

The C Terminus of the *Escherichia coli* RecA Protein Modulates the DNA Binding Competition with Single-stranded DNA-binding Protein*

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The nucleation step of *Escherichia coli* RecA filament formation on single-stranded DNA (ssDNA) is strongly inhibited by prebound *E. coli* ssDNA-binding protein (SSB). The capacity of RecA protein to displace SSB is dramatically enhanced in RecA proteins with C-terminal deletions. The displacement of SSB by RecA protein is progressively improved when 6, 13, and 17 C-terminal amino acids are removed from the RecA protein relative to the full-length protein. The C-terminal deletion mutants also more readily displace yeast replication protein A than does the full-length protein. Thus, the RecA protein has an inherent and robust capacity to displace SSB from ssDNA. However, the displacement function is suppressed by the RecA C terminus, providing another example of a RecA activity with C-terminal modulation. RecAΔC17 also has an enhanced capacity relative to wild-type RecA protein to bind ssDNA containing secondary structure. Added Mg²⁺ enhances the ability of wild-type RecA and the RecA C-terminal deletion mutants to compete with SSB and replication protein A. The overall binding of RecAΔC17 mutant protein to linear ssDNA is increased further by the mutation E38K, previously shown to enhance SSB displacement from ssDNA. The double mutant RecAΔC17/E38K displaces SSB somewhat better than either individual mutant protein under some conditions and exhibits a higher steady-state level of binding to linear ssDNA under all conditions.

RecA protein catalyzes homologous DNA pairing and strand exchange reactions that are at the heart of recombination processes in all cells. Strand exchange is initiated when RecA binds to single-stranded DNA (ssDNA)¹ within a gap or an ssDNA terminal extension. RecA protein binds DNA in at least two steps. The first is a slow nucleation step (1–3), and this is followed by a rapid, cooperative binding of additional monomers to lengthen the filament uniquely in a 5′ to 3′ direction (4, 5). The resulting RecA-ssDNA complex has an extended, helical conformation, with ~6 RecA monomers and 18 nucleotides (nt) of DNA per right-handed helical turn (18.6 base pairs per turn

in RecA filaments with bound ATPγS) (6). This nucleoprotein filament can pair the bound single strand with the complementary strand of an incoming duplex, resulting in homologous recombination.

The ssDNA-binding protein from *Escherichia coli*, SSB, affects formation of the nucleoprotein filament in several ways. *In vitro*, under standard reaction conditions that generally include 8–12 mM Mg²⁺, SSB stimulates filament formation on ssDNA substrates derived from bacteriophages by binding to and denaturing regions of secondary structure in the ssDNA that would otherwise hinder RecA filament extension (7). SSB is then displaced by the growing RecA filament (8). SSB thus permits the formation of a contiguous extended filament on the DNA. However, RecA and SSB bind ssDNA competitively *in vitro*, such that when SSB is prebound to ssDNA, it inhibits the nucleation stage of RecA protein filament formation (9, 10). Subsequent binding of RecA to ssDNA results either when SSB transiently vacates a region of ssDNA or when the RecOR mediator proteins facilitate RecA nucleation onto SSB-coated ssDNA (5, 11, 12).

Genetic studies also indicate that SSB inhibits RecA filament formation and subsequent homologous recombination. Mutations in *recF*, *recO*, or *recR* genes, which belong to the same epistasis group, result in defects in the repair of stalled replication forks (13–18). These defects are probably due at least in part to the inability of RecA protein to displace SSB from the single-stranded region of the stalled fork. In support of this model, overproduction of SSB leads to a sensitivity to UV-inflicted DNA damage that is similar to the phenotype of *recF* mutants (19). Additional evidence supports a competition between RecA and SSB for ssDNA *in vivo*. The *recF*, *recO*, or *recR* phenotypes can be partially suppressed, and the suppressor mutations map to the *recA* gene. These *recA* mutants include *recA803* (V37M) (20), *recA2020* (T121I) (21, 22), *recA441* (E38K/I298V) (23–25), and *recA730* (E38K) (26, 27). These mutant RecA proteins would need to be able to compensate in some way for the loss of the RecFOR proteins, and indeed RecA803, RecA441, and RecA E38K proteins all exhibit an enhanced ability to compete with SSB *in vitro* (8, 28). RecA E38K protein competes best with SSB, followed by RecA441 and then RecA803 (8).

SSB protein has multiple DNA binding modes, and interconversion between them is mediated by salt concentrations (29–31). At relatively low divalent salt concentrations (10–100 μM), SSB binds with a site size of 35 nt and exhibits a smooth contour in the electron microscope. At higher salt concentrations, SSB transitions to binding modes with site sizes of 56 (1–10 mM salt) and then 65 nt (0.1–1 M salt). The SSB₆₅ binding mode is characterized by a beaded appearance in the electron microscope. The cooperativity of SSB binding to ssDNA is enhanced at the lower salt concentrations.

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¹ The abbreviations used are: ssDNA, single-stranded DNA; nt, nucleotides; ATPγS, adenosine 5′-O-(thiotriphosphate); SSB, single-stranded binding protein; RPA, replication protein A.

The RecA protein of *Escherichia coli* (M_r 37,842) has a structure featuring three distinct domains (32–34). The core domain (residues 31–269) includes the ATP and DNA binding sites. The core is flanked by smaller N- and C-terminal domains. The C-terminal domains (residues 270–352) appear as distinct lobes on the surface of RecA filaments, which shift position markedly in response to the presence of different bound nucleotides (35, 36). The far C terminus of the RecA protein (defined here as the C-terminal 25 amino acid residues) exhibits a preponderance of negatively charged amino acids, with seven Glu or Asp residues in the terminal 17 residues. This function of this region has been explored with the use of C-terminal deletions (37–42). Several of these studies documented the *in vitro* effects of deleting all or most of the C-terminal 25 amino acid residues. The major effects were an improvement of binding to dsDNA (37–39) and an evident alteration of the conformation of the core domain (42).

To more systematically explore the function of the C terminus, C-terminal deletions removing 6, 13, and 17 amino acids were constructed and characterized in detail (43, 44). The truncated proteins dramatically alter the pH-reaction profile for DNA strand exchange (43) and exhibit a progressive reduction in the requirement for free Mg^{2+} in the same reaction (44). For the RecA Δ C17 mutant protein, there is no measurable requirement for Mg^{2+} in excess of that required to coordinate the ATP used in a given experiment (44). At Mg^{2+} concentrations above their respective optima, reactions promoted by the mutant proteins are somewhat inhibited and produce DNA species that do not migrate into the gel. For RecA Δ C17, this inhibition is observed at Mg^{2+} concentrations (*i.e.* 10 mM) that are optimal for strand exchange with wild-type RecA protein. The new DNA species are thought to represent DNA networks that form when RecA protein is able to bind to the outgoing strand and pair the strand again with a duplex DNA (45). This trait was previously observed in strand exchange reactions with RecA441, which has an enhanced capacity to compete with SSB for the displaced single strand during strand exchange reactions (46). This suggested to us that the C-terminal deletion mutants of RecA might also have an enhanced capacity to displace SSB protein. In this study, we demonstrate that C-terminal deletions of RecA protein greatly enhance the capacity of the protein to compete with SSB and that the effect increases with progressive truncation out to at least 17 amino acid residues.

EXPERIMENTAL PROCEDURES

Proteins and Biochemicals—*E. coli* SSB was purified as described (47). SSB was stored in a buffer containing 20 mM Tris-HCl (pH 8.3), 1 mM EDTA, 50% glycerol, 1 mM β -mercaptoethanol, and 500 mM NaCl. The concentration of the purified SSB protein was determined from the absorbance at 280 nm using the extinction coefficient of $2.83 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (48). *Saccharomyces cerevisiae* replication protein A (RPA) was purified as described (49). The concentration was determined by the absorbance at 280 nm using the extinction coefficient of $8.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (50). The wild-type RecA, RecA Δ C6, RecA Δ C13, and RecA Δ C17 proteins were purified as described (43). RecA 441 was purified using the following modification to the wild-type RecA procedure previously described (43). The initial polyethyleneimine pellet was washed with R buffer (20 mM Tris-HCl (80% cation, pH 7.5), 1 mM dithiothreitol, 0.1 mM EDTA, and 10% (w/v) glycerol) and 50 mM ammonium sulfate and extracted two times with R buffer plus 150 mM ammonium sulfate. The RecA E38K (RecA 730) mutant protein was purified like the wild-type RecA protein, except that the final fraction was subjected to an additional step. The protein was loaded onto a PBE 94 column equilibrated with R buffer, and the column was developed with a linear gradient from 0 to 1.0 M KCl. The RecA E38K mutant was eluted at \sim 600 mM KCl. The eluted protein was dialyzed extensively against R buffer and concentrated as for the wild-type protein. The RecA Δ C17/E38K double mutant protein was purified using the same protocol as the RecA Δ C17 protein (43). The concentration of each RecA and RecA variant protein

was determined using the extinction coefficient of wild-type RecA, $2.23 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (51). Unless otherwise noted, all reagents were purchased from Fisher. Lactate dehydrogenase, pyruvate kinase, phosphoenolpyruvate, NADH, and ATP were purchased from Sigma. PBE 94 resin was purchased from Amersham Biosciences. Dithiothreitol was purchased from Research Organics, Inc.

DNA Substrates—All DNA concentrations are given in terms of total nucleotides. Poly(dT) was purchased from Amersham Biosciences, and the approximate average length is 229 nt. The concentration of poly(dT) was determined by UV absorption at 260 nm using an extinction coefficient of $8.73 \text{ mM}^{-1} \text{ cm}^{-1}$. M13mp8 bacteriophage circular ssDNA was prepared as described (52). The concentration of M13mp8 ssDNA was determined by UV absorption at 260 nm using the extinction coefficient $9.03 \text{ mM}^{-1} \text{ cm}^{-1}$.

ATP Hydrolysis Assays—A coupled spectrophotometric enzyme assay (53, 54) was used to measure the ssDNA-dependent ATPase activities of the wild-type RecA, RecA Δ C6, RecA Δ C13, and RecA Δ C17 proteins. The regeneration of ATP from phosphoenolpyruvate and ADP was coupled to the oxidation of NADH and monitored by the decrease in absorbance of NADH at 380 nm. The 380-nm wavelength was used, so that the signal remained within the linear range of the spectrophotometer for the duration of the experiment. The assays were carried out on a Varian Cary 300 dual beam spectrophotometer equipped with a temperature controller and a 12-position cell changer. The cell path length and band pass were 0.5 cm and 2 nm, respectively. The NADH extinction coefficient at 380 nm of $1.21 \text{ mM}^{-1} \text{ cm}^{-1}$ was used to calculate the rate of ATP hydrolysis.

The reactions were carried out at 37 °C in 25 mM Tris-OAc (80% cation), 1 mM dithiothreitol, 3 mM potassium glutamate, 5% (w/v) glycerol, an ATP regeneration system (10 units/ml pyruvate kinase, 1.92 mM phosphoenolpyruvate), and a coupling system (3 mM NADH and 10 units/ml lactate dehydrogenase). The concentrations of DNA (M13mp8 ssDNA or poly(dT)), RecA protein (wild-type RecA, RecA Δ C6, RecA Δ C13, RecA Δ C17, RecA 441, RecA E38K, or RecA Δ C17/E38K), $Mg(OAc)_2$, and SSB or RPA are indicated in the figure legends. To initiate the assay, the ssDNA was preincubated with either a RecA protein or an ssDNA binding protein (SSB or RPA) for 10 min at 37 °C. Then SSB or an RecA protein, respectively, was added. ATP was added to 3 mM final concentration, either with the RecA protein or with SSB or RPA, as indicated. Data collection was then begun. In reactions in which no ssDNA-binding protein is included, SSB storage buffer is added instead. In the salt titrations, conditions were the same as above on poly(dT), except without SSB or SSB storage buffer. After a steady-state rate was achieved, aliquots of concentrated NaCl were added, allowing the reactions to come to steady state between additions.

RESULTS

Experimental Rationale—The goal of these experiments was to compare the abilities of wild-type RecA and C-terminally truncated RecA mutant proteins to compete with SSB for binding to ssDNA. RecA protein hydrolyzes ATP when bound to DNA, and the ATPase rate is proportional to the amount of RecA bound under most conditions (7, 53, 55). The rate of ATP hydrolysis can be measured using a coupled enzyme assay described previously (53, 54). Although indirect, this technique has been used extensively to measure RecA binding to DNA (3, 5, 53, 56). Importantly, this assay was also shown to represent a reliable and real time method to monitor SSB displacement by RecA when SSB is prebound to ssDNA (8). In that study, the time-dependent increase in the rate of ATP hydrolysis was shown to correlate with the displacement of SSB, as measured using the change in the fluorescence of SSB upon dissociation from DNA.

SSB Competition with Wild-type RecA Protein for Binding to M13mp8 ssDNA—The outcome of the competition between SSB and RecA for binding to circular bacteriophage M13mp8 ssDNA strongly depends on the relative concentrations of the proteins, on which protein is preincubated with the ssDNA, and on the Mg^{2+} concentration (7, 9). As Fig. 1 illustrates, when the RecA protein is present at concentrations stoichiometric with its available DNA binding sites (3 nt of ssDNA/RecA monomer), RecA displaces prebound SSB from M13mp8 ssDNA quite slowly at 3 mM Mg^{2+} . This Mg^{2+} concentration is

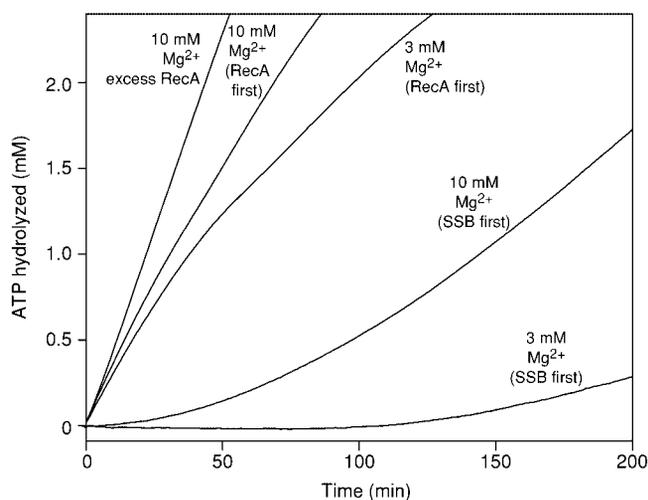


FIG. 1. Effects of SSB on wild-type RecA protein binding to circular ssDNA, with either RecA or SSB preincubated with the DNA at 3 and 10 mM Mg^{2+} . DNA binding was monitored indirectly by following the DNA-dependent ATPase activity of RecA protein. Reactions were carried out as described under "Experimental Procedures" and contained 5 μM M13mp8 ssDNA, 1.67 or 2.5 μM RecA protein, 0.5 μM SSB protein, 3 mM ATP, and 3 or 10 mM $Mg(OAc)_2$, as indicated. Either RecA or SSB was preincubated with the ssDNA for 10 min before the final protein addition (RecA first or SSB first reactions, respectively). RecA was added to 2.5 μM only in the reaction indicated as containing excess RecA, with RecA prebound at 10 mM Mg^{2+} ; otherwise, the RecA was present at 1.67 μM . In each experiment, ATP was added with the SSB.

stoichiometric to the ATP concentration used in the experiment. At 10 mM Mg^{2+} , the SSB is displaced much more quickly upon the RecA addition. If, however, RecA is preincubated with the M13mp8 ssDNA at 3 nt/monomer, some RecA protein is gradually displaced in the presence of SSB, but the initial rates of ATP hydrolysis indicate a high level of RecA binding. The rates decline somewhat with time but remain much higher than the initial rates observed when SSB was preincubated with the ssDNA. RecA protein again competes with SSB somewhat better at 10 mM Mg^{2+} than at 3 mM Mg^{2+} . The decline in rates indicates that the DNA is not completely bound by RecA when RecA is present at these stoichiometric levels, affording SSB significant access to the DNA. When the RecA concentration is increased 50% (2 nt/monomer), so that the excess RecA ensures coverage of the DNA, RecA is not displaced by SSB when the RecA is bound to the DNA prior to the SSB (Fig. 1).

SSB Competes with Wild-type RecA Protein for Binding to Poly(dT) ssDNA—A complication in the study of SSB effects on wild-type RecA binding to M13mp8 ssDNA at various Mg^{2+} concentrations is that SSB can remove Mg^{2+} -induced secondary structure in M13mp8 ssDNA, which otherwise inhibits RecA binding (7, 57). Thus, SSB has a stimulatory effect on RecA filament formation as well as a competitive, inhibitory effect. In order to eliminate this stimulatory contribution from SSB, poly(dT) ssDNA was used in place of M13mp8 ssDNA. Previously, it was shown that much more prebound RecA is displaced from poly(dT) by SSB than from M13mp8 ssDNA (7). This occurs to a large extent because of end-dependent disassembly of RecA filaments (5, 56, 58), which is readily observed only on linear DNAs, where filament extension at one end does not compensate for disassembly at the other. Here we show that at 10 mM Mg^{2+} , wild-type RecA protein (3 nt/monomer) is almost completely displaced from poly(dT) by SSB in a process that is complete in less than 10 min (Fig. 2). At 3 mM Mg^{2+} , the displacement of RecA by SSB is considerably faster. RecA added to poly(dT) prebound with SSB is not able to appreciably displace the SSB at 3 or 10 mM Mg^{2+} , indicating that binding

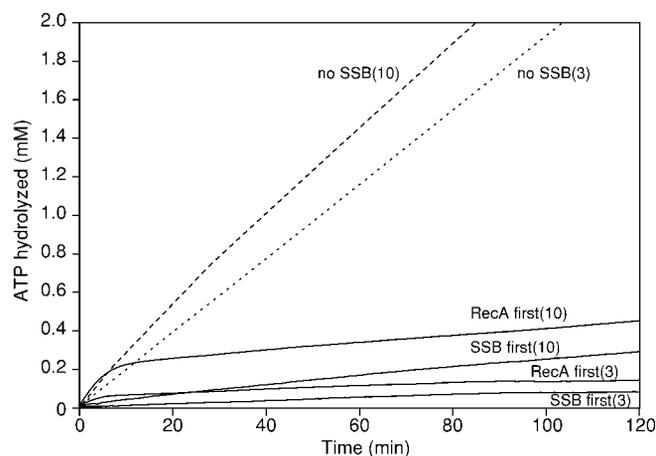


FIG. 2. Effects of SSB on wild-type RecA protein binding to poly(dT) ssDNA with either RecA or SSB preincubated with the DNA at 3 and 10 mM Mg^{2+} . DNA binding was monitored as described in the legend to Fig. 1. Reactions contained 5 μM poly(dT) ssDNA, 1.67 μM RecA protein, 0.7 μM SSB, 3 mM ATP, and 3 or 10 mM $Mg(OAc)_2$, as indicated in parentheses. Either RecA or SSB was preincubated with the ssDNA for 10 min before the final protein addition. For the controls, denoted by dashed lines, SSB was replaced with SSB storage buffer. In each experiment, ATP was added with the SSB or SSB storage buffer.

of SSB to poly(dT) is highly favored over RecA binding to the linear poly(dT). Unlike the case with M13mp8 cssDNA, the resulting steady-state rates are the same regardless of whether RecA or SSB is preincubated with the ssDNA. In these experiments, the ATP is added with the SSB, and thus ATP is not included in the RecA preincubation in the experiments where RecA is added first. However, including ATP in the RecA preincubation had no discernible effect on the results shown (data not shown). As previously observed, a small net disassembly occurs in the absence of SSB, as indicated by the slight decline in the rate of ATP hydrolysis with time (Fig. 2, dashed lines), and disassembly and reassembly of filaments is doubtless occurring at a steady state (5, 56, 58). The rate of hydrolysis by RecA protein in the absence of SSB is somewhat higher at 10 mM Mg^{2+} than at 3 mM.

The Capacity of RecA Protein to Compete with SSB Is Enhanced with Progressive Deletion of the C-terminal Amino Acids—The C terminus of RecA contains a number of acidic residues. As shown in Fig. 3A, deletions of 6, 13, or 17 amino acids from the C terminus progressively remove a total of 3, 6, or 7 glutamate and aspartate residues. We tested the ability of these mutant proteins to compete with SSB, compared with that of wild-type RecA protein. At 3 mM Mg^{2+} , where wild-type RecA has the least capacity to compete with SSB for binding to poly(dT), RecA Δ C13 and, to an even greater extent, RecA Δ C17, bind well to the poly(dT) that has been prebound with SSB (Fig. 3B). At 3 mM Mg^{2+} , RecA Δ C6 is unable to compete with SSB.

The ATPase reactions of each protein in the absence of SSB are also displayed in Fig. 3B to show that at 3 mM Mg^{2+} , in the absence of SSB, each protein binds to poly(dT) to approximately the same extent. The apparent k_{cat} for ATP hydrolysis by wild-type RecA (calculated by assuming that all of the DNA is bound by RecA protein) in these reactions is 11.5 min^{-1} , quite comparable with rates with poly(dT) observed previously (7). However, as shown below, this rate is lower than the nearly 30 min^{-1} rate observed when RecA is carefully titrated onto circular ssDNA. The lower rate almost certainly reflects incomplete binding of the DNA and perhaps an equilibrium state in which filaments are undergoing steady-state end-dependent assembly and disassembly.

SSB Displacement by the Variant RecA Proteins Is Enhanced by Mg^{2+} —All of the RecA proteins including RecA Δ C6, com-

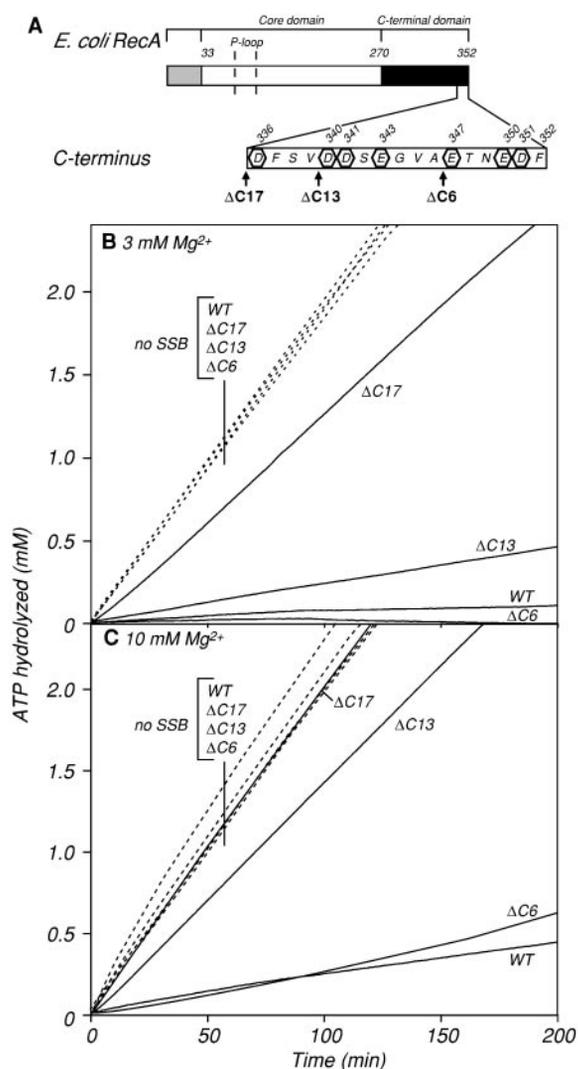


FIG. 3. Displacement of SSB from poly(dT) by wild-type RecA protein and C-terminally truncated RecA proteins $\Delta C6$, $\Delta C13$, and $\Delta C17$ at 3 and 10 mM Mg^{2+} . DNA binding was monitored indirectly by following the DNA-dependent ATPase activity of RecA protein. A highlights the C-terminal region of RecA protein. The core domain, which includes the P-loop (ATP binding motif), is shown in white. The shaded and black regions of the sequence correspond to the N-terminal and C-terminal domains, respectively. The primary structure of the C-terminal 17 amino acids of the RecA protein is diagrammed below the linear sequence. The hexagons highlight the high concentration of negatively charged amino acids in this region. The arrows indicate points of truncation in the deletion mutants: RecA $\Delta C6$, RecA $\Delta C13$, and RecA $\Delta C17$. For the data shown in B and C, reactions contained 0.7 μM SSB, 1.67 μM RecA protein, 5 μM poly(dT) ssDNA, 3 mM ATP, and either 3 mM (B) or 10 mM (C) $Mg(OAc)_2$. In all reactions, ATP and SSB were preincubated with the ssDNA for 10 min before the addition of the RecA protein indicated. The controls without SSB for each RecA protein variant are shown as dashed lines and substituted SSB storage buffer for the SSB. At the point where these dashed lines are bisected by the vertical labeling line, the listing of the proteins (top to bottom) corresponds to the top to bottom positioning of the dashed lines (the top line is the reaction of the wild-type RecA protein, etc.). WT, wild-type RecA protein.

pete more effectively with SSB at 10 mM Mg^{2+} , and at 10 mM Mg^{2+} it is more evident that the ability of RecA to compete with SSB increases with progressive deletion of the RecA C terminus (Fig. 3C). The RecA $\Delta C17$ mutant protein exhibits no detectable lag in binding, indicating a particularly rapid SSB displacement process. Each RecA protein binds poly(dT) efficiently in the absence of SSB at 10 mM Mg^{2+} .

When M13mp8 ssDNA is used, the outcome of the wild-type RecA-SSB competition is highly dependent on which protein is

preincubated with the ssDNA (Fig. 1) (7, 9). However, when poly(dT) is used, the resulting steady-state rates are the same no matter which protein is preincubated with the ssDNA (Fig. 2). We find that for RecA $\Delta C17$ protein, the steady-state rates are also the same whether RecA $\Delta C17$ or SSB is preincubated with poly(dT) (Fig. 3 and data not shown). When RecA $\Delta C17$ was preincubated with poly(dT) and ATP and then challenged with SSB, the challenge had no measurable effect on the rate of ATP hydrolysis at 10 mM Mg^{2+} and, by inference, on the state of RecA $\Delta C17$ binding. At 3 mM Mg^{2+} , there is a slow decline in ATPase rate occurring over ~ 60 min after the challenge, after which the rate seen in Fig. 3B is observed (data not shown). This suggests a slow displacement of the mutant RecA protein by the SSB.

The Capacity of RecA Protein to Compete with RPA Is Progressively Enhanced as Deletion of the C Terminus of RecA Is Increased—Both the wild-type and C-terminally truncated RecA proteins are better able to compete with SSB at 10 mM Mg^{2+} than at 3 mM Mg^{2+} (Figs. 1 and 3, B and C). This difference could be due to effects of Mg^{2+} either on the RecA proteins or on SSB. Mg^{2+} is known to affect the cooperativity of the different binding modes of SSB (59). An increase in Mg^{2+} could lower the cooperativity of binding of SSB, which in turn could affect its ability to compete with RecA protein. We therefore investigated whether Mg^{2+} stimulated the competition of RecA with RPA, a ssDNA binding protein whose binding, unpublished studies indicate, is not affected by Mg^{2+} .² Additionally, we asked whether the inhibition of wild-type RecA protein was due to a specific protein-protein interaction with SSB mediated through the C-terminal region of RecA. The removal of this region would then result in the ability of C-terminally truncated RecA protein to compete with SSB, as shown in Fig. 3, B and C. However, as illustrated in Fig. 4A, the same result obtained with SSB is seen with RPA. At 3 and 10 mM Mg^{2+} , wild-type RecA has a limited capacity to compete with RPA for binding to poly(dT). In contrast, RecA $\Delta C17$ does bind to poly(dT) coated with RPA and is especially proficient at 10 mM Mg^{2+} (Fig. 4A). This suggests that Mg^{2+} has a significant enhancing effect on RecA protein function in SSB displacement even when the C terminus is removed.

Additional experiments were carried out with the wild-type RecA protein and M13mp8 circular ssDNA to examine the effects of Mg^{2+} under conditions in which filament disassembly at filament ends would minimally affect the results. SSB was preincubated with the ssDNA. With this circular DNA substrate, the RecA protein can slowly displace the SSB at 3 mM Mg^{2+} (Fig. 4B). The displacement is considerably faster at 10 mM Mg^{2+} .

Displacement of SSB by RecA C-terminal truncation mutants is apparently not due to a higher inherent affinity for ssDNA. The ability of RecA protein to bind DNA in the presence of increasing NaCl concentration correlates with its inherent DNA affinity (49). Binding to poly(dT) was monitored indirectly with the DNA-dependent ATPase assay in the absence of SSB and as a function of increasing NaCl concentration (data not shown). The half-maximal binding point (taken as the NaCl concentration where the ATPase activity is halved relative to the maximum) is seen at ~ 750 mM NaCl for the wild-type protein, decreasing slightly to about 700 mM under these conditions for the RecA $\Delta C17$ mutant.

RecA $\Delta C17$ Protein Binds M13mp8 ssDNA with Secondary Structure Better than Does Wild-type RecA Protein—RecA E38K and RecA803, which have been demonstrated to compete with SSB for binding to ssDNA more effectively than wild-type

² M. Wold, personal communication.

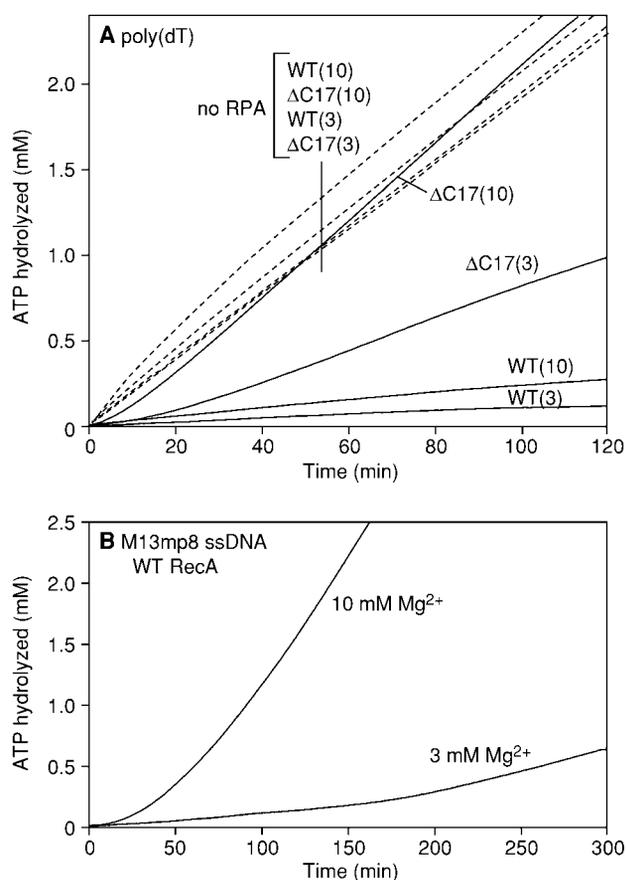


FIG. 4. Displacement of RPA from ssDNA by wild-type RecA protein (WT) and C-terminally truncated RecA proteins ΔC6, ΔC13, and ΔC17. DNA binding by RecA and RecA variants was monitored indirectly by following the DNA-dependent ATPase activity of RecA protein. Reactions in *A* contained 0.6 μM RPA, 1.67 μM RecA protein, 5 μM poly(dT) ssDNA, 3 mM ATP, and either 3 mM or 10 mM Mg(OAc)₂ as indicated by the number in parentheses. RPA was preincubated with the ssDNA for 10 min before the addition of the RecA protein indicated. Controls carried out in the absence of RPA (dashed lines) are labeled as indicated in the legend to Fig. 3. *B*, two reactions with wild-type RecA carried out under the same conditions as in *A* but with M13mp8 ssDNA replacing the poly(dT).

RecA, appear to also bind to regions of secondary structure in ssDNA better than the wild-type protein (8). We investigated whether RecAΔC17 was more capable than wild-type RecA at binding M13mp8 ssDNA that contains secondary structure, induced by 10 mM Mg²⁺, in the absence of SSB. In these experiments, the RecA was present at 2 nt/monomer to prevent RecA displacement in the control experiments that contained SSB. As illustrated in Fig. 5, the rate of ATP hydrolysis of wild-type RecA protein rebound to M13mp8 ssDNA in the presence of SSB and 10 mM Mg²⁺ drops significantly when SSB is omitted, to 30% of the rate in the presence of SSB (dashed lines in Fig. 5). This is consistent with a considerably reduced binding to the ssDNA under these conditions, as observed previously for RecA binding to ssDNA with appreciable secondary structure in the absence of SSB (7). In contrast, the ATPase rate of RecAΔC17 at 10 mM Mg²⁺ drops only a little upon omission of SSB, to 84% of the rate seen with SSB (Fig. 5). In sum, the data indicate that RecAΔC17 is better able to bind DNA that contains secondary structure than is wild-type RecA.

Improved SSB Displacement and Increased Steady-state DNA Binding Are Observed When a 17-Residue C-terminal Truncation Is Combined with the RecA E38K Mutation—Several other RecA mutations have been shown to enhance displacement of SSB, with RecA E38K being the best of those

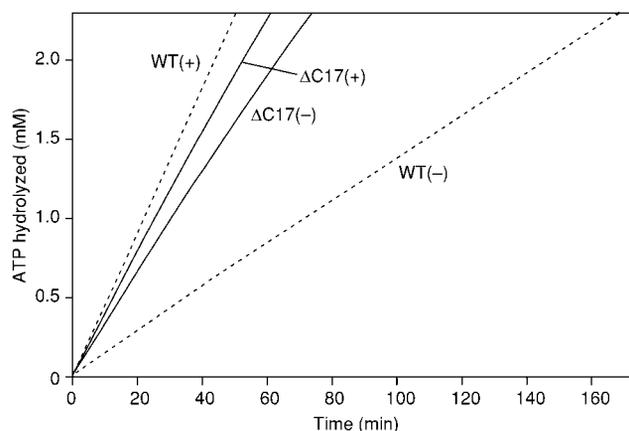


FIG. 5. Comparison of the capacity of wild-type RecA protein (WT) to bind to secondary structure-containing M13mp8 ssDNA with that of the RecAΔC17 protein. DNA binding by wild-type and C-terminally truncated RecA protein was monitored indirectly by following the DNA-dependent ATPase activity of RecA protein. Reactions were carried out as described under “Experimental Procedures” and contained 5 μM M13mp8 ssDNA, 2.5 μM RecA protein, 3 mM ATP, and 10 mM Mg(OAc)₂. Some reactions also contained 0.5 mM SSB as indicated (plus signs in parentheses). Wild-type RecA protein or RecAΔC17 were preincubated the ssDNA for 10 min, before the addition of ATP and either SSB (+) or SSB storage buffer (-). The reactions with wild-type RecA protein are shown with dashed lines to highlight the contrast with the reactions with the RecAΔC17 mutant, which exhibit a much reduced effect of SSB addition.

examined to date (8). The RecA441 double mutant (E38K/I298V) also includes the E38K mutation, but the second mutation seems to moderate its effects (8). We wished to compare the effects of these previously characterized mutant proteins with the RecA C-terminal deletion mutants. The results are shown in Fig. 6. At 3 mM Mg²⁺, the RecA441 mutant was similar to the RecAΔC17 mutant in its capacity to displace SSB. The RecA E38K mutant was the best of the individual mutants in this activity. A short but discernible lag in reaching a steady state of ATP hydrolysis was observed for each of the individual mutants. At 10 mM Mg²⁺, the activity of the RecA441 mutant was similar to that of RecAΔC13 and less than that of RecAΔC17. The RecA E38K mutant was still the best individual mutant in SSB displacement. The lag in reaching an apparent steady state was reduced for all of the individual mutant proteins. When the RecAΔC17 and E38K mutations were combined in a single protein, a further enhancement was observed in SSB displacement and ssDNA binding. At the low Mg²⁺ concentration, the double mutant protein was more effective at SSB displacement than either single mutant. There was no discernible lag in ATP hydrolysis with the double mutant protein under any condition tested, and there was a substantially higher steady-state rate of ATP hydrolysis (with an apparent k_{cat} in excess of 20 min⁻¹). We note that the ATP hydrolysis was higher in the presence of SSB than in its absence, a property observed with no other RecA variant.

The higher rates of ATP hydrolysis could reflect a higher intrinsic rate of ATP hydrolysis by the double mutant protein. Alternatively, they could reflect a greater steady-state level of binding to the DNA. On the linear poly(dT) DNA substrates used here, net DNA binding reflects the balance between filament assembly and end-dependent disassembly (5, 56, 58). We evaluated the intrinsic rate of ATP hydrolysis by comparing mutants side by side with excess protein on M13mp8 circular ssDNA and with SSB added after the RecA protein (data not shown). Under these conditions, there is no net filament disassembly (no ends on the circular DNA), and the DNA binding is saturated for each mutant. The rates of ATP hydrolysis for the

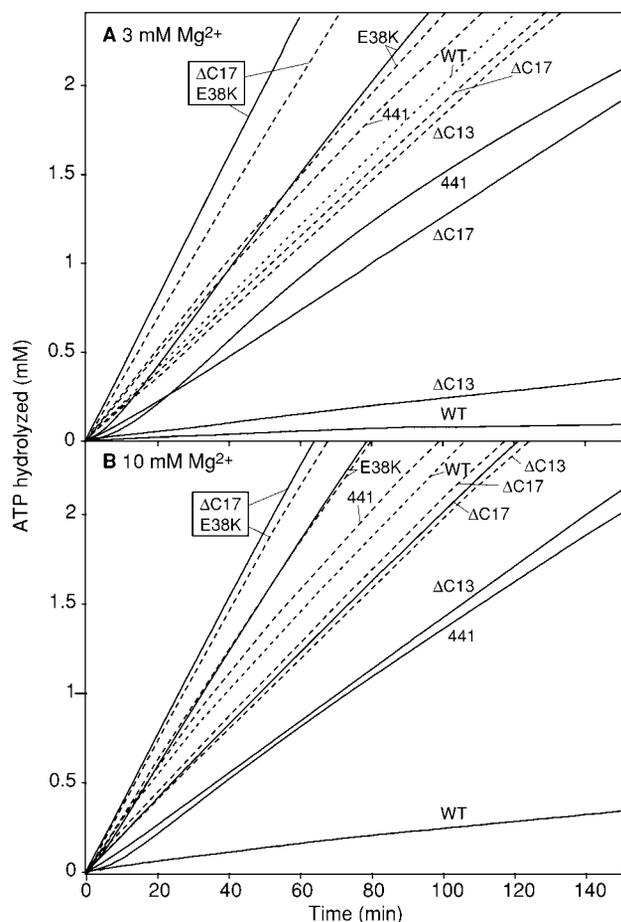


FIG. 6. Comparison of the SSB displacement and ssDNA binding activities of various RecA mutants proteins added to SSB-prebound poly(dT) ssDNA: RecA (wild-type; WT), RecA Δ C13, RecA Δ C17, RecA 441, RecA E38K, and RecA RecA Δ C17/E38K. DNA binding by RecA and RecA mutant proteins was monitored indirectly by following the DNA-dependent ATPase activity of RecA protein. Reactions contained $0.7 \mu\text{M}$ SSB, $1.67 \mu\text{M}$ RecA or RecA mutant protein, $5 \mu\text{M}$ poly(dT) ssDNA, 3 mM ATP, and either 3 mM (A) or 10 mM (B) Mg^{2+} . In all reactions, SSB and ATP were preincubated with the ssDNA for 10 min before the addition of the RecA protein indicated. The controls without SSB for each RecA protein variant are shown as dashed lines.

double mutant and the wild-type proteins were identical (apparent $k_{\text{cat}} = 33 \text{ min}^{-1}$) in this trial.

DISCUSSION

We conclude that the C-terminal region of wild-type RecA protein negatively modulates the capacity of wild-type RecA to compete with SSB for binding to ssDNA. Progressive removal of 6, 13, and 17 amino acids from the C terminus of RecA results in a progressive increase in the capacity of RecA to displace SSB. RecA Δ C17 also displaces RPA from ssDNA much more readily than wild-type RecA. The binding of RecA Δ C17 to DNA containing secondary structure is enhanced relative to wild-type RecA. This result reinforces a pattern previously established for RecA E38K and RecA803 mutant proteins (8). The capacity of these C-terminally truncated mutants to compete with SSB is quite high, especially in the case of RecA Δ C17. The RecA Δ C17 truncation and the E38K mutation work together in a double mutant to eliminate a discernible lag in SSB displacement under some conditions and to increase the steady-state level of DNA binding by RecA.

The inhibition of RecA protein binding to ssDNA by SSB could be mediated by specific protein-protein interactions, or it could reflect a simple competition for DNA binding sites. For

example, SSB could inhibit the binding of wild-type RecA protein by means of specific protein-protein interactions with the RecA C terminus, which would be progressively eliminated in the C-terminally truncated RecA proteins. Such a mechanism would imply species specificity. We examined this possibility by substituting RPA, the ssDNA-binding protein from *S. cerevisiae*, for SSB. We find that RPA competes efficiently with wild-type RecA for binding to ssDNA and that the C-terminally truncated RecA proteins exhibit a progressively enhanced capacity to displace RPA. This indicates that the function of the RecA C terminus does not involve a species-specific interaction.

Excess Mg^{2+} (above that required to coordinate with ATP) has a stimulatory effect on the displacement of SSB by RecA protein, over and above the enhancement conferred by the C-terminal deletions, as previously observed for wild-type RecA (7). For all of the RecA variants and for wild-type RecA, SSB displacement is more facile in the presence of 10 mM Mg^{2+} than it is at 3 mM Mg^{2+} . This could reflect an alteration of the binding state of SSB or an effect on RecA protein itself. SSB has multiple salt-dependent DNA binding modes (30, 59) that might differentially affect the ability of SSB to compete with RecA protein for ssDNA binding sites. The experiments that substituted RPA for SSB had a second purpose, to attempt to address the source of the stimulation by Mg^{2+} . Unpublished experiments suggest that RPA does not have multiple salt-dependent DNA binding modes of the sort observed with SSB and that Mg^{2+} does not stimulate the binding of ssDNA by RPA.² We find that the ability of the C-terminally truncated RecA proteins and wild-type RecA to compete with RPA is stimulated by the higher Mg^{2+} concentration, suggesting that the excess Mg^{2+} is directly affecting the RecA protein. Mg^{2+} may be acting on SSB, as well, to alter its function in this system. We hypothesized in the previous paper (44) that a Mg^{2+} interaction site might exist in the *E. coli* RecA C terminus, where it could be mediated by the many glutamate and aspartate residues present there. To the extent that Mg^{2+} does not affect RPA binding, the Mg^{2+} effects in experiments with SSB appear to be due largely to effects of Mg^{2+} on the RecA protein. Since this is true for even the RecA Δ C17 variant, there may be a Mg^{2+} interaction site or sites on RecA protein outside of the C terminus that affect the capacity of RecA to displace SSB. In contrast, an interaction of Mg^{2+} with these sites does not appear to be required for the strand exchange reaction, since the excess Mg^{2+} requirement in that reaction was largely eliminated for RecA Δ C17 (44).

Another study also found a link between the capacity of RecA to promote strand exchange in the absence of excess Mg^{2+} and the protein's ability to compete with SSB for ssDNA-binding sites. The additions of the volume-occupying agents polyethylene glycol and polyvinyl alcohol to RecA-mediated strand exchange reactions both greatly reduced the excess Mg^{2+} requirement in strand exchange and increased the ability of RecA to compete with SSB for ssDNA (60). This result, combined with our similar results using RecA Δ C17 protein, suggests that volume-occupying agents may stabilize a conformation of the C terminus of RecA that does not inhibit these activities. This conformation may be the same one induced by the addition of excess Mg^{2+} . The removal of the C terminus also alleviates the inhibition. The addition of Mg^{2+} , addition of volume-occupying agents, or removal of the C terminus may expose the RecA DNA binding site in such a way that RecA is better able to compete with SSB and RPA or is better able to extract ssDNA from the surface of these binding proteins.

It is useful to compare the properties of the C-terminally truncated mutant RecA proteins with those of other RecA mutant proteins shown to have an increased ability to compete

with SSB for ssDNA. A primary question is the mechanism by which RecA mutants might have an enhanced ability to compete with SSB. Using a salt titration midpoint assay, which reflects the equilibrium DNA affinity of RecA (61), we found that the inherent DNA affinity of RecA Δ C17 for poly(dT) is not appreciably changed from that of wild-type RecA (data not shown). Previous studies of RecA mutant proteins that compete better with SSB than wild-type RecA have shown that the properties of these proteins, RecA803 V37M, RecA441 (E38K/I298V), and RecA E38K, are also not due to an increased ssDNA binding affinity (8, 28, 62). The capacity of these previously characterized RecA mutant proteins to compete with SSB was shown to correlate with their rate of association with DNA, a characteristic also found with wild-type RecA protein in the presence of volume-occupying agents (60).

In this study, we also show that the SSB displacement and steady-state DNA binding of the 17-residue C-terminal deletion mutant protein are improved further by the E38K mutation. The double mutant displaces SSB with no lag that is measurable in our experiments and provides a higher steady-state level of DNA binding on linear poly(dT) in the presence of SSB than any mutant studied to date. Since the intrinsic ATPase activity of the double mutant is the same as that of the individual mutant proteins, we attribute the increase in steady-state ATP hydrolysis to an increase in the steady-state level of DNA binding. These results suggest that SSB displacement and/or overall DNA binding is modulated by several different parts of the RecA protein. In the previous paper (44), we proposed that the negative charges of the C terminus were part of a regulatory network of protein surface salt bridges. The increase in SSB displacement and overall DNA binding observed when the deletion of the C-terminal 17 residues and the replacement of an acidic residue with a basic residue at position 38 are combined could also reflect particular disruptions of an extensive salt bridge network. We note that the double mutant appears to bind to DNA better in the presence of SSB than in its absence. We do not presently have an explanation for this effect.

RecA Δ C17 shares an additional property with RecA E38K and RecA803, an increased proficiency compared with wild-type RecA of competing with secondary structure for ssDNA binding sites (8, 28). Whereas the rates of ATP hydrolysis for RecA E38K and RecA803 on M13mp8 ssDNA at 10 mM Mg²⁺ in the presence of SSB were similar to that of wild-type protein, the rates of the mutant proteins in the absence of SSB were much higher than that of wild-type RecA. In addition, the RecA E38K, RecA803, and wild-type proteins all bound equally well to etheno M13mp8 DNA, which does not contain secondary structure. We obtained a similar result for RecA Δ C17. The ATPase rate for wild-type RecA on M13mp8 ssDNA at 10 mM Mg²⁺ in the absence of SSB is 30% of the rate in the presence of SSB. In contrast, the ATPase rate of RecA Δ C17 at 10 mM Mg²⁺ drops only a moderate amount upon omission of SSB, to 84%. In sum, the data indicate that RecA Δ C17, like RecA803 and RecA E38K, is better able to bind DNA that contains secondary structure than is wild-type RecA. Notably, this property is not intrinsic to RecA mutant proteins that compete more efficiently with SSB. RecA441, which is more proficient at competing with SSB than RecA803, binds DNA with secondary structure only as well as wild-type RecA (62).

Regulation of RecA DNA binding ability and/or its ability to compete with SSB would allow RecA to gain access to SSB-coated DNA only at the appropriate time, such as after a replication fork stalls. The binding of RecA to ssDNA that has been previously bound with SSB is facilitated by the RecO and RecR mediator proteins (5, 63). These studies suggested that

these mediator proteins alter the binding of SSB to ssDNA, creating a nucleation site for RecA filament formation. The work presented here indicates that RecA possesses an inherent and robust capacity to displace SSB but that this capacity is suppressed by the C terminus. This suggests another potential mechanism of mediator protein action. The RecO and RecR proteins might interact directly with RecA protein during the filament nucleation process, altering RecA conformation so that the C terminus is no longer inhibitory.

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REFERENCES

- Kowalczykowski, S. C., Clow, J., and Krupp, R. A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 3127–3131
- Pugh, B. F., and Cox, M. M. (1988) *J. Mol. Biol.* **203**, 479–493
- Pugh, B. F., and Cox, M. M. (1987) *J. Biol. Chem.* **262**, 1326–1336
- Register, J. C., III, and Griffith, J. (1985) *J. Biol. Chem.* **260**, 12308–12312
- Shan, Q., Bork, J. M., Webb, B. L., Inman, R. B., and Cox, M. M. (1997) *J. Mol. Biol.* **265**, 519–540
- Ogawa, T., Yu, X., Shinohara, A., and Egelman, E. H. (1993) *Science* **259**, 1896–1899
- Kowalczykowski, S. C., and Krupp, R. A. (1987) *J. Mol. Biol.* **193**, 97–113
- Lavery, P. E., and Kowalczykowski, S. C. (1992) *J. Biol. Chem.* **267**, 20648–20658
- Kowalczykowski, S. C., Clow, J., Somani, R., and Varghese, A. (1987) *J. Mol. Biol.* **193**, 81–95
- Cox, M. M., and Lehman, I. R. (1982) *J. Biol. Chem.* **257**, 8523–8532
- Umez, K., Chi, N. W., and Kolodner, R. D. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 3875–3879
- Bork, J. M., Cox, M. M., and Inman, R. B. (2001) *EMBO J.* **20**, 7313–7322
- Courcelle, J., Carswell-Crumpton, C., and Hanawalt, P. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 3714–3719
- Smith, K. C., and Sharma, R. C. (1987) *Mutat. Res.* **183**, 1–9
- Galitski, T., and Roth, J. R. (1997) *Genetics* **146**, 751–767
- Asai, T., and Kogoma, T. (1994) *J. Bacteriol.* **176**, 7113–7114
- Steiner, W. W., and Kuempel, P. L. (1998) *J. Bacteriol.* **180**, 6269–6275
- Cox, M. M. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 8173–8180
- Moreau, P. L. (1988) *J. Bacteriol.* **170**, 2493–2500
- Madiraju, M. V., Templin, A., and Clark, A. J. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 6592–6596
- Wang, T. C., and Smith, K. C. (1986) *J. Bacteriol.* **168**, 940–946
- Wang, T. C. V., Madiraju, M. V. V. S., Templin, A., and Clark, A. J. (1991) *Biochimie (Paris)* **73**, 335–340
- Thomas, A., and Lloyd, R. G. (1983) *J. Gen. Microbiol.* **129**, 681–686
- Volkert, M. R., Margossian, L. J., and Clark, A. J. (1984) *J. Bacteriol.* **160**, 702–705
- Knight, K. L., Aoki, K. H., Ujita, E. L., and McEntee, K. (1984) *J. Biol. Chem.* **259**, 11279–11283
- Wang, T. C., Chang, H. Y., and Hung, J. L. (1993) *Mutat. Res.* **294**, 157–166
- Ennis, D. G., Levine, A. S., Koch, W. H., and Woodgate, R. (1995) *Mutat. Res.* **336**, 39–48
- Madiraju, M. V., Lavery, P. E., Kowalczykowski, S. C., and Clark, A. J. (1992) *Biochemistry* **31**, 10529–10535
- Lohman, T. M., Bujalowski, W., and Overman, L. B. (1988) *Trends Biochem. Sci.* **13**, 250–255
- Bujalowski, W., Overman, L. B., and Lohman, T. M. (1988) *J. Biol. Chem.* **263**, 4629–4640
- Lohman, T. M., and Ferrari, M. E. (1994) *Annu. Rev. Biochem.* **63**, 527–570
- Story, R. M., Weber, I. T., and Steitz, T. A. (1992) *Nature* **355**, 318–325
- Roca, A. I., and Cox, M. M. (1997) *Prog. Nucleic Acid Res. Mol. Biol.* **56**, 129–223
- Lusetti, S. L., and Cox, M. M. (2002) *Annu. Rev. Biochem.* **71**, 71–100
- VanLoock, M. S., Yu, X., Yang, S., Lai, A. L., Low, C., Campbell, M. J., and Egelman, E. H. (2003) *Structure* **11**, 1–20
- Yu, X., Jacobs, S. A., West, S. C., Ogawa, T., and Egelman, E. H. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 8419–8424
- Benedict, R. C., and Kowalczykowski, S. C. (1988) *J. Biol. Chem.* **263**, 15513–15520
- Tateishi, S., Horii, T., Ogawa, T., and Ogawa, H. (1992) *J. Mol. Biol.* **223**, 115–129
- Rusche, J. R., Konigsberg, W., and Howard-Flanders, P. (1985) *J. Biol. Chem.* **260**, 949–955
- Yarranton, G. T., and Sedgwick, S. G. (1982) *Mol. Gen. Genet.* **185**, 99–104
- Larminat, F., and Defais, M. (1989) *Mol. Gen. Genet.* **216**, 106–112
- Yu, X., and Egelman, E. H. (1991) *J. Struct. Biol.* **106**, 243–254
- Lusetti, S. L., Wood, E. A., Fleming, C. D., Modica, M. J., Korth, J., Abbott, L., Dwyer, D. W., Roca, A. I., Inman, R. B., and Cox, M. M. (2003) *J. Biol. Chem.* **278**, 16372–16380
- Lusetti, S. L., Shaw, J. J., and Cox, M. M. (2003) *J. Biol. Chem.* **278**, 16381–16388
- Chow, S. A., Rao, B. J., and Radding, C. M. (1988) *J. Biol. Chem.* **263**, 200–209
- Lavery, P. E., and Kowalczykowski, S. C. (1990) *J. Biol. Chem.* **265**, 4004–4010
- Shan, Q., Cox, M. M., and Inman, R. B. (1996) *J. Biol. Chem.* **271**, 5712–5724
- Lohman, T. M., and Overman, L. B. (1985) *J. Biol. Chem.* **260**, 3594–3603
- Egler, A. L., Inman, R. B., and Cox, M. M. (2002) *J. Biol. Chem.* **277**, 39280–39288

50. Sugiyama, T., Zaitseva, E. M., and Kowalczykowski, S. C. (1997) *J. Biol. Chem.* **272**, 7940–7945
51. Craig, N. L., and Roberts, J. W. (1981) *J. Biol. Chem.* **256**, 8039–8044
52. Neuendorf, S. K., and Cox, M. M. (1986) *J. Biol. Chem.* **261**, 8276–8282
53. Lindsley, J. E., and Cox, M. M. (1990) *J. Biol. Chem.* **265**, 9043–9054
54. Morrical, S. W., Lee, J., and Cox, M. M. (1986) *Biochemistry* **25**, 1482–1494
55. Shan, Q., and Cox, M. M. (1996) *J. Mol. Biol.* **257**, 756–774
56. Arenson, T. A., Tsodikov, O. V., and Cox, M. M. (1999) *J. Mol. Biol.* **288**, 391–401
57. Muniyappa, K., Shaner, S. L., Tsang, S. S., and Radding, C. M. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 2757–2761
58. Bork, J. M., Cox, M. M., and Inman, R. B. (2001) *J. Biol. Chem.* **276**, 45740–45743
59. Bujalowski, W., and Lohman, T. M. (1986) *Biochemistry* **25**, 7799–7802
60. Lavery, P. E., and Kowalczykowski, S. C. (1992) *J. Biol. Chem.* **267**, 9307–9314
61. Menetski, J. P., and Kowalczykowski, S. C. (1985) *J. Mol. Biol.* **181**, 281–295
62. Lavery, P. E., and Kowalczykowski, S. C. (1988) *J. Mol. Biol.* **203**, 861–874
63. Umezu, K., and Kolodner, R. D. (1994) *J. Biol. Chem.* **269**, 30005–30013