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RecA Protein Dynamics in the Interior of RecA Nucleoprotein Filaments

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We characterize aspects of the conformation and dynamic state of RecA filaments when bound to dsDNA that are specifically linked to the presence of the second of the two bound DNA strands. Filaments bound to dsDNA exhibit a facile exchange between free and bound RecA monomers or oligomers in the filament interior that is not seen on ssDNA. The RecA mutant K72R, which binds but does not hydrolyze ATP, forms mixed filaments with wild type RecA protein under some conditions. In the presence of dATP, mixed filaments are formed on dsDNA or ssDNA in which the RecA K72R content approximately reflects the proportion of the K72R mutant in the total RecA protein present when the filament is formed. In the presence of ATP, mixed filaments are formed on dsDNA, but the mutant protein strongly inhibits the binding of wtRecA protein to single-stranded DNA. When RecA K72R is added to pre-formed filaments containing only wild-type RecA protein on single-stranded DNA, little of the mutant protein exchanges into the filament. Exchange occurs readily, however, when the filament is bound to double-stranded DNA. The presence of a second DNA strand in RecA-dsDNA filaments produces as altered and more dynamic filament state relative to filaments formed on single-stranded DNA. The results point to a substantial alteration in filament state when synapsis occurs during RecA protein-mediated DNA strand exchange.

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Introduction

The bacterial RecA protein is critical to the processes of recombinational DNA repair, homologous recombination, induction of the S.O.S. response to DNA damage, and the partitioning of chromosomes at cell division (Clark & Sandler, 1994; Cox, 1994; Kowalczykowski *et al*., 1994; Roca & Cox, 1990, 1995; West, 1992; Zyskind *et al*., 1992). The *Escherichia coli* RecA protein is a 352 amino acid polypeptide chain with a predicted molecular mass of 37,842 Da. RecA is an ancient protein present in virtually all bacteria, with structural and functional homologues in eukaryotes ranging from yeast to humans (Ogawa *et al*., 1993; Sung, 1994; A. I. Roca & M. M. Cox, unpublished data).

In vitro, RecA protein promotes a set of DNA strand exchange reactions that mimic its presumed role in recombination and recombinational DNA repair. The reactions can involve either three or four DNA strands (Figure 1). RecA first forms a nucleoprotein filament on the single-stranded or gapped DNA substrate (DNA1). This DNA is then aligned with a homologous linear duplex DNA (DNA2). A strand switch then occurs within the filament producing a nascent region of hybrid DNA, which is extended to generate the products shown. In a normal reaction, the strand exchange reaction proceeds unidirectionally, 5' to 3' relative to the single-stranded DNA (or the single-stranded region of the gapped DNA) to which the RecA first binds.

RecA protein is a DNA-dependent ATPase, with a monomer k_{cat} of 30 min^{-1} when bound to single-stranded DNA (ssDNA; dATP is hydrolyzed

Abbreviations used: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; wtRecA, the wild-type RecA protein; EDTA, ethylenediaminetetraacetic acid; OAc, acetate on; Fl DNA, supercoiled closed-circular form of plasmid of bacteriophage DNA as isolated from *E. coli* cells; FIII DNA, linear double-stranded DNA; ATPγS, adenosine 5'-*O*-(3-thio)triphosphate; SBB, the single-stranded DNA binding protein of *E. coli*; LDH, lactate dehydrogenase; NADH, the reduced form of nicotinamide adenine dinucleotide.

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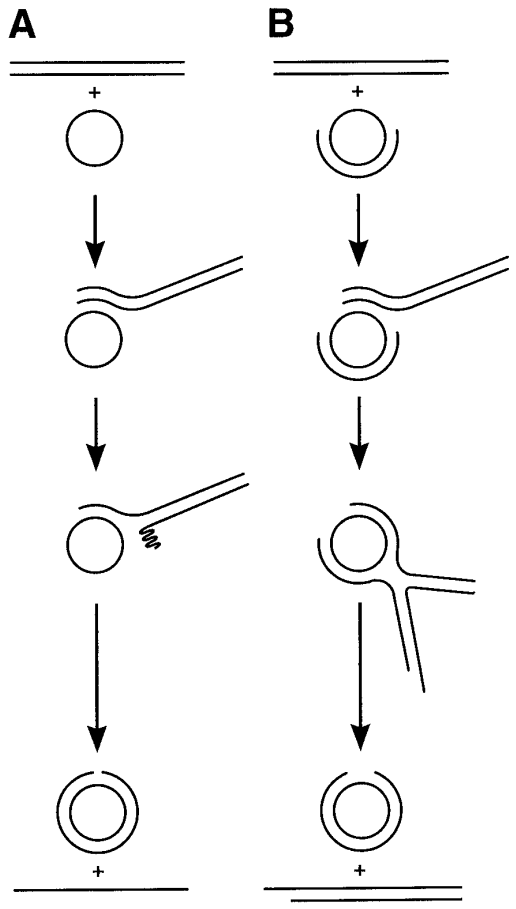

Figure 1. RecA protein-mediated DNA strand exchange reactions. Typical reactions involving three (A) and four (B) DNA strands are shown.

at rates about 10 to 20% higher). ATP is hydrolyzed uniformly throughout the nucleoprotein filament (Brenner *et al*., 1987). When a homologous duplex DNA is added to the reaction, the k_{cat} drops abruptly to about 20 min^{-1}, and remains at that level throughout the ensuing DNA strand exchange reaction. This ATP hydrolysis is coupled to the final stage of DNA strand exchange in which a nascent stretch of hybrid DNA is extended unidirectionally (Bedale & Cox, 1995; Shan *et al*., 1995). ATP hydrolysis is also required for filament disassembly (Lindsley & Cox, 1990). RecA hydrolyzes ATP in the absence of DNA only in the presence of molar concentrations of a variety of salts (Pugh & Cox, 1988a,b).

RecA protein can promote a limited and undirected DNA strand exchange reaction under some conditions in the presence of ATPγS, an ATP analog that is not readily hydrolyzed by RecA (Jain *et al*., 1994; Kim *et al*., 1992a,b; Menetski *et al*., 1990; Rosselli & Stasiak, 1990). A similar strand exchange reaction can be promoted by the RecA mutant K72R (Rehrauer & Kowalczykowski, 1993; Shan *et al*., 1995). The Lys → Arg substitution occurs in a well-conserved nucleotide binding fold, and the mutant protein binds but does not hydrolyze ATP. The mutant functions best with dATP (Rehrauer & Kowalczykowski, 1993). Although the RecA K72R protein binds to DNA, binds to ATP, and promotes a degree of DNA strand exchange *in vitro*, the K72R mutation produces a recA null phenotype *in vivo* (Konola *et al*., 1994; R. Devoret, personal communication).

A complete understanding of RecA nucleoprotein filament function requires an understanding of the pathways for assembly and disassembly of the filaments. Filaments assemble on ssDNA in the 5' to 3' direction (Register & Griffith, 1985). The assembly of filaments containing over 2000 monomers is complete in one to two minutes, indicating assembly rates of greater than 1000 monomers min^{-1}. Disassembly of filaments from ssDNA is not generally observed as long as ATP is regenerated. There is no direct coupling of ATP hydrolysis and dissociation of RecA monomers from the filaments, and little measurable exchange of RecA monomers between free and ssDNA-bound pools, although some direct transfer between filaments can be observed (Neuendorf & Cox, 1986). Direct transfer between two oligonucleotides *via* a ternary intermediate has also been described (Menetski & Kowalczykowski, 1987a,b).

Disassembly from linear dsDNA is the best-characterized disassembly pathway (Lindsley & Cox, 1989, 1990). Net disassembly of filaments is localized to the filament end opposite to that at which assembly occurs (Lindsley & Cox, 1990). End-dependent disassembly of filaments from double-stranded DNA (dsDNA) is highly pH-dependent, with rates increasing from no measurable disassembly at pH 6.0 to a maximum rate above pH 8.0 of about 240 monomers min^{-1} (assuming one bound RecA monomer per three base-pairs) (Lindsley & Cox, 1989). End-dependent filament disassembly from dsDNA also requires ATP hydrolysis (Lindsley & Cox, 1989, 1990). Dissociation or transfer of RecA monomers or oligomers at interior positions of RecA filaments bound to dsDNA has not been characterized under conditions in which ATP is regenerated.

When ATP is not regenerated, filament assembly and disassembly processes can be modulated by ATP and ADP levels (Lee & Cox, 1990b). Filaments disassemble from either ssDNA or dsDNA if ADP levels build up, and filament formation is precluded when ADP concentrations equal or exceed the ATP concentration (Cox *et al*., 1983; Lee & Cox, 1990a).

Published models for the role of ATP hydrolysis in the final stages of DNA strand exchange can be divided into three categories, distinguished by (1) a requirement for filament disassembly as strand exchange proceeds, with or without reassembly on the displaced DNA strand (Howard-Flanders *et al*., 1984; Konforti & Davis, 1992; Morel *et al*., 1994), (2) a redistribution of RecA monomers within a filament (Kowalczykowski & Krupp, 1995; Menetski *et al*., 1990; Rehrauer & Kowalczykowski, 1993), or (3) no disassembly or redistribution (Burnett *et al*., 1994; Cox, 1994). Efficient DNA strand exchange

takes place under conditions in which RecA remains quantitatively bound to the hybrid DNA product (Ullsperger *et al*., 1995), showing that a net disassembly of the filament is not a mechanistic requirement. However, a redistribution of RecA monomers within the filament remains a formal possibility, and there is little published data to support a case for or against. In this study, we demonstrate the formation of mixed filaments of the RecA mutant K72R and wild-type RecA protein (wtRecA), and characterize the effects on RecA-mediated ATP hydrolysis. These data forms the basis for challenge experiments documenting an exchange between free and bound RecA monomers in RecA filaments formed on dsDNA.

Results

Experimental design

The goal of these experiments is to document any exchange of RecA monomers into or out of RecA nucleoprotein filaments that is independent of filament ends. Two independent and complementary approaches were used to follow the exchange processes. In both cases the idea was to define changes in the properties of filaments of wtRecA protein when the mutant RecA K72R was present or added to the solution. The first approach was to follow ATP or dATP hydrolysis by wtRecA protein. Since the mutant does not hydrolyze ATP, substitution of the mutant for wild-type protein in the filament must bring about a decrease in the observed rates of ATP hydrolysis. The decrease brought about when filament formation is initiated in the presence of varying amounts of the mutant protein was first monitored under conditions in which total RecA protein (wild-type + mutant) is in large excess relative to DNA, so that either protein is present in amounts sufficient to saturate the available DNA binding sites. This situation should exacerbate any tendency for one protein to exclude the other from the DNA. These results were then compared to those obtained when the DNA is in excess so that all available RecA monomers have a binding site. An inhibitory effect due to simple exclusion of one protein from the DNA in the situation where protein is in excess should be relieved when all the protein can bind DNA. However, if an observed inhibition is a cooperative effect reflecting a random mixing of the two proteins, the inhibition conferred by the mutant might still exist even when DNA is in excess. We examined filaments formed on both ssDNA and dsDNA. Since the limited function of RecA K72R is enhanced by dATP, we examined both the dATPase and ATPase activities of wtRecA protein in the presence of the mutant. This data set formed the basis for a series of challenge experiments in which wtRecA or the mutant was added to pre-formed filaments of the other protein to see if an increase or decrease in ATP hydrolysis signaling an exchange reaction could be detected. All reactions contained 3 mM ATP or dATP.

The second approach was sucrose gradient sedimentation utilizing ^{35}S-labeled wtRecA protein. The amount of radiolabeled wtRecA bound to DNA can be quantitatively followed by scintillation counting. The total amount of protein in the peaks corresponding to bound and free fractions, labeled and unlabeled, can also be estimated by SDS-PAGE.

Sucrose gradient sedimentation assay is a relatively direct measure of RecA protein binding to DNA, and exchange between bound and free protein. 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, and it is subject to possible artifacts associated with the requirement that adenosine 5'-*O*-(3-thio)triphosphate (ATPγS) be added at the end of experiments to maintain the integrity of bound complexes during centrifugation. On the other hand, the coupled ATPase assay is an indirect measure of RecA protein binding to DNA, and there are inherent ambiguities associated with the interpretation of some experiments. However, the ATPase assay offers convenience, high accuracy and reproducibility, and detection of dynamic changes in real time. When combined, the two approaches complement each other and provide a reasonably complete picture of the status of the RecA filaments under most conditions. A few caveats still apply, as noted below.

RecA K72R does not inhibit wtRecA-mediated NTP hydrolysis in the absence of DNA

The wtRecA protein exhibits a substantial DNA-independent ATPase activity in the presence of 1 to 2 M levels of a variety of salts (Pugh & Cox, 1988b). The DNA-independent ATPase and dATPase activities of wtRecA in 1.8 M NaCl were examined in the presence of RecA K72R protein. The total RecA protein (wild-type + mutant) was maintained at 5 μM. The rate of dATP hydrolysis is a linear function of the concentration of wtRecA protein in the mixture, with no evidence of inhibition or interference by the mutant protein (Figure 2A). The observed dependence of the rate of ATP hydrolysis on the concentration of wtRecA protein is very slightly concave (Figure 2B), suggesting a minor inhibitory effect of the mutant protein. With 100% wtRecA protein, the rate of ATP hydrolysis is 116.8 μM min^{-1}, and the rate of dATP hydrolysis is 148.0 μM min^{-1} (each measurement represents an average of two assays). The reported K_m for ATP under these conditions is 830 μM (Pugh & Cox, 1988b). The ATP or dATP hydrolytic activity of the wtRecA protein present (derived from the data such as that in Figure 2 and converted to turnover of ATP per wtRecA monomer) remains constant as the fraction of protein present as wtRecA changes, suggesting that any interaction that might occur between the wild-type and mutant

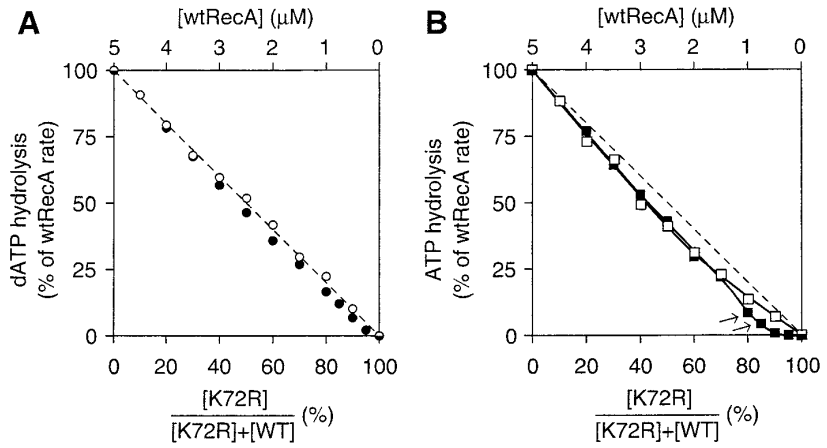

Figure 2. Effects of RecA K72R on the DNA-independent NTP hydrolytic activity of wtRecA protein at high salt concentrations. Reactions were carried out as described in Methods in a standard reaction buffer containing 1.8 M NaCl and 5 mM dATP or ATP. A, Reactions with dATP. B, reactions with ATP. Symbols are: (○ or □), mixtures of wtRecA and RecA K72R proteins, with total protein concentration kept constant at 5 μM (bottom axes); (● or ■), reactions with different concentrations of wtRecA protein alone (0 to 5 μM).

proteins under these conditions has little or no effect on the intrinsic capacity of wtRecA to hydrolyze ATP or dATP in the presence of high salt concentrations.

The results are somewhat different when the concentration of wtRecA protein is varied without compensatory additions of mutant protein. In this case, the derived turnover of ATP (but not dATP) by wtRecA is no longer constant but increases with protein concentration, leveling off only above 2 μM (Pugh & Cox, 1988b; Figure 2). The part of the curve affected is indicated by arrows in Figure 2B. The presence of the mutant protein in amounts sufficient to bring the total protein to 5 μM clearly eliminates the decline in ATP turnover seen at low concentrations of wtRecA. The fraction of the total RecA protein that is wild-type always hydrolyzes ATP as though 5 μM wtRecA protein were present, indicating that it must be interacting with the mutant protein.

RecA K72R forms mixed filaments with wtRecA protein on ssDNA

RecA protein filaments were formed on ssDNA, beginning with protein mixtures containing varying amounts of RecA K72R. The total RecA protein was again kept constant at 5 μM. The ssDNA concentration was set at 3 μM, enough to bind only 20% of the RecA protein. The results obtained in the presence of 3 mM dATP and 300 nM SSB are shown in Figure 3A. As the wtRecA protein is replaced by RecA K72R, the observed rate of dATP hydrolysis decreases. The decrease is a linear function of the

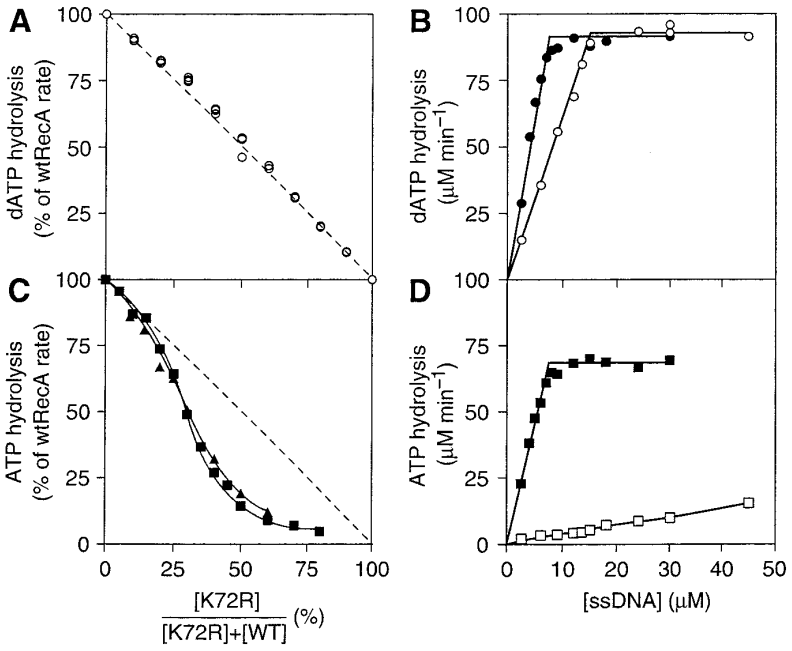

Figure 3. Effects of RecA K72R on the ssDNA-dependent NTP hydrolytic activity of wtRecA protein; formation of mixed filaments. Reactions were carried out as described in Methods, using M13mp8 circular single-stranded DNA. SSB was present at a concentration providing one SSB monomer per ten nucleotides of ssDNA. A, Reactions with 3 mM dATP and mixtures of wtRecA and RecA K72R proteins, with total protein kept constant at 5 μM. The ssDNA concentration was 3 μM, or enough to bind 20% of the total RecA protein. For all of the protein mixtures, overlapping data points are shown for at least three independent experiments. B, Effect of increasing ssDNA concentration on dATP hydrolysis rates. Symbols represent reactions with 2.5 μM wtRecA protein either in the presence (○) or absence (●) of 2.5 μM RecA K72R protein. C, Reactions with 3 mM ATP and mixtures of wtRecA and RecA K72R proteins, with total protein kept constant at 5 μM. The ssDNA concentration was 3 μM (■) or 30 μM (▲). D, Effect of increasing ssDNA concentration on ATP hydrolysis, rates. Symbols represent reactions with 2.5 μM wtRecA protein either in the presence (□ or absence (■) of 2.5 μM RecA K72R protein.

concentration of wtRecA protein present. In all of the protein mixtures ranging from 20 to 80% RecA K72R, enough of either protein is present to saturate the available DNA binding sites. The results indicate that RecA K72R competes on an equal basis with wtRecA for binding sites in the ssDNA, and its presence in the filaments has no effect on the dATP hydrolytic activity of wtRecA protein.

The effect of increasing ssDNA concentration on dATP hydrolysis rates observed with 2.5 μM wtRecA in the presence or absence of 2.5 μM RecA K72R protein was measured (Figure 3B). Either in the presence or absence of RecA K72R mutant protein, the dATP hydrolysis rates exhibited saturation with respect to the concentration of ssDNA. The maximum dATP hydrolysis rate in either case was about 90 μM min^{-1}. This yields an apparent k_{cat} of 36 min^{-1} for hydrolysis of dATP by bound wtRecA protein that compares well with previous measurements (Shan *et al*., 1995). However, the ssDNA concentration-dependent dATPase rate increase (the slope of the linear region) in the absence of K72R mutant (12.1 μM dATP min^{-1} μM^{-1} ssDNA) is twice that observed in the presence of K72R mutant (5.89 μM dATP min^{-1} μM^{-1} ssDNA). The apparent stoichiometry between wtRecA and ssDNA generated from these data is 2.97 bases per wtRecA in the absence of K72R mutant, and 6.28 bases per wtRecA in the presence of an equal concentration of the K72R mutant. This result indicates that RecA K72R shares the same binding properties (including the same binding stoichiometry) as wtRecA when binding to ssDNA. The dATP hydrolytic activity of wtRecA is inhibited by RecA K72R through simple competition for ssDNA binding sites, and this inhibition is completely relieved when ssDNA is in excess to the total RecA protein. The results in Figure 3A could reflect the formation of separate, uniform filaments of either the mutant or wtRecA protein, with either filament forming with an equal probability. Alternatively, the results could reflect the activity of mixed filaments. Experiments presented below indicate that the wild-type and mutant proteins have a substantial capacity for interaction, leading us to favor the latter interpretation.

The results obtained when dATP is replaced by ATP are quite different (Figure 3C). In this case, with protein in excess, a sharper drop is seen as the proportion of RecA K72R in the total pool is increased. When 50% of the RecA is RecA K72R, the observed ATPase activity of wtRecA is decreased by almost 90%. This could reflect either an enhanced binding of the K72R mutant relative to wtRecA in the presence of ATP, or cooperative interactions with randomly distributed RecA K72R in the filament that produce an inhibition of ATP hydrolysis in nearby wtRecA K72R monomers. When the ssDNA is provided in excess so that all available RecA protein can bind, the inhibition pattern is exactly the same as when protein is in excess (Figure 3C). This suggests that the RecA K72R is distributed in a similar manner within the filament whether all RecA or only a fraction of it can bind to the DNA, and the presence of the mutant in the filament affects the function of the wild-type protein *via* protein–protein interactions. An alternative possibility is that mixtures of the mutant and wild-type proteins cannot bind to the ssDNA.

Figure 3D shows the ssDNA concentration dependence of the ATPase activity of a mixture of wtRecA and RecA K72R mutant (2.5 μM each) along with 2.5 μM wtRecA control. For the wtRecA control, the ATP hydrolysis rate increased with ssDNA concentration to a maximum of 68.5 μM min^{-1} with an apparent stoichiometry of 2.96 bases per RecA monomer. However, the rates observed for the wild-type + mutant mixture were no more than 15.7 μM min^{-1} and increased only slightly with increasing ssDNA concentration. There was no clear break in the plot to define the apparent stoichiometry. It was also noted that the ATP hydrolysis of an equal mixture of wtRecA and RecA K72R is a dynamic process. The initial rates were about twice the steady state rates plotted in Figure 3D, with the ATP hydrolysis rates gradually decreasing to the steady state rates over a period of at least 30 minutes (data not shown). This phenomenon indicates that a slow adjustment of interactions or activity occurs in the mixed filament in the presence of ATP.

RecA protein filament formation on ssDNA was also examined by sucrose gradient sedimentation (Figure 4). The reactions all contained 12 μM ssDNA and a RecA protein mixture containing a total of 8 μM RecA protein. First, 4 μM ^{35}S-wtRecA and 4 μM either unlabeled wtRecA (Figure 4A) or RecA K72R mutant (Figure 4B) were mixed in the presence of dATP. There are enough DNA binding sites to bind 4 μM total RecA protein, so 50% of the labeled protein should be in the bound fraction if binding of labeled and unlabeled protein is equivalent and random. With unlabeled wtRecA protein present, 49.1% of radiolabeled wtRecA was found in the bound RecA pools (Figure 4A). In the presence of unlabeled RecA K72R, there appeared to be a small drop in the binding of the labeled fraction (Figure 4B) to 44.1% bound. We estimate that the binding data derived from the gradient assays are accurate only to about $\pm$10%, and in many runs the amount of RecA bound tended to be somewhat less than that expected based on other assays. The results are therefore consistent with the dATPase measurements, and indicate that both wtRecA protein and the RecA K72R mutant compete on an approximately equal basis for binding to ssDNA in the presence of dATP.

In the gradient assay, there is a possibility that anomalous RecA protein binding might be induced when ATPγS is added to halt the experiment. In the case of binding to ssDNA in the presence of dATP, the extensive dATPase assays provide good evidence that essentially saturating amounts of RecA protein (wild-type + mutant) are bound to the ssDNA at the time ATPγS is added.

As might be expected from the ATPase assays, the

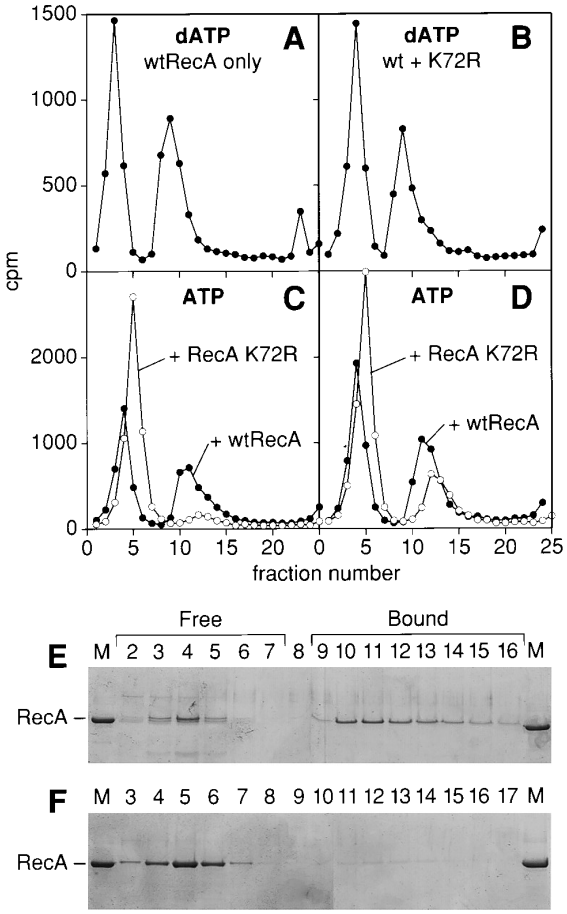

Figure 4. Sucrose gradient sedimentation analysis of RecA protein filaments formed on ssDNA. Reactions were carried out as described in Methods, using 12 μM M13mp8 circular single-stranded DNA, 1.2 μM SSB protein, and 3 mM dATP or ATP. In these gradients and those in subsequent figures, the top of the gradient is at the left and the first peak is the unbound (free) RecA. A, Reaction with 3 mM dATP and a RecA mixture containing 4 μM of ^{35}S-labeled wtRecA and 4 μM unlabeled wtRecA protein. The fraction of labeled protein bound to the DNA is 49.1%, calculated as described in Methods. B, Reaction with 3 mM dATP and a RecA mixture containing 4 μM of ^{35}S-labeled wtRecA and 4 μM RecA K72R. The bound labeled protein is 44.1% of the total. C, Reaction with 3 mM ATP and a RecA mixture containing 4 μM of ^{35}S-labeled wtRecA and 4 μM unlabeled wtRecA (●; 47.9% bound) or RecA K72R (○; 7.4% bound). D, Reaction with 3 mM ATP and a RecA mixture containing 5.33 μM of ^{35}S-labeled wtRecA and 2.67 μM unlabeled wtRecA (●; 45.7% bound) or RecA K72R (○; 23.7% bound). E, Sucrose gradient fractions (no. 2 to 16) from reaction in panel C with 3 mM ATP and a wtRecA mixture containing 4 μM of ^{35}S-labeled. wtRecA and 4 μM unlabeled wtRecA (●) visualized by 11% SDS–PAGE. F, Sucrose gradient fractions (no. 2 to 16) from reaction with 3 mM ATP and a RecA mixture containing 4 μM of ^{35}S-labeled wtRecA and 4 μM unlabeled K72R (○) visualized with 11% SDS-PAGE. M, RecA protein marker.

results obtained in the presence of ATP are distinctly different. Two different ratios of labeled and unlabeled RecA proteins were examined in the presence of ATP. Figure 4C shows the sucrose gradient profiles when 4 μM ^{35}S-labeled wtRecA and 4 μM either unlabeled wtRecA or RecA K72R mutant were used in the RecA mixture. When the reaction contained only wtRecA, 47.9% of the radioactivity was in the bound fractions, consistent with the expected equal distribution between free and bound pools. However, only 7.4% of the radioactivity was in the bound fractions when RecA K72R replaced the unlabeled wtRecA. Similar experiments were also carried out using a RecA mixture containing 5.33 μM ^{35}S-labeled wtRecA and 2.67 μM either unlabeled wtRecA or K72R mutant (Figure 4D). In this case, the labeled protein should still be evenly distributed between free and bound forms, and 45.7% of radioactivity was in the bound fractions when the RecA mixture only contained wtRecA. However. the amount of bound wtRecA was again reduced, to 23.7% of the total radiolabeled protein, when the unlabeled protein was the K72R mutant.

These results could indicate that the RecA K72R protein displaces the wtRecA protein in the presence of ATP. Alternatively, the two proteins together could form complexes incapable of binding to ssDNA. The fractions from the sucrose gradient shown in Figure 4C were also analyzed by SDS-PAGE (Figure 4E and F). The wtRecA and RecA K72R proteins (labeled or unlabeled) are indistinguishable on these gels, so that we are merely determining the presence or absence of RecA of any form in the bound and free fractions. When the protein mixture included unlabeled wtRecA protein, RecA protein was readily seen in both the free and bound fractions (Figure 4E). However, when the unlabeled protein was RecA K72R, most RecA was found in the free RecA pool and very little RecA protein was seen in the bound fractions (Figure 4F). This result indicates that mixtures of wtRecA protein and RecA K72R interfere with binding to ssDNA in the presence of ATP. The inhibitory effect of RecA K72R on ssDNA-dependent ATP hydrolysis reflects this reduction in DNA binding.

The results obtained with ssDNA in the presence of ATP indicate a strong tendency for the wtRecA and RecA K72R proteins to interact. They also indicate that ATP hydrolysis can be greatly affected by cooperative interactions between RecA monomers.

Mixed filaments formed in the presence of ATP or dATP are affected differently by the addition of SSB

The effects of the single-stranded DNA binding protein of *E. coli* (SSB) on the function of the mixed filaments was evaluated by measuring the rates of dATP or ATP hydrolysis before and after SSB addition, under conditions in which RecA protein (wild-type + mutant) was in excess. As shown in Figure 5A, the rate of dATP hydrolysis was always enhanced by SSB addition by about 1.5-fold,

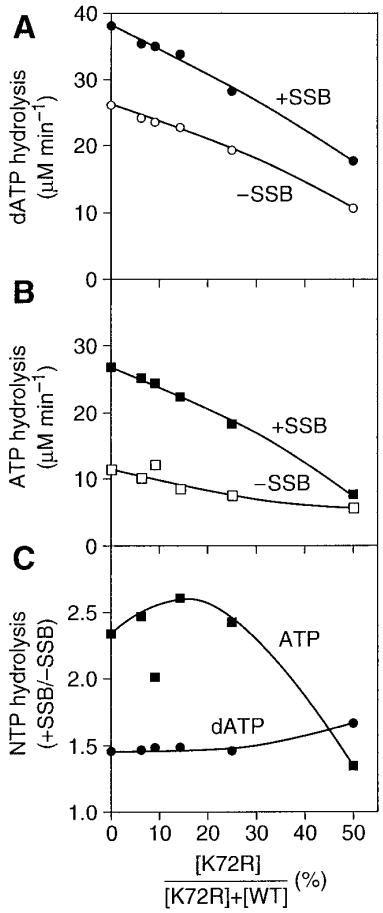

Figure 5. Effects of SSB on the activity of mixed filaments of wtRecA and RecA K72R. Reactions were carried out as described in Methods, using 3 μM M13mp8 circular single-stranded DNA, 0.3 μM SSB protein, and 3 mM dATP or ATP. Reactions contained the indicated mixtures of wtRecA and RecA K72R proteins, with total protein kept constant at 5 μM. Reactions with RecA proteins and ssDNA were initiated by the addition of nucleotide. After monitoring for at least 20 minutes, SSB was added and monitoring continued. A, Rates of dATP hydrolysis before and after SSB additions. B, rates of ATP hydrolysis before and after SSB addition. C, the change observed after SSB addition (+SSB/−SSB); summary of data in panels A and B.

regardless of how much RecA K72R was present (Figure 5B and C). When ssDNA was in excess, the stimulation was reduced to about 1.2-fold (data not shown). In the latter case, most of the RecA protein can presumably bind to the DNA, and the effect of SSB in removing secondary structure in the DNA to provide more contiguous RecA binding sites is not so prominent.

The results with ATP were again different. As the concentration of RecA K72R was increased, the enhancement by SSB declined. When DNA was in excess and RecA K72R represented 50% of the RecA protein present, the addition of SSB actually resulted in a decline in ATP hydrolysis (data not shown). The results are consistent with those in Figure 4 and indicate that the mixed filaments have a reduced capacity to compete with SSB for ssDNA binding when formed in the presence of ATP.

RecA K72R protein forms mixed filaments with wtRecA on dsDNA

The NTPase activity of mixed filaments on dsDNA was examined at pH 6.3, where the lag in RecA protein binding to dsDNA is minimal (Figure 6). The effects of mixing RecA K72R with wtRecA protein on dsDNA-dependent dATPase are examined in Figure 6, panels A and B. When the total RecA protein (5 μM) is in excess relative to the DNA, the replacement of wtRecA by RecA K72R in the protein mixture produces a degree of inhibition that is proportionally greater than the reduction in wtRecA protein concentration (Figure 6A). This inhibition reflects the formation of mixed filaments of RecA K72R and wtRecA on dsDNA in which the mutant protein inhibits the dATPase activity of adjacent or nearby wild-type monomers. This is indicated by the fact that the inhibition is not relieved when excess DNA is added, as shown in Figure 6B. When increasing amounts of DNA are added to a solution containing 2.5 μM wtRecA protein, a proportional increase in the rate of dATPase is observed until the concentration of DNA reaches approximately 15 μM (3 bp per RecA monomer), and then levels off. When the 2.5 μM wtRecA is 50% of a protein mixture with the mutant (total RecA = 5 μM), the rate of dATPase does not level off until over 30 μM DNA (6 bp per RecA monomer, enough to permit binding of the wild-type and mutant proteins) is present. More significantly, the maximum hydrolytic rate observed is more than twofold lower (Figure 6B). Excess DNA does not relieve the inhibition imparted by the mutant protein. A similar inhibition of the ATPase activity by RecA K72R was observed (Figure 6C and D). The very similar levels of inhibition found when either protein or DNA was in excess, regardless of whether ATP or dATP was the cofactor, suggested that the distribution of the mutant protein in the mixed filaments on dsDNA was random to a first approximation.

RecA protein filament formation on dsDNA in the presence of dATP was also analyzed by sucrose gradient sedimentation (Figure 7). An equal mixture of 3 μM ^{35}S-labeled wtRecA and 3 μM unlabeled wtRecA or K72R mutant was used to bind to 18 μM M13mp8 RFIII DNA. In this experiment, the DNA is sufficient to bind a total of 3 μM RecA protein, and the labeled protein should again be distributed 50/50 between free and bound forms if binding is complete and unaffected by the identity of the unlabeled RecA. As shown in Figure 7A, 42.4% of the labeled RecA was found in the bound fractions in the reaction containing unlabeled wtRecA; 33.9% of the labeled RecA was found in the bound RecA fractions when K72R is 50% of the RecA mixture. Figure 7B shows RecA binding to dsDNA where a RecA mixture containing 4.8 μM labeled wtRecA and 1.2 μM unlabeled RecA was used to bind to

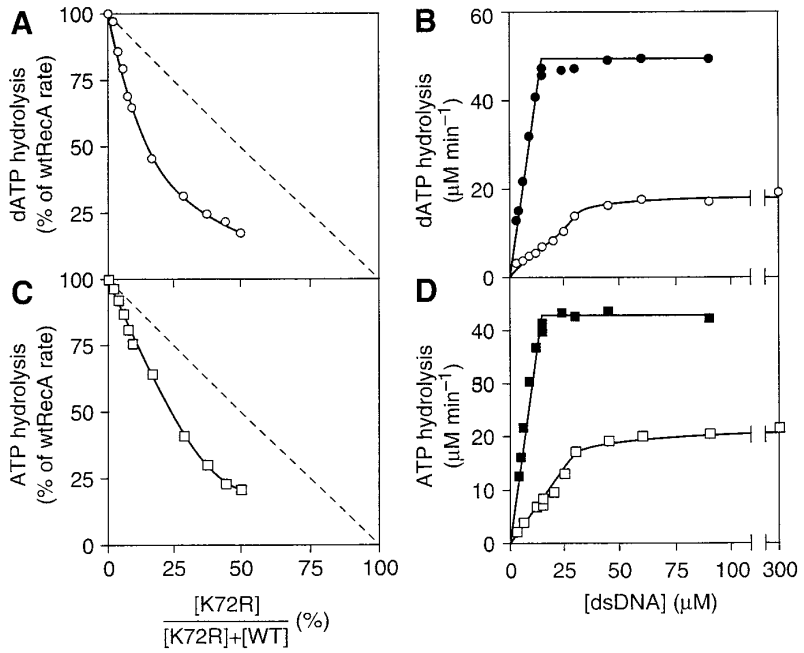

Figure 6. Effects of RecA K72R on the dsDNA-dependent NTP hydrolytic activity of wtRecA protein; formation of mixed filaments. Reactions were carried out as described in Methods, using linear M13mp8 dsDNA cleaved by *Sma*I (panels A and C) or pUC18 dsDNA cleaved by *Pst*I panels B and D). A, Reactions with 3 mM dATP and mixtures of wtRecA and RecA K72R proteins, with total protein kept constant at 5 μM. The dsDNA concentration was 6 μM, or enough to bind 20% of the total RecA protein. B, Effect of increasing dsDNA concentration on dATP hydrolysis rates. Symbols represent reactions with 2.5 μM wtRecA protein either in the presence (○) or absence (●) of 2.5 μM RecA K72R protein. C, Reactions with 3 mM ATP and mixtures of wtRecA and RecA K72R proteins, with total protein kept constant at 5 μM. The dsDNA concentration was 6 μM. D, Effect of increasing dsDNA concentration on ATP hydrolysis rates. Symbols represent reactions with 2.5 μM wtRecA protein either in the presence (□) or absence (■) of 2.5 μM RecA K72R protein.

18 μM M13mp8 RFIII DNA. Radioactivity found in the bound fractions was 46.5% and 35.1% for the RecA mixture containing unlabeled wtRecA or RecA K72R, respectively. These results indicate that either the filaments are somewhat less stable on dsDNA when RecA K72R is present, or the mutant has some tendency to displace the wtRecA protein. The apparent instability or displacement, however, does not fully explain the very large reduction in dATPase activity on dsDNA that occurs in the presence of the mutant protein. Both the gradients and the saturation behavior noted in the dsDNA titration (Figure 6B) suggest that substantial amounts of the wtRecA protein remains bound to the DNA under these conditions, and that at least some of the inhibition of dATP hydrolysis conferred by the mutant protein reflects an interaction between mutant and wtRecA proteins in mixed filaments.

In this case, we cannot eliminate the possibility that the amount of bound RecA protein seen in the gradients was increased by the addition of ATPγS to stop the reactions. The titration curve generated with the wild-type–K72R mixture in Figure 6B levels off at a point where about one protein monomer per 3 bp of DNA is present, and this provides the best evidence that substantial and perhaps saturating amounts of protein were bound to the dsDNA before the addition of ATPγS. It remains possible, however, that the bound protein is biased in favor of the mutant protein. The results of the gradient assay do eliminate the possibility that a mixture of mutant and wild-type proteins precludes binding even in the presence of ATPγS, as is seen with ssDNA binding in the presence of ATP. The gradient assays were not carried out for dsDNA binding in the presence of ATP. The NTPase titrations in Figure 6B and D suggest that significant binding of mixed filaments to dsDNA occurs with either dATP or ATP.

There is little ATP or dATP-mediated dissociation of RecA monomers from wtRecA filaments formed on ssDNA

If RecA monomers or oligomers dissociate transiently from interior positions in a RecA filament, a challenge with the K72R mutant should allow replacement of some wtRecA with the mutant. The resulting mixed filaments should be manifested by decreases in the rate of dATP or ATP hydrolysis due to either protein–protein interactions with the mutant in the filament or a general reduction of wtRecA protein binding to the DNA. Regardless of the molecular basis of the reduction in NTP hydrolysis, a pre-formed and completely saturating filament of wtRecA protein should not be affected by a challenge unless some of the monomers in the filament dissociate to leave binding opportunities for the challenging protein. The coupled spectrophotometric assay for dATP or ATP hydrolysis allows the observation of any changes in NTP hydrolysis in real time.

Filaments were pre-formed on ssDNA in the presence of dATP with wtRecA protein in excess (5 μM) relative to ssDNA (3 μM). Reactions were monitored for 30 minutes, achieving a steady state rate of dATP hydrolysis of 34 μM min^{-1} (an average of six assays). Varying amounts of RecA K72R were then added, and the rates of dATP hydrolysis monitored for an additional 45 minutes. As seen in

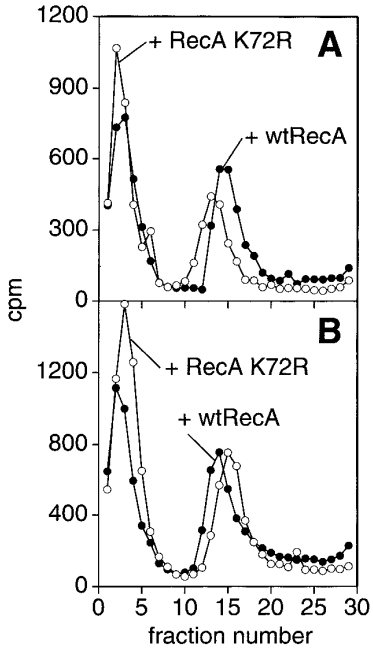

Figure 7. Sucrose gradient sedimentation analysis of RecA protein filaments formed on dsDNA. Reactions were carried out as described in Methods, using 18 μM M13mp8 linear double-stranded DNA and 3 mM dATP. A, Reaction with a RecA mixture containing 3 μM of ^{35}S-labeled wtRecA and 3 μM unlabeled wtRecA (●; 42.4% of the labeled protein bound) or RecA K72R (○; 33.9% bound). B, Reaction with a RecA mixture containing 4.8 μM of ^{35}S-labeled wtRecA and 1.2 μM unlabeled wtRecA (●; 46.5% bound) or RecA K72R (○; 35.1% bound).

Figure 8A, little change in the rate of dATP hydrolysis was observed upon addition of 0 to 5 μM RecA K72R protein. The results indicate that little, if any, dissociation of wtRecA occurs that would permit incorporation of the mutant protein into the filament.

The reverse order experiment is shown in Figure 8B. Addition of varying amounts of wtRecA to pre-formed mutant filaments leads to an immediate initiation of dATP hydrolysis at levels that increased with the amount of wild-type protein added. The rate of dATP hydrolysis observed after the challenge (12.6 μM min^{-1} for a challenge with 5 μM wtRecA) was somewhat less than the rate observed when an equal mixture of wtRecA and RecA K72R was used at levels in excess to the ssDNA (about 16 μM min^{-1}; Figure 3A). A control experiment, in which RecA K72R was omitted and 5 μM wtRecA was added to 3 μM ssDNA in the presence of SSB, yielded a rate of dATP hydrolysis of 31.2 μM min^{-1}. The results of the challenge with wtRecA indicate either that wtRecA can displace the mutant protein under these conditions, or that the mutant protein makes incomplete filaments that leave loading sites for the wtRecA protein. The filaments formed after the challenge with wtRecA were not equivalent to those formed by pre-mixing the wild-type and mutant proteins, at least with respect to dATP hydrolysis. The data for the challenge experiments with filaments on ssDNA and dATP are summarized in Figure 8C.

Similar experiments were carried out with ATP. Results are shown in Figure 8D. The rate of ATP hydrolysis in the filaments changed little for 20 minutes following the challenge, after which a slow transition to a slower rate was observed. The extent of the decrease was again a function of the amount of RecA K72R in the challenge. A challenge of mutant filaments with the wtRecA protein produce much smaller increases in rates of ATP hydrolysis than was the case for the dATP hydrolysis (Figure 8E). This almost certainly reflects the reduced capacity of the mixed filaments to displace SSB and bind to ssDNA in the presence of ATP as shown in Figures 4 and 5. The challenge experiments with ssDNA and ATP as summarized in Figure 8F.

RecA-ssDNA filaments challenged with either the K72R mutant or wtRecA were also examined by sucrose gradient sedimentation (Figure 9). In these experiments, protein concentrations were adjusted so that most of the labeled wtRecA protein present prior to the challenge would be bound to the DNA, allowing changes wrought by the challenge to be evident. RecA filaments were formed with 5 μM ^{35}S-labeled wtRecA protein and 12 μM ssDNA. A maximum of 80% of the protein may bind to the DNA under these conditions. The pre-formed RecA-ssDNA filaments were then challenged by an equal amount (5 μM) of unlabeled K72R mutant or wtRecA protein. No more than 40% of the protein present after the challenge can be bound to the ssDNA, and the fraction of the labeled protein bound should be reduced with time to at least that level if there is an efficient exchange between free and bound RecA protein.

In the presence of dATP (Figure 9, left column), 77.1 or 75.3% of labeled RecA was in the bound RecA pool in the control reactions where RecA storage buffer was substituted for challenging RecA protein (panel A) or ATPγS was added before the challenging RecA protein (panel D), respectively. When unlabeled protein was added as a challenge, 72 or 68.3% of labeled RecA was still found in the bound RecA pool even 50 minutes after addition of RecA K72 mutant (panel B) or wtRecA protein (panel C), respectively. These results demonstrate that less than 10% (and perhaps none) of the RecA bound to ssDNA dissociates so as to allow replacement by unlabeled protein over a 50-minute period when dATP is the nucleotide cofactor.

Similar challenging experiments were also carried out using ATP as cofactor (Figure 9). Plots in the middle and right columns show the status of labeled wtRecA protein in the filaments at 20 and 50 minutes after the addition of the unlabeled proteins. In the control experiments (panels A and D), only about 2/3 of the labeled RecA protein was found in the bound fractions (as opposed to the 80% predicted maximum). This may reflect an intrinsic decrease in the stability of the filaments in the

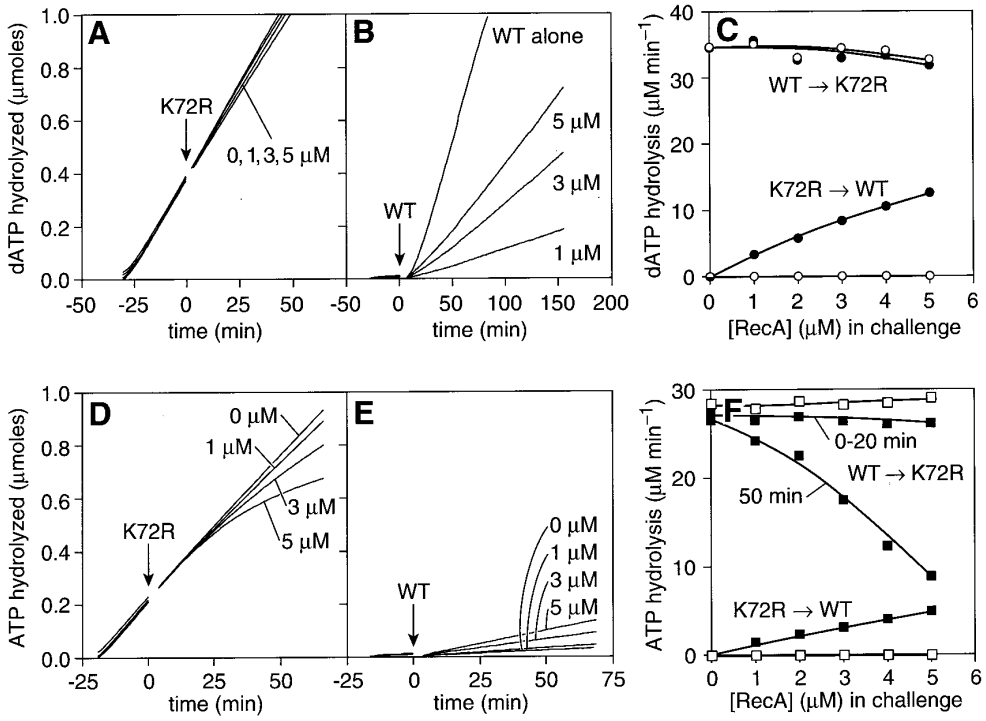

Figure 8. Lack of RecA exchange in filaments formed on ssDNA; challenge experiments. Reactions were carried out as described in Methods. Reactions contained 3 μM circular M13mp8 ssDNA, 0.3 μM SSB, and 3 mM dATP (panels A to C) or ATP (panels D to F). Reactions with 5 μM wtRecA protein (panels A and D) or 5 μM RecA K72R (panels B and E) were initiated by the addition of SSB and nucleotide and monitored at steady state. Reactions were challenged at a time defined as zero time (arrow) with the indicated concentration of the second protein, and monitoring was continued. In panels A, D, and E, the 0 RecA K72R challenge control reaction substituted RecA storage buffer for the challenging protein. In panel B, one reaction was initiated without RecA K72R, and 5 μM wtRecA protein was added at zero time (wtRecA control). The challenge resulted in less than a 3% change in reaction volume in all cases. Results for dATP (panels A and B) are summarized in panel C. The upper two lines summarize the data for the K72R challenge of a wtRecA reaction, and the bottom two lines summarize the data for the inverse challenge in panel B. Rates shown in each case are those before (○) and after (●) the challenge. The ATP results are summarized in panel F. The upper three lines summarize the data for the K72R challenge of a wtRecA reaction, and the bottom two lines summarize the data for the inverse challenge in panel E. Rates shown in each case are those before (□) and after (■) the challenge. In the K72R challenge of the wtRecA reaction, rates are given at both 20 minutes and 50 minutes after the challenge.

gradient when they are formed with ATP, even though ATPγS is added to stabilize them prior to centrifugation. In the RecA K72R challenge experiment, ^{35}S-labeled RecA retained in the bound RecA pool was gradually decreased to 32.8% after 20 minutes (panel B, middle column) and 6.1% after 50 minutes (panel B, right column). This is generally consistent with the slow decline in ATP hydrolysis seen in Figure 8. In the reaction where unlabeled wtRecA was used in the challenge (panel C, middle and right columns), the observed reduction in the labeled wtRecA protein in the bound fractions was much reduced. The bound fractions observed with ATP in panel A (middle column) and B (middle and right column) were also checked by SDS-PAGE (data not shown). The amount of RecA protein in the 20-minute challenge reaction decreased significantly compared to that in panel A. Little RecA protein can be detected in the bound fractions 50 minutes after the K72R challenge in the presence of ATP, consistent with the results shown in Figure 4. Evidently, a slow replacement of labeled with unlabeled protein in the presence of ATP leads to a catastrophic reduction of filament stability when the mutant protein is used in the challenge. The relative stability of the filaments in the presence of ATP when unlabeled wtRecA is used as the challenge is consistent with previous observations by Neuendorf & Cox (1986), showing that ssDNA-dependent ATP hydrolysis results in little exchange of RecA between free and bound forms. In this earlier work (Neuendorf & Cox, 1986), ATPγS was also included in the gradients, which may serve to further reduce the observed instability of the filaments formed in the presence of ATP.

Filaments formed on dsDNA are more dynamic (in terms of monomer exchange in and out) than those formed on ssDNA

A set of challenge experiments were also carried out with filaments formed on dsDNA at pH 6.3 (Figure 10). Conditions were otherwise similar to those in Figure 8, except that the 3 μM ssDNA was replaced by 6 μM dsDNA. When RecA K72R was added to wtRecA filaments in the presence of dATP,

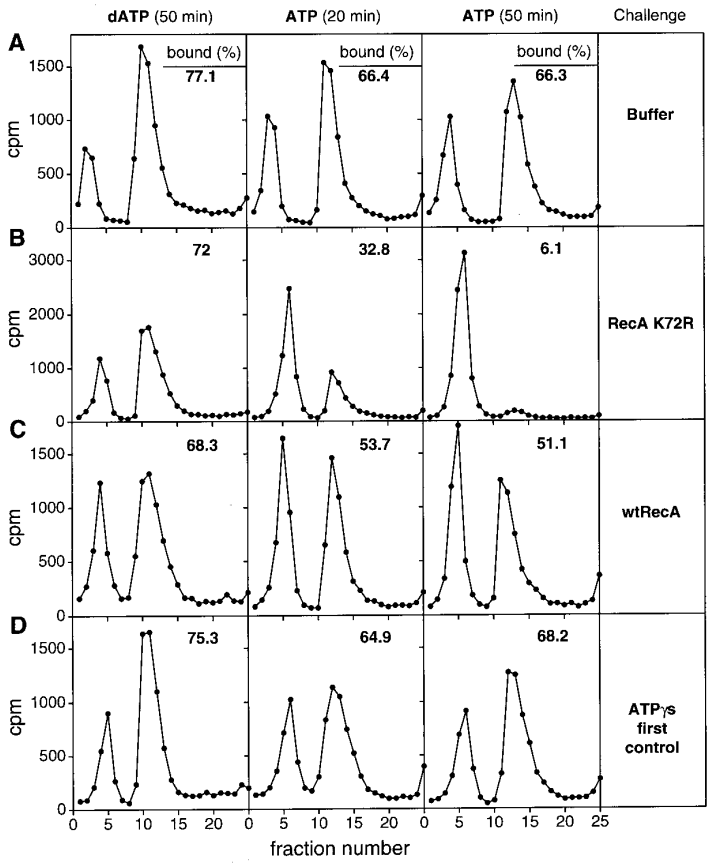

Figure 9. RecA exchange in filaments formed on ssDNA; challenge experiments analyzed by sucrose gradient sedimentation. Reactions were carried out as described in Methods. Reactions contained 12 μM circular M13mp8 ssDNA, 1.2 μM SSB, and 5 μM of ^{35}S-labeled wtRecA. The three columns reflect three similar reactions differing only in the nucleotide cofactor used (3 mM dATP or ATP as indicated) and the length of time after the challenge at which the reaction was stopped by addition of ATPγS (times indicated at the top of each column). For each set of experiments, panels B and C show the pre-formed labeled RecA protein filaments, challenged by 5 μM unlabeled RecA K72R (B) or wtRecA (C). Buffer control reactions in panel A substitute RecA storage buffer for the challenging RecA protein. In panel D, ATPγS was added first to the performed RecA-ssDNA filament (1 mM final concentration) before the challenging RecA K72R was added. In the panel D reactions, the indicated incubation was still carried out at 37°C after the challenge. The fraction of labeled RecA protein in the bound fraction is indicated in each panel and was calculated as described in Methods.

a transition to a lower rate of dATP hydrolysis was observed beginning three to five minutes after the challenge and continuing for up to 20 minutes (Figure 10A). Unlike the changes seen in challenge experiments involving ssDNA, the final rates of dATP hydrolysis were comparable to those observed when the same amounts of wild-type and mutant proteins were mixed prior to filament formation (Figure 10B). There is no direct correlation between the hydrolysis of dATP in these experiments and the replacement of wtRecA with mutant protein. In most of the challenge experiments, every monomer in the wtRecA filaments hydrolyzes over 60 dATPs (three minutes of reaction at k_{cat}) before a change in the hydrolytic rate is evident. Similar results obtained from experiments with ATP are shown in Figure 10, panels C and D. The challenge by the mutant protein again results in a decrease in ATP hydrolysis, indicating substitution of the mutant into the filament. In this case, the changes occur somewhat more rapidly, and the final rates observed after the challenge are in most cases significantly less than the rates observed when the same amounts of wild-type and mutant were mixed prior to filament formation.

In the reverse order experiments, where the mutant is pre-incubated with dATP (or ATP) and DNA prior to a challenge with wtRecA protein, the rates of dATP or ATP hydrolysis observed after the challenge are similar in magnitude to those seen for pre-mixed proteins in Figure 6, although the changes from no hydrolysis to the new rates are nearly instantaneous (data not shown). We have no evidence that the mutant protein binds to dsDNA in the absence of the wild-type protein, and the results may reflect the rapid formation of a mixed filament where none was present prior to the wtRecA challenge.

The fate of RecA-dsDNA filaments after a challenge in the presence of dATP was also analyzed with the sucrose gradient (Figure 11). When 4.8 μM ^{35}S-labeled RecA protein and 18 μM RFIII M13mp8 DNA were used to form RecA-dsDNA filaments, up to 62.5% of the RecA can bind to the dsDNA. The pre-formed filaments were challenged by unlabeled wtRecA or RecA K72R proteins for 20 minutes. In the control reactions (panels A and D), 55 to 61% of labeled RecA was in the bound RecA pool. Panels B and C show the status of the labeled RecA in the filaments 20 minutes after a challenge with 4.8 μM RecA K72R or wtRecA, respectively. If the labeled and unlabeled proteins compete equally well for DNA binding sites, and the proteins exchange rapidly between free and bound pools, about 31% of the labeled protein should remain bound to the DNA at

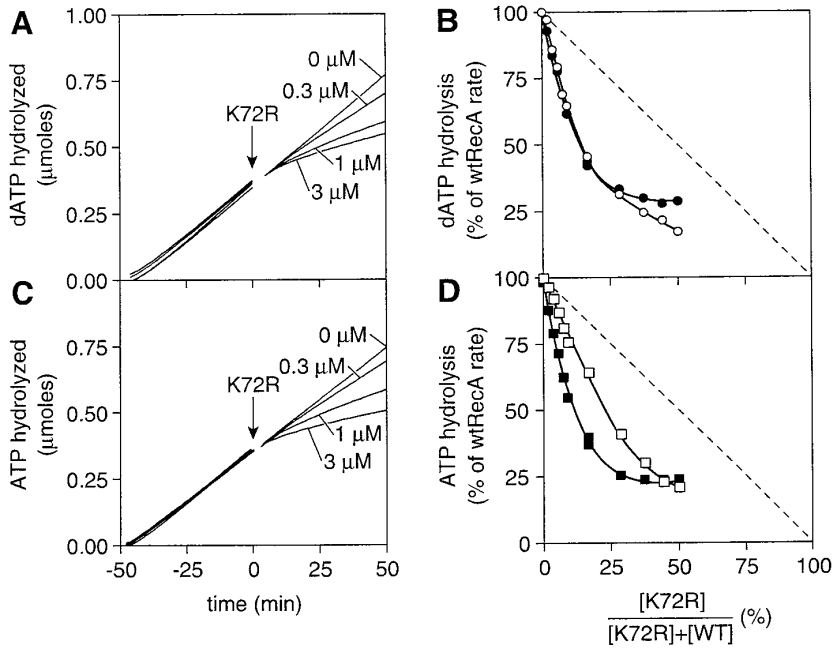

Figure 10. Exchange of RecA K72R into wtRecA filaments formed on dsDNA; challenge experiments. Reactions were carried out as described in Methods, and contained 6 μM linear M13mp8 dsDNA (cleaved by *Sma*I) and 5 μM wtRecA were initiated by the addition of 3 mM dATP (A and B) or 3 mM ATP (C and D) and monitored at steady state. In panels A and C, reactions were challenged at a time defined as zero time (arrow) with the indicated concentration RecA K72R protein, and monitoring was continued. In panels A and C, the zero RecA K72R challenge control reaction substituted RecA storage buffer for the challenging RecA K72R. The challenge resulted in less than a 3% change in the reaction volume in all cases. The final rates of dATP and ATP hydrolysis observed after the challenge are summarized in panels B (●) and D (■), respectively. For comparison, the data obtained when wtRecA and K72R were mixed prior to initiating a reaction (from Figure 5A and C) are superimposed in panels B and D for dATP (○) and ATP (□), respectively, Note that the total RecA protein (wild-type + mutant) in the challenge reactions varies, but is held constant at 5 μM in the data from Figure 5. Protein is in large excess relative to DNA binding sites in both cases, so that the data reflect the proportion of the bound protein that is wild-type.

equilibrium. In both cases, about 25% of labeled RecA remained in the bound RecA pool after 20 minutes. Given the small reductions in bound RecA in the controls relative to expectations, these results suggest that the exchange reaction in the presence of dATP comes to equilibrium or close to it in 20 minutes. These results demonstrate that RecA filaments formed on dsDNA are much more dynamic than those formed on ssDNA. Addition of ATPγS before adding the unlabeled RecA almost completely abolished the exchange of RecA protein, indicating that the RecA protein exchange process requires ATP or dATP hydrolysis.

The exchange of RecA monomers into and out of filaments formed on dsDNA is independent of the length of the DNA substrate, the concentration of DNA ends, or pH values between 6.3 and 7.5

If RecA protein filaments extend uninterrupted for the length of available DNA substrates, and if exchange of RecA monomers or oligomers occurs only on filament ends, the results of challenge experiments should be affected by the length of the DNA substrate and by the concentration of DNA ends. Challenge experiments similar to those in Figure 10 were carried out except that three different dsDNA substrates, all derived from ϕX174 DNA, were used. One was nicked circular duplex ϕX174 DNA, the second was full length ϕX174 DNA (5386 bp) cleaved by *Pst*I restriction enzyme, and the third was ϕX174 DNA digested with *Hae*III. The last of these three substrates contained 11 different dsDNA fragments ranging from 72 bp to 1323 bp. The total concentration of dsDNA (in bp) was identical in all of the experiments, but the DNA fragments produced by the *Hae*III digest are smaller and the concentration of DNA ends is 11 times greater. When RecA K72R protein was added to the pre-formed wtRecA filaments, the time courses for changes in wtRecA's ATPase or dATPase activities were identical for the three different dsDNA substrates (Figure 12A and B). The ATP or dATP hydrolysis rates before and after the RecA K72R challenge, and the relative rate change upon challenge, are summarized in Table 1. The results suggest that RecA protein exchanges occur in the interior of nucleoprotein filaments formed on dsDNA.

The experiments described above for RecA-dsDNA filaments, were carried out at pH 6.3 to avoid complications arising from the slow nucleation step encountered in dsDNA binding at neutral pH (Pugh & Cox, 1987, 1988a). Since most of the published data on RecA protein-mediated reactions were obtained at pH values from 7 to 8, we wished to determine if the exchange of RecA monomers out of RecA filaments on dsDNA occurred at neutral pH values. There are several ways to circumvent the slow nucleation step in dsDNA binding by RecA, and lowering the pH is only one of them. Incorporating a single-stranded gap in the dsDNA substrate is just as effective (Pugh & Cox, 1987, 1988a). We therefore formed RecA filaments on a circular dsDNA substrate (GD1037) with 7299 bp of

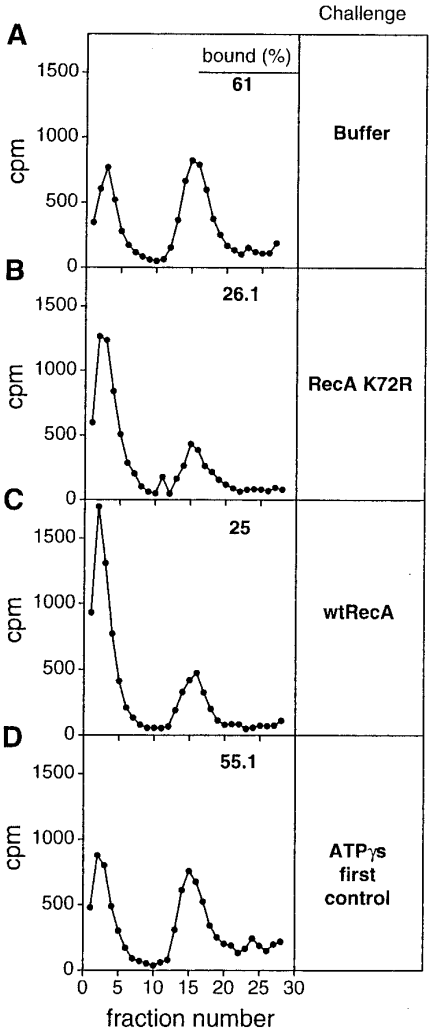

Figure 11. RecA exchange in filaments formed on dsDNA; challenge experiments analyzed by sucrose gradient sedimentation. Reactions were carried out as described in Methods. Reactions contained 18 μM linear M13mp8 dsDNA, 4.8 μM of ^{35}S-labeled wtRecA, and 3 mM dATP. The challenge experiments were carried out for 20 minutes. In panels B and C, pre-formed labeled RecA protein filament formed on dsDNA was challenged by 4.8 μM unlabeled RecA K72R (B) or wtRecA (C). Buffer control reactions in panel A substituted RecA storage buffer for the challenging RecA protein. In panel D, ATPγS was added first to the pre-formed RecA-dsDNA filament (1 mM, final concentration) before challenging RecA K72R was added, then the incubation was continued for 20 minutes. The fraction of labeled RecA protein in the bound fraction is indicated in each panel and was calculated as described in Methods.

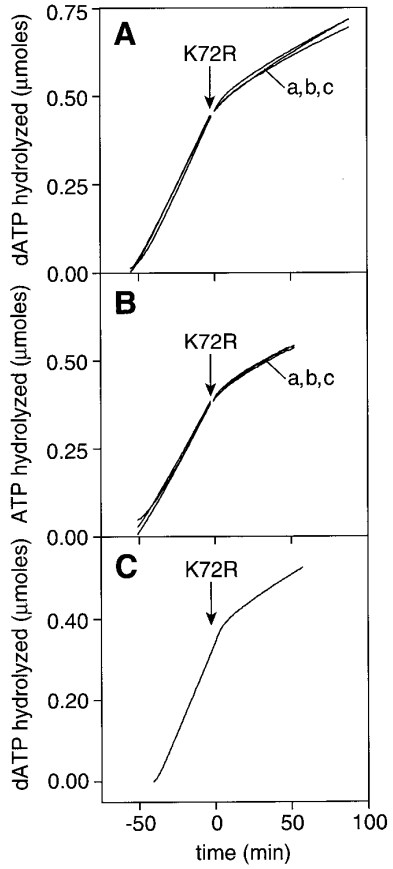

Figure 12. RecA protein exchange with filaments formed on dsDNA; independence of DNA length or concentration of DNA ends. Reactions were carried out under conditions described in Methods, contained 6 μM dsDNA and 5 μM wtRecA, were initiated by the addition of 3 mM dATP (A) or 3 mM ATP (B), and monitored at steady state. Three different dsDNA substrates derived from ϕX174 DNA were used in panels A and B. One was nicked circular duplex ϕX174 DNA (5386 bp, a), the second was full length ϕX174 DNA (5386 bp) cleaved by *Pst*I restriction enzyme (b), and the third was ϕX174 DNA digested with *Hae*III, which contains 11 different dsDNA fragments ranging from 72 bp to 1323 bp (c). Reactions were challenged at a time defined as zero time (arrow) with 3 μM RecA K72R and monitoring was continued. The challenge resulted in less than a 3% change in the reaction volume. The ATP or dATP hydrolysis rates before and after the RecA K72R challenge, and the relative rate decrease upon challenge, are summarized in Table 1. In panel C, RecA protein filaments were formed on GD1037 DNA. Reactions contained 6 μM of the gapped DNA and 5 μM wtRecA in a Tris-acetate buffer (pH 7.5) as described in Methods, and were again initiated by the addition of 3 mM dATP. The challenge was carried out as in panels A and B, at a time defined as zero time (arrow) with 3 μM RecA K72R and monitoring was continued. The rates of dATP hydrolysis before and after the challenge were 22.2 and 5.9 μM min^{-1}, respectively.

duplex DNA contiguous with a 1037 nucleotide single-stranded region. DNA and protein concentrations were the same as those in panels A and B of Figure 12, but a Tris-acetate buffer at pH 7.5 replaced the pH 6.3 buffer. As shown in Figure 12C, the challenge with RecA K72R results in a virtually identical decline (from 22.2 to 5.9 μM min^{-1}) in the rate of dATP hydrolysis. This provides evidence that the RecA filaments formed on dsDNA exhibit similar properties with respect to monomer exchange at pH 6.3 and 7.5.

Table 1. RecA protein exchange with filaments formed on dsDNA; independence of DNA length or concentration of DNA ends

	dATPase (μM min^{-1})			ATPase (μM min^{-1})		
	Before	After	Decrease (%)	Before	After	Decrease (%)
φX174 (*RF*II)	22.5	6.7	70.2	19.9	5.2	74.0
φX174 (*Pst*I)	22.3	5.8	74.0	21.3	5.4	74.8
φX174 (*Hae*III)	23.7	6.2	73.8	20.5	5.5	73.2

Reactions are those shown in Figure 12, panels A and B. The before and after column headings refer to the measured rates of dATP or ATP hydrolysis before and after the challenge with RecA K72R protein. The % decrease refers to the decline in hydrolytic activity resulting from the challenge.

Discussion

There are three primary conclusions arising from this study. First, the RecA K72R mutant forms mixed filaments with wtRecA protein under at least some conditions. The presence of the mutant protein generates an inhibition of the wtRecA ATP or dATP hydrolytic activity under many conditions that appears to reflect a cooperative interaction between RecA monomers in the filament. Characterization of the mixed filaments facilitates the interpretation of challenge experiments in which the mutant protein is added to pre-formed filaments of wild-type protein and *vice versa*. The challenge experiments lead to the second conclusion, that RecA filaments formed on dsDNA are more dynamic, with respect to exchange of RecA protein between free and bound pools, than those formed on ssDNA. We do not know if the species undergoing exchange is a RecA monomer or an oligomer. A transient dissociation of wtRecA monomers or oligomers from filaments formed on dsDNA is manifested by a decrease in ATP or dATP hydrolysis and a corresponding decrease in bound labeled RecA protein. Unlike the end-dependent disassembly of filaments observed at higher pH (Lindsley & Cox, 1989, 1990), this exchange occurs in the interior of the filament, and is not affected by changes in the length of the dsDNA or the concentration of DNA ends. The third conclusion is that the increased monomer exchange indicates a change in filament state brought on by the incorporation of a second DNA strand into the filament. Occupation of one of the three DNA strand binding sites postulated to be present in the RecA filament groove (Cox, 1995) yields a stable RecA-ssDNA nucleoprotein filament, and occupation of a second binding site by a complementary DNA strand has a substantial effect on the properties of that filament.

The use of ATP or dATP hydrolysis to monitor the binding of wild-type RecA protein in the filaments has an inherent ambiguity. A decline in the hydrolytic activity observed when the mutant protein is present could be due simply to replacement of an amount of wild-type protein proportional to the decline. However, the decline could be further affected by inhibitory effects mediated by cooperative protein–protein interactions in the filaments that either decrease NTP hydrolysis in the bound filament or impede binding altogether. We have attempted to sort this out in part by measuring the activities of the protein mixtures under two conditions, with either protein or DNA in excess. Inhibition observed when RecA protein (wild-type + mutant) is in excess suggests that both proteins compete well for DNA binding sites. Inhibition that remains when DNA is in excess can be attributed to effects of protein–protein interactions in mixed filaments. In many cases, the degree of inhibition observed does not change when DNA is in excess as opposed to when RecA protein is in excess. Ambiguities remain, however, and we have refined the interpretation of the experiments by carrying out a set of parallel experiments with ^{35}S-labeled RecA, separating the bound and free forms of the protein on sucrose density gradients. The combination of methods provides a fairly complete view of the dynamic state of RecA filaments bound to DNA.

The RecA K72R mutant protein is surprisingly competent in DNA strand exchange reactions when dATP is used as a cofactor (Shan *et al*., 1995), and it forms filaments on ssDNA with a striated structure similar to that formed by the wild-type RecA protein (Q.S. & R. B. Inman, unpublished results). There appears to be little discrimination between the wild-type and mutant proteins in binding to DNA and forming filaments, at least in the presence of dATP. The proteins compete for DNA binding sites and exhibit the same binding site size on ssDNA in the presence of dATP. Some of the inhibition patterns observed under other conditions indicate a direct interaction between them. The formation of mixed filaments containing this mutant and wild-type RecA protein provides an opportunity to test many aspects of the role of ATP hydrolysis in DNA strand exchange reactions. Interpretation of results requires an understanding of the properties of the mixed filaments. The DNA substrate and the nucleotide cofactor are revealed in this study as key variables.

For reasons not completely understood, RecA protein-mediated DNA strand exchange reactions

are enhanced when dATP replaces ATP (Menetski & Kowalczykowski, 1989). The RecA K72R mutant also functions much better with dATP, although no hydrolysis occurs (Rehrauer & Kowalczykowski, 1993; Shan *et al*., 1995). Perhaps not surprisingly, the results obtained with mixed filaments are more readily interpreted when dATP is the nucleotide cofactor. On ssDNA, there is a simple competition for DNA binding sites, whether protein or ssDNA is present in excess. When protein is in excess, the inhibition of dATP hydrolysis exactly reflects the fraction of total protein made up by the mutant. The results are at least qualitatively confirmed by the gradient sedimentation assay. No inhibition is observed when ssDNA is in excess, suggesting that the presence of mutant protein in the filaments has little effect on the activity of adjacent wild-type monomers. When wild-type filaments are challenged by the mutant protein, no decline in dATP hydrolysis (or the labeled RecA protein in bound fractions in the sucrose gradients) is seen that would indicate a protein exchange, although the inverse experiment suggests that the mutant protein can be displaced to a degree by the wild-type protein (or that there are gaps in the filament formed by the mutant protein that can be filled by wtRecA). With dATP, there is no indication of a cooperative interaction between RecA monomers on ssDNA that is manifested in the inhibition patterns observed when the mutant protein is added.

In contrast, on dsDNA the mixed filaments exhibit an enhanced inhibition of dATP hydrolysis most readily explained by an interaction between the mutant and wild-type proteins in the filament. The inhibition is not relieved at all when dsDNA is in excess, suggesting that the filaments are indeed mixed and the mutant protein affects the activity of adjacent wtRecA monomers even when all the protein can bind to DNA. The complementary experiments with sucrose density gradients suggest that somewhat more wtRecA protein has been displaced than would occur if the wild-type and mutant proteins competed equally for binding sites, but also indicate that the reduction in bound wtRecA protein is insufficient to explain the reduction in dATP hydrolysis (with caveats noted in Results). When combined with data showing the rate of dATP hydrolysis in mixed filament exhibits a saturation behavior with respect to dsDNA concentration that is consistent with nearly stoichiometric binding of protein to the DNA, the results indicate that at least some of the inhibition of dATP hydrolysis in mixed filaments on dsDNA can be attributed to direct interactions between wild-type and mutant proteins.

In contrast to the situation observed on ssDNA, challenge experiments readily reveal an exchange between mutant and wild-type proteins in filaments formed on dsDNA in the presence of dATP. The exchange endpoint (reflected by a new steady state rate of dATP hydrolysis) is generally consistent with that observed when wild-type and mutant proteins are pre-mixed in the same amounts. Every dATP hydrolytic event is not obligatorily coupled to the dissociation of a wtRecA monomer and its replacement with another mutant or wild-type monomer. Upon challenge with the mutant protein, the transition to a lower dATPase rate occurs over 10 to 20 minutes, so that hundreds of dATPs are hydrolyzed for every wtRecA monomer that is replaced by a mutant protein.

The results are more complicated when ATP replaces dATP. Cooperative interactions between adjacent RecA monomers seem evident even on ssDNA (Figure 3C and D). The results obtained with the sucrose gradients show that mixtures of the mutant and wild-type proteins do not readily bind to ssDNA when ATP is the nucleotide cofactor, explaining the strong inhibition of the rates of ATP hydrolysis when the mutant protein is present. The inhibition of ssDNA binding in these experiments provides yet another indication of strong interactions between the wild-type and mutant proteins. The results obtained with mixed filaments on dsDNA in the presence of ATP are similar to those obtained with dsDNA in the presence of dATP. Surprisingly, the mixed filaments formed in the presence of ATP appear to bind to dsDNA more readily than to ssDNA. In general, filaments of wild-type protein appear to be more stable, predictable, and resistant to substitution by the mutant protein when dATP is used.

The same evidence that indicates a strong interaction between wild-type and mutant proteins implicates those interactions as key factors affecting the ATP or dATP hydrolytic cycle. Cooperative interactions between RecA monomers that affect the RecA NTPase activity have seemed likely, but only a few studies have provided evidence for them (Menge & Bryant, 1988; Lindsley & Cox, 1989; Cox, 1994). The emerging picture is one in which ATP hydrolysis in an individual monomer does not happen in isolation. Instead, a hydrolytic event in one monomer triggers some step in the hydrolytic cycle of its neighbor, perhaps resulting in waves of ATP or dATP hydrolysis traveling unidirectionally down a filament at some established rate (Cox, 1994). ATP hydrolysis may not be possible in a lone RecA monomer, and further work may lead to definition of a minimum oligomeric unit for optimal ATP hydrolysis.

Little or no RecA protein dissociation or exchange is observed with pre-formed filaments of wild-type RecA protein on ssDNA, even though each RecA monomer is hydrolyzing ATP or dATP with a k_{cat} of 30 min^{-1} or more. This agrees with earlier results (Neuendorf & Cox, 1986). Exchange is facilitated when dsDNA is bound within the filament. The second DNA strand must be complementary to the first to elicit this effect, since the inhibition of dATP hydrolysis seen in mixed filaments formed in the presence of excess dsDNA (Figure 6B) is not observed in the presence of excess (non-complementary) ssDNA (Figure 3B). The addition of this second complementary DNA strand in wtRecA filaments also results in a rate of dATP or ATP

hydrolysis that is decreased about 30% relative to that observed for the same filaments on ssDNA (Cox, 1994). The same changes in filament state may also occur when DNAs are first paired during DNA strand exchange. We previously characterized an abrupt and homology-dependent decrease in the rate of ATP hydrolysis when homologous duplex DNA is added to RecA filaments formed on ssDNA (Schutte & Cox, 1987). The ATPase rate observed following this synapsis-associated decrease is identical to that observed for RecA filaments bound to dsDNA. The altered state of RecA filaments on dsDNA may be relevant to filament function during DNA strand exchange.

The exchange of RecA monomers out of filaments bound to dsDNA is distinct from the end-dependent disassembly of filaments on dsDNA at neutral pH characterized previously (Lindsley & Cox, 1989, 1990). The process documented in the current study occurs readily at pH 6.3, where no net disassembly of filaments occurs. The present exchange also does not exhibit any dependence on the concentrations of filament ends or on the length of the dsDNA, as the end-dependent disassembly process does. The exchange also occurs at pH 7.5, and is therefore likely to be relevant to the DNA strand exchange process.

Does an exchange of RecA monomers or oligomers between free and DNA-bound pools play a mechanistic role in DNA strand exchange? The exchange observed for filaments bound to dsDNA meets one requirement of the mechanistic proposal of Kowalczykowski and colleagues that ATP hydrolysis during strand exchange is required to re-distribute RecA monomers (Kowalczykowski & Krupp, 1995; Menetski *et al*., 1990; Rehrauer & Kowalczykowski, 1993). However, there is as yet no evidence that the RecA protein exchange is a mechanistic requirement for strand exchange. The data included in this report provide one result potentially relevant to the question of the function of monomer exchange. While the use of dATP in place of ATP enhances the DNA strand exchange reaction, it tends to suppress the exchange of RecA monomers into and out of RecA filaments. This suggests that RecA protein exchange may be irrelevant or actually detrimental to the strand exchange process.

Materials and Methods

Enzymes and biochemicals

Escherichia coli RecA protein was purified as described previously (Cox *et al*., 1981). The RecA K72R protein was purified as described (Shan *et al*., 1995). Labeling of wtRecA protein with ^{35}S was carried out using a published procedure (Soltis & Lehman, 1983) and it was purified in the same manner as unlabeled wtRecA protein. The specific activity of labeled wtRecA protein was 6.1×10^7 cpm μmol^{-1}. RecA protein was stored in R buffer (20 mM Tris-acetate (80% cation, pH 7.5), 1 mM DTT, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 10% (w / v) glycerol). The ssDNA-dependent ATPase and three-strand DNA exchange activity of the labeled RecA protein were comparable to those of the unlabeled RecA protein. All RecA protein preparations were more than 95% pure and free of detectable nucleases. The wtRecA, labeled RecA, and RecA K72R protein concentrations were determined by absorbance at 280 nm using an extinction coefficient of $\epsilon_{280} = 0.59 A_{280}$ mg^{-1} ml (Craig & Roberts, 1981). *E. coli* single-stranded DNA binding protein was purified as described (Lohman *et al*., 1986) with the minor modification that a DEAE-Sepharose column was added to ensure removal of single-strand exonucleases. The concentration of SSB protein was determined by absorbance at 280 nm using an extinction coefficient of $\epsilon_{280} = 1.5$ A_{280} mg^{-1} ml (Lohman & Overman, 1985). Restriction endonucleases, phage T4 polynucleotide kinase were purchased from New England Biolabs. Terminal transferase was purchased from Promega. Tris buffer was from Fisher Scientific. ATP, proteinase K, lactate dehydrogenase (LDH), pyruvate kinase, phosphoenolpyruvate, and nicotinamide adenine dinucleotide (reduced form, NADH) were purchased from Sigma. Ultrapure dATP, DEAE-Sepharose resin and a MonoQ column were from Pharmacia Biotech Inc. Hydroxylapatite resin was from Bio-Rad. $H_2[^{35}S]O_4$ was purchased from New England Nuclear.

DNA

Duplex and ssDNA substrates were derived from bacteriophage ϕX174 (5386 bp), pUC18 (2686 bp), and M13mp8 (7229 bp; Messing, 1983). The ϕX174 supercoiled circular duplex DNA, nicked circular duplex DNA (RFII; about 90% in nicked circular form), and viral circular ssDNA were purchased from New England Biolabs. The ϕX174 dsDNA digested with *Hae*III restriction endonuclease (11 fragments ranging from 72 to 1353 bp) was purchased from Gibco BRL. Supercoiled circular duplex DNA and circular single-stranded DNA from bacteriophage M13mp8 were prepared as described previously (Davis *et al*., 1980; Messing, 1983; Neuendorf & Cox, 1986). Plasmid pUC18 was purified as described (Davis *et al*., 1980). Gapped duplex DNA (GD1037) was prepared using a large scale RecA reaction and isolated from agarose gel as described (Shan *et al*. 1995). The (+) strand of GD1037 is circular M13mp8. 1037 ssDNA (8266 nucleotides in length; Shan, *et al*., 1995). The complementary strand has 7229 nucleotides derived from M13mp8. The GD1037 DNA contains a 7229 bp duplex region and a 1037 nucleotide single-stranded gap. 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 DNA substrates were generated by complete digestion of supercoiled DNA by appropriate restriction endonucleases. The protein was removed by extraction with phenol / chloroform / isoamyl alcohol (25:24:1, by vol.) and chloroform / isoamyl alcohol (24:1, v / v) followed by ethanol precipitation.

ATPase and dATPase assays

NTP hydrolysis activities were measured by a coupled enzyme assay (Lindsley & Cox, 1990; 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. 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, 1990; Morrical *et al*., 1986).

Rates of DNA-independent ATP or dATP hydrolysis were measured at 37°C in a solution (400 μl) containing 50 mM Tris-acetate (80% cation, pH 7.5), 17.5 mM $Mg(OAc)_2$, 2% (w / v) glycerol, 0.1 mM EDTA, 1 mM DTT, 1.8 M NaCl, and 5 mM ATP or dATP. The ATP and dATP regeneration used in these reactions contained 24 mM phosphoenolpyruvate, 100 units ml^{-1} pyruvate kinase, and 3 mM K-glutamate. A coupling system containing 3 mM NADH and 80 units ml^{-1} lactate dehydrogenase was also included. The final pH at room temperature after addition of all reaction components was 7.5.

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, an ATP regeneration system (3 mM phosphoenolpyruvate, 5 units ml^{-1} pyruvate kinase, and 3 mM K-glutamate (Lindsley & Cox, 1990)) or a dATP regeneration system (7.5 mM phosphoenolpyruvate, 20 units ml^{-1} pyruvate kinase, and 3 mM K-glutamate (Menetski & Kowalczykowski, 1989)), and a coupling system (3 mM NADH and 4.5 units ml^{-1} lactate dehydrogenase). Rates of dsDNA-dependent ATP or dATP hydrolysis were carried out under identical conditions except that 25 mM 2-(*N*-morpholino)ethanesulphonic acid (Mes)NaOH (56% anion pH 6.3) replaced the Tris-acetate unless otherwise indicated. In all reactions, the nucleotide cofactor concentration (ATP or dATP) was 3 mM. The final pH after addition of all reaction components was 7.6, or 6.3 for reactions with dsDNA. Concentrations of DNA and proteins are reported in the text and Figure legends.

Unless otherwise specified, the ssDNA used in ssDNA-dependent ATP or dATPase assays was circular ssM13mp8 DNA. RecA protein was incubated with ssDNA at 37°C for ten minutes before a mixture of ATP or dATP and SSB was added to initiate the reaction. The concentration of SSB was 1/10 of the ssDNA concentration. In dsDNA-dependent ATP or dATPase assays, unless otherwise specified, the dsDNA was M13mp8 linearized with a unique restriction endonuclease. RecA protein was incubated with dsDNA at 37°C for ten minutes before ATP or dATP was added to initiate the reaction. When wtRecA and RecA K72R were used as a protein mixture, they were mixed at least 30 minutes prior to reaction and stored on ice. When wtRecA and RecA K72R were used separately in challenge experiments, the challenging RecA protein (either wtRecA or RecA K72R) was added to an ongoing reaction after a steady-state rate of ATP or dATP hydrolysis had been achieved and monitored for at least 20 minutes.

Data analysis

The NTPase assays reported here were carried out over a ten month period using three wtRecA and two RecA K72R preparations. The data presented in a given Figure were generally collected within one week. The error in determining absolute ATP or dATP hydrolysis rates by the coupled assay was less than 10%. Up to six NTP hydrolysis rates can be determined at the same time in the spectrophotometer, and in many cases several sets of six assays were combined in a single Figure. NTP hydrolysis by mixtures of wtRecA and RecA K72R was always measured in parallel with a control (one of the six reactions) containing a concentration of wtRecA protein equal to the total concentration of mutant + wtRecA protein in the other five experiments. Rates from the various wtRecA control reactions obtained under a given set of conditions were averaged, and rates from different sets of NTP hydrolysis assays were combined only if the rates from the respective wtRecA control reactions fell within $\pm 5\%$ of the averaged wtRecA rate. Under all conditions used in this investigation and a previous investigation (Shan *et al*., 1995), the RecA K72R protein did not hydrolyze ATP or dATP at a level detectable by the coupled spectrophotometric assay. Rates of RecA K72R-mediated ATP or dATP hydrolysis were assumed to be zero for purposes of calculating NTPase rates for the mixed filaments.

Reaction conditions for sucrose gradient sediment assays

DNA binding experiments

Reaction conditions for forming RecA-ssDNA or RecA-dsDNA filaments were essentially the same as those used in ssDNA or dsDNA-dependent NTPase assays, except that NADH and LDH was not included. The DNA substrates used were the indicated full-length versions of M13mp8 unless another DNA is specified. The ssDNA and dsDNA concentrations were increased relative to those in the ATP and dATPase assays in order to increase the signal in the gradients. The ssDNA binding reactions (120 μl) contained 12 μM circular ssDNA, 8 μM RecA, and 1.2 μM SSB protein; the dsDNA binding reactions (120 μl) contained 18 μM linear dsDNA and 6 μM RecA protein. Reactions were carried out at 37°C for 20 minutes and stopped by adding ATPγS to a final concentration of 1 mM, before being analyzed by sucrose gradient sedimentation as described below.

Challenge experiments

RecA-ssDNA filaments or RecA-dsDNA filaments were formed as described above, but contained 5 μM ^{35}S-labeled RecA protein and 12 μM circular ssDNA or 4.8 μM ^{35}S-labeled RecA protein and 18 μM linear dsDNA. The regeneration system used in challenge experiments with ATP or dATP contained 7.5 mM phosphoenolpyruvate and 20 units / ml pyruvate kinase. Equal amounts of unlabeled wtRecA protein or RecA K72R was added and incubation was continued for either 20 or 50 minutes. Reactions were stopped by adding ATPγS to 1 mM and the amount of labeled RecA protein remaining bound to DNA was analyzed by sucrose gradient sedimentation. Two control reactions were also included in the each set of RecA protein challenge experiments. In one, RecA storage buffer replaced the challenging RecA protein. In the other control reaction, ATPγS (1 mM, final concentration) was added to the reaction before the challenging protein was added. In independent experiments, we confirmed that the ATP regeneration system used was

sufficient to maintain the fraction of nucleotide cofactor present as ADP or dADP at less than 5% for the entire time course of the reaction shown.

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–ss-DNA complexes from free RecA protein the gradients also contained 25 mM Tris-acetate (80% cation, pH 7.5), 1 mM DTT, and 1 mM $Mg(OAc)_2$. To separate RecA–ds-DNA complexes, 25 mM Mes(NaOH) (56% anion, pH 6.3) replaced Tris-acetate in the gradient. 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 either by collecting drops from a 21-gauge needle that had been pushed through the bottom of the centrifuge tube, or by using a density gradient fractionator (Isco, Model 183). Fractions were collected in 1.5 ml decapped Eppendorf tubes, and average fraction size was about 180 µl. A 100 µl aliquot from each fraction was spotted on a Whatman 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 fraction defining the background, plus one fraction before and after the peak fractions thus defined. The radioactivity in fractions is 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. 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 irreproducible and were ignored. The quantitation is reported as % of radioactivity in the free or bound RecA peak, defined in each case as the % of counts in one peak divided by the total counts in both peaks. Some of these gradients were also analyzed by SDS-PAGE to determine total RecA content.

SDS-PAGE

A 50 µl aliquot from each fraction was mixed with 7 µl protein gel loading buffer (100 mM Tris-HCl, pH 6.8, 16% SDS, 40% glycerol, 5.6 M β-mercaptoethanol, 0.4% bromophenol blue) and 50 µl of the mixture was loaded onto a mini-protein gel (BioRad). The protein gel was stained with Orange G (Molecular Probes, Eugene, Oregon) and imaged by FluroImager (Molecular Dynamics). The staining procedure can detect up to 1 ng of protein (Molecular Probes Technical Bulletin), which is at least as sensitive as silver stain.

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