyme assay (Lindsley & Cox, 1990a; Morrical *et al.*, 1986). A Perkin-Elmer Lambda 7 double-beam recording spectrophotometer equipped with two thermojacketed cuvette holders, each capable of holding six cuvettes, was used for absorbance measurements. The cell path length and band pass were 0.5 cm and 2 nm, respectively. The regeneration of ATP or dATP from ADP or dADP and phosphoenolpyruvate with the oxidation of NADH can be followed by the decrease in the absorbance at 380 nm. Absorbances were measured at 380 nm rather than 340 nm (the absorbance maximum for NADH), to remain within the linear range of the spectrophotometer over an extended time course. An increase in the concentration of ATP or dATP or any of the coupling system components did not change the observed ATP or dATPase rate; the data obtained reflect the initial velocity of ATP hydrolysis at all times (Lindsley & Cox, 1990a; Morrical *et al.*, 1986).

Rates of ssDNA-dependent ATP and dATP hydrolysis were measured at 37°C in a reaction mixture (400 µl) containing 25 mM Tris acetate (80% cation, pH 7.5), 10 mM $Mg(OAc)_2$, 5% (w/v) glycerol, 1 mM dithiothreitol. A regeneration system for both ATP and dATP (11.85 mM phosphoenolpyruvate, 20 units ml^{-1} pyruvate kinase, and 3 mM potassium glutamate) and a coupling system (3 mM NADH and 4.5 units ml^{-1} lactic dehydrogenase) were also included. In all reactions, the nucleotide cofactor concentration (ATP or dATP) was 3 mM. The final pH after addition of all reaction components was pH 7.6. Concentrations of DNA and proteins are reported in the text and Figure legends.

RecA protein (5 µM) was incubated with ssDNA (3 µM) at 37°C for ten minutes before a mixture of ATP or dATP and SSB was added to initiate the reaction. Unless otherwise specified, the concentration of SSB (0.3 µM) was 1/10 of the ssDNA concentration (measured in total nucleotides) and the concentrations of RecO (75 nM) and RecR (180 nM) were 1/40 and 1/16.7 of the ssDNA concentration, respectively, in this assay and the following assays. When present together, RecO and RecR proteins were always added to the reactions as a mixture. Excess RecA protein was used in these assays to ensure saturation of all available ssDNA binding sites. The ATP/ADP ratio was monitored in selected experiments with added (α-^{32}P)-labeled ATP and thin layer chromatography (Marrione & Cox, 1995) as controls to document that the ATP regeneration system was sufficient to preclude significant build-up of ADP for lengths of time at least equivalent to the times over which data were gathered in this study.

Reaction conditions for sucrose gradient sedimentation assays

Reaction conditions for forming RecA-ssDNA filaments were essentially the same as those used in the ssDNA-dependent NTPase assays, except that NADH and LDH were not included. The ssDNA concentration was increased relative to those used in ATPase assays in order to increase the signal in the bound fractions. The ssDNA binding reactions (120 µl) contained 12 µM M13mp8.1037 ssDNA, 5 µM ^{35}S-labeled RecA, and 1.2 µM SSB protein. When RecO and RecR proteins were also used in the reaction, the final concentrations for the RecO and RecR proteins were 0.3 µM and 0.72 µM, respectively. Reactions were carried out at 37°C for various times as indicated in the text and Figure legends and stopped by adding ATPγS to a final concentration of 1 mM, before analysis by sucrose gradient sedimentation as described below.

Sucrose gradient sedimentation

Pre-formed sucrose gradients (5% to 15%, 5 ml) were poured and stored at 4°C in a cold room for no more than two hours before use. To separate the RecA-ssDNA complexes from free RecA protein, the gradients also contained 25 mM Tris acetate (80% cation, pH 7.5), 1 mM DTT and 1 mM $Mg(OAc)_2$. A 100 µl sample from each individual reaction was loaded on top of the gradient and centrifuged in a Beckman SW50.1 rotor at 40,000 rpm, 4°C, for one hour. Gradients were fractionated by using a density gradient fractionator (Isco, model 183). Fractions were collected in 1.5 ml decapped Eppendorf tubes, and the average fraction size was about 180 µl. A 100 µl aliquot from each fraction was spotted on a GF/A filter. These filters were then dried, added to scintillation vials containing 5 ml scintillation cocktail (Ready Safe, New England Nuclear), and assayed for radioactivity by liquid scintillation (Beckman LS 3801). The background was defined as the lowest point in each individual gradient profile. Measured gradient peaks included all contiguous fractions having at least twice the radioactivity as the fraction defining the background, plus one fraction before and after the peak fractions thus defined. The radioactivity in fractions was plotted from the top to the bottom of the gradient from left to right in the gradient profiles shown. The free RecA protein migrates as a peak near the top of the gradient and bound RecA protein migrates as a distinct peak in the middle of the gradient. An agarose gel was used to detect DNA in the sucrose gradient fractions, and fractions containing DNA were designated as the bound peak. The measured radioactivity in all fractions defining each of these two peaks was added to determine the number of counts reflecting either bound or free labeled RecA protein. Occasional small peaks at other points in the gradients were not reproducible and were ignored. The quantitation is reported as percent of radioactivity in the free or the bound RecA peak, defined in each case as the percent of counts in one peak divided by the total counts in both peaks. Since RecA protein is present in excess relative to the ssDNA in these experiments, no more than 80% of the labeled RecA protein can appear in the ssDNA-bound fraction. Proteins from fractions in some of the gradient profiles shown were also analyzed by SDS-PAGE and/or Western blot (Sambrook *et al.*, 1989).

Electron microscopy

Reaction conditions for electron microscopy were essentially the same as those used in the sucrose gradient assay. Reactions contained 12 µM M13mp8.1037 ssDNA, 5 µM RecA protein, 1.2 µM SSB, 300 nM RecO protein, and 720 nM RecR protein. In some experiments, the RecA concentration was lowered to 4 µM from 5 µM in order to get a cleaner background as necessary for quantitation. Reactions were carried out at 37°C for various times as indicated in the text and Figure legends.

Reactions were stopped by adding ATPγS to 1 mM and incubation at 37°C for three minutes, then spread by a modified adsorption procedure (Webb *et al.*, 1995). The samples were diluted 60-fold into 200 mM ammonium acetate containing 15% glycerol and adsorbed to carbon films on an EM grid for three minutes. The grids were washed by floating on two 1 ml drops of 50 mM ammonium acetate containing 15% glycerol for two and three minutes, respectively. Glycerol (15%, w/v) was included in the uranyl acetate staining solution and subsequent water washes. The grids were dried under a heat lamp for ten minutes before shadowing. The above protocol was designed to visualize complete reaction mixtures. We elected to use this strategy, rather than to visualize purified products, because we could be more certain of not missing unexpected products and preserving solution structure. However, the strategy results in higher background noise (due to unreacted substrate and ATP regeneration constituents) and consequently lower resolution.

The presence of SSB protein on the ssDNA projecting from one end of RecA filaments was established by binding protein A-gold (10 nm, Sigma Co) to SSB antibody raised in rabbit (a kind gift from Tim Lohman) which in turn binds to SSB protein if present on the complex. This procedure was modified by actually carrying out the protein A-gold–antibody reactions on reaction mixtures already deposited and dried on activated carbon films. This modification therefore completely avoids the problem of aggregation that can occur when these types of reactions are carried out in true solution. Full details of this procedure will be presented in a future publication but can presently be obtained on the World Wide Web at URL: http://phage.bocklabs.wisc.edu/.

Judgment of RecA filament length

We observed that RecA-coated ssDNA often had one uncoated end which appeared to contain ssDNA bound to SSB. Furthermore, there appeared to be a reciprocal relationship between RecA filament length and the amount of ssDNA complexed with the presumed SSB at one end. We cannot prove in these experiments that this is true because of the 1.5-fold length extension caused by RecA and the very large foreshortening of ssDNA by SSB. In fact, the exact path of the ssDNA within the SSB-bound end sections was generally much too convoluted to be resolved with any confidence. In Figures 4 and 8 we have made filament length comparisons by focusing on the RecA filament length alone and ignoring the end segment. Since direct measurement of filaments is sufficiently tedious to make the accumulation of large and statistically significant data sets impractical, a procedure was used in which judgments are made by eye, similar to that applied in earlier studies (e.g. see Shan *et al.*, 1996). Observed RecA filament lengths are related to a short standard line marked directly on the electron microscope screen. For the data in Figure 8, the expected lengths of filaments assembled onto gapped linear DNA (3′ to 5′ *versus* 5′ to 3′ assembly) were determined as a fraction of judged full-length gapped circles completely coated with RecA protein that were present in the same samples. The judgment process was checked by direct measurement of seven partially RecA-coated linear gapped DNA molecules and four fully coated circular gapped DNA molecules. We found that for the partial filaments (those shown in Figure 8 between 0 and 10 judged length units) there was an average discrepancy of ±0.5 unit with a maximum of 0.9 unit between judgments and measurements. For fully coated circles, the average discrepancy was ±2.2 units with a maximum of 3.5 units. In the critical experiments shown in Figure 4, a different control was carried out to check the judgment process. Grids were assigned an undescriptive identifier (by J.M.B.) and judgments were then made ''blind'' (by R.B.I.) on two grids from each sample. All four grids were correctly identified on the basis of the RecA filament distribution. Additionally, each experiment was repeated in its entirety and judgments made to verify that all reactions were repeatable.

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