THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 18, Issue of May 2, pp. 16381–16388, 2003

Printed in U.S.A.

Magnesium Ion-dependent Activation of the RecA Protein Involves the C Terminus*

Received for publication, December 18, 2002, and in revised form, February 10, 2003
Published, JBC Papers in Press, February 20, 2003, DOI 10.1074/jbc.M212916200

Shelley L. Lusetti, Jeffrey J. Shaw, and Michael M. Cox‡

From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

Optimal conditions for RecA protein-mediated DNA strand exchange include 6–8 mM Mg^{2+} in excess of that required to form complexes with ATP. We provide evidence that the free magnesium ion is required to mediate a conformational change in the RecA protein C terminus that activates RecA-mediated DNA strand exchange. In particular, a "closed" (low Mg^{2+}) conformation of a RecA nucleoprotein filament restricts DNA pairing by incoming duplex DNA, although single-stranded overhangs at the ends of a duplex allow limited DNA pairing to occur. The addition of excess Mg^{2+} results in an "open" conformation, which can promote efficient DNA pairing and strand exchange regardless of DNA end structure. The removal of 17 amino acid residues at the *Escherichia coli* RecA C terminus eliminates a measurable requirement for excess Mg^{2+} and permits efficient DNA pairing and exchange similar to that seen with the wild-type protein at high Mg^{2+} levels. Thus, the RecA C terminus imposes the need for the high magnesium ion concentrations requisite in RecA reactions *in vitro*. We propose that the C terminus acts as a regulatory switch, modulating the access of double-stranded DNA to the presynaptic filament and thereby inhibiting homologous DNA pairing and strand exchange at low magnesium ion concentrations.

The RecA protein of *Escherichia coli* plays a central role in the processes of homologous DNA recombination and DNA repair. RecA is a DNA-dependent ATPase that catalyzes an *in vitro* DNA strand exchange reaction between single-stranded (ssDNA)[1] and homologous double-stranded DNA (dsDNA) molecules. The DNA strand exchange reaction takes place in several stages (Fig. 1). The RecA protein forms a nucleoprotein filament that completely encompasses the circular ssDNA. This filament then aligns the bound single strand with a homologous duplex DNA to form a DNA pairing intermediate often referred to as a joint molecule. 1000 base pairs of DNA can be aligned and exchanged in a joint molecule under the empirically defined optimal reaction conditions, which typically include 1–3 mM ATP and about 10 mM magnesium ion. All steps to this point, including the formation of joint molecules, require ATP but not ATP hydrolysis. ATP hydrolysis is needed only to complete the late stages of strand exchange of long DNA substrates, often derived from bacteriophage DNAs. Whereas DNA pairing, leading to joint molecule formation, can occur at either end of a linear duplex, the subsequent and ATP hydrolysis-dependent extension of the nascently paired regions is unidirectional, proceeding 5′ to 3′ relative to ssDNA initially bound in the filament. Thus, exchange proceeds in one direction along the linear duplex, and joints formed at the "wrong" end in the pairing phase are eliminated. In a DNA strand exchange involving quite long DNAs that leads to nicked circular product formation, the ends of the duplex where the exchange begins and ends are referred to as proximal and distal, respectively (Fig. 1) (1, 2).

Examination of the conditions for an optimal RecA protein-catalyzed DNA strand exchange reaction reveals an unexplored enigma. Magnesium forms a relatively strong 1:1 complex with ATP (3) and should be required at concentrations equal to the ATP added to the reaction. However, optimal rates and yields in RecA-mediated DNA strand exchange require an additional 6–8 mM of "free" Mg^{2+} (4–6). Some of this magnesium ion is associated with the DNA, but the DNA concentration in most experiments is on the order of a few (1–20) μM in total nucleotides (and backbone phosphate) and thus could not complex more than a small fraction of the available magnesium ion. Lower magnesium concentrations, more stoichiometric with the added ATP, are sufficient for primary DNA binding. Some homologous DNA pairing occurs in the presence of ATPγS, an analog that is not appreciably hydrolyzed, but the higher magnesium ion concentrations are required for the generation of long hybrid DNA products with ATP (7). The extra Mg^{2+} is contributing something significant to the reaction, but the effects have not been explained.

The question is intriguing, since the free magnesium ion requirements for RecA protein-mediated DNA strand exchange *in vitro* appear to exceed what is available *in vivo*. Mg^{2+} is present at about 100 mM in a bacterial cell (8–10), but almost all of this is bound up in ribosomes and DNA. The level of free magnesium ion is thought to be on the order of 1 or 2 mM at most (11). Based on *in vitro* data, the RecA protein should be almost inactive in the cell, although the crowding effect of the *in vivo* environment may moderate the effects of low available Mg^{2+} (12). There is a decreased requirement for magnesium ion in the presence of volume-occupying agents such as polyethylene glycol or polyvinyl alcohol, utilized to approximate the crowded environment of the cell, suggesting that an active conformation of RecA protein can be stabilized by either excess magnesium ion or the presence of crowding reagents at low magnesium (12).

The RecA protein has a small C-terminal domain extending from residue 270 to the protein terminus at residue 352, the function of which has not been fully explored (1, 13). The last 24

* This work was supported by National Institutes of Health Grant GM32335. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biochemistry, University of Wisconsin, 433 Babcock Dr., Madison, WI 53706-1544. Tel.: 608-262-1181; Fax: 608-265-2603; E-mail: cox@biochem.wisc.edu.

[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ATPγS, adenosine 5′-*O*-(thiotriphosphate); SSB, single-stranded DNA-binding protein; MES, 4-morpholineethanesulfonic acid.

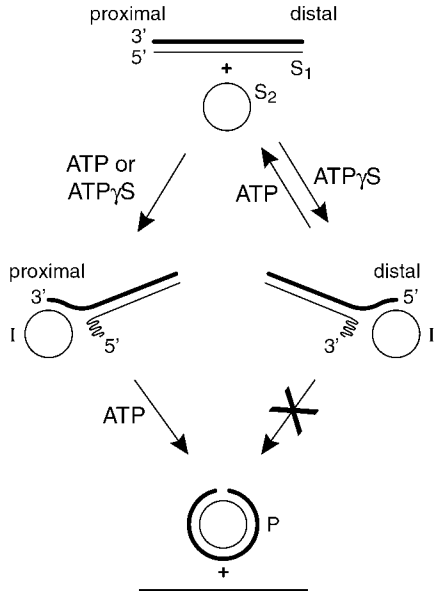

FIG. 1. **DNA strand exchange promoted by the RecA protein *in vitro*.** The DNA strand exchange reaction used in this report is illustrated. DNA strand exchange occurs in multiple stages. A RecA filament first forms on the single-stranded DNA (not shown). A linear duplex is then aligned with the bound single strand, and a strand exchange is initiated. The reaction can occur on either end of the duplex, and intermediates formed at either end can be observed when an ATP analogue that is not hydrolyzed (ATPγS) is used. The subsequent extension of the hybrid DNA proceeds unidirectionally when ATP is hydrolyzed, 5′ to 3′ relative the single-stranded circle. Thus, intermediates formed on one end (proximal) proceed to products, whereas those formed on the other (distal) end are eliminated when ATP is hydrolyzed. The branched reaction intermediates are referred to as joint molecules. The product used most often to analyze reaction progress is the nicked circular duplex. The S_1, S_2, I, and P labels identify DNA species that are similarly labeled in the data figures.

amino acid residues of the RecA protein are disordered in the published RecA protein crystal structures (14–16). We refer to this region as the RecA C terminus (as opposed to the entire C-terminal domain). Within this region, 7 of 25 amino acid residues are negatively charged. In addition to a general lack of structural information about the RecA C terminus, there has been little indication that this part of the protein has functional significance. Several C-terminal deletion mutants of the *E. coli* RecA protein have been characterized. Deletion from the C terminus of either 25 amino acid residues (17), or a fragment making up about 15% of the RecA polypeptide (18), resulted in faster nucleation, leading to filament formation on dsDNA. A proposal was advanced that the effect could be attributed to the elimination of electrostatic repulsion between the negatively charged residues in the C terminus and the phosphates in the DNA (17, 18). Both C-terminal deletion mutants promoted DNA strand exchange under at least some conditions, and the larger deletion exhibited an enhanced DNA strand exchange in the absence of single-stranded DNA-binding protein (SSB) (17, 18). The larger deletion exhibited ATPase and ssDNA binding activities similar to wild-type (18). The 25-residue deletion bound to ssDNA more tightly in some assays (17). A 25-residue C-terminal deletion of the RecA protein of *Proteus mirabilis* also exhibited improved binding to dsDNA (19).

Additional C-terminal deletions of RecA protein have also been constructed and studied, but without detailed biochemical characterization. A construct that removes most of the C-terminal domain (75 residues), when expressed with wild-type RecA, interferes with recombinational DNA repair and increases UV sensitivity slightly (20). A 17-residue C-terminal deletion mutant was shown not to affect UV resistance, induction of the SOS response, recombination, or Weigle reactivation when expressed on its own (21). A small effect on conjugational recombination was observed when the same mutant and wild-type proteins were both present *in vivo* (21). Removal of 18 residues from the C terminus resulted in a substantial conformational change in RecA filaments bound to dsDNA, suggesting an allosteric relationship between the C terminus and the RecA core domain (22).

In the previous paper (23), we characterized a set of RecA C-terminal deletion mutant proteins designed to systematically test the role of the acidic amino acids located in the C terminus of the RecA protein. Removal of the last 13 (RecAΔC13), 17 (RecAΔC17), or 25 amino acid residues (RecAΔC25) increased the rate of binding to dsDNA. The C-terminal deletions also produced a profound effect on the pH dependence of RecA protein-promoted DNA strand exchange reactions relative to the wild-type protein. Ionizable groups in the C-terminal region and others elsewhere in the protein appear to contribute to the pH reaction profile.

In the present study, we show that the negatively charged C terminus of the RecA protein has a modulating function on the DNA strand exchange activity of RecA protein. In the absence of free magnesium ion, the C terminus locks the protein in a conformation in which the initiation of DNA strand exchange is inhibited. When the free magnesium ion concentration is increased to 6–8 mM, the DNA strand exchange function of RecA is activated. The results suggest that magnesium ion interacts directly with RecA protein, altering the conformation of the C terminus.

EXPERIMENTAL PROCEDURES

Enzymes and Biochemicals—The wild-type *E. coli* RecA, RecAΔC6, RecAΔC13, and RecAΔC17 proteins were purified as described (23). A plasmid containing the *recA E343K* mutant (pEAW166) was constructed using PCR site-directed mutagenesis. The RecA E343K point mutant protein was expressed and purified as described for the wild-type RecA protein (23). The concentrations of the purified RecA proteins were determined from the absorbance at 280 nm using the extinction coefficient of 2.23×10^4 M^{-1} cm^{-1} (24). *E. coli* SSB was purchased from Sigma. The concentration of the purified SSB protein was determined from the absorbance at 280 nm using the extinction coefficient of 2.83×10^4 M^{-1} cm^{-1} (25). Unless otherwise noted, all reagents were purchased from Fisher. ATPγS was purchased from Roche Molecular Biochemicals. Dithiothreitol was obtained from Research Organics. Phosphoenolpyruvate, pyruvate kinase, ATP, bromphenol blue, and NADH were purchased from Sigma. Restriction endonucleases *Eco*RI, *Pst*I, and *Sma*I were obtained from New England Biolabs.

Bacteriophage M13 DNA Substrates—Circular single-stranded and supercoiled circular duplex DNAs from bacteriophage M13mp8 (7229 bp) were prepared using previously described methods (26–28). Except where specifically noted otherwise, full-length linear duplex DNA was generated by the digestion of M13mp8 supercoiled bacteriophage DNA with the *Eco*RI restriction endonuclease, using conditions suggested by the enzyme supplier. The digested DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), followed by ethanol precipitation. The concentrations of ssDNA and dsDNA solutions were determined by absorbance at 260 nm, using 36 and 50 μg ml^{-1} A_{260}^{-1}, respectively, as conversion factors. All DNA concentrations are given in μM nucleotides.

DNA Three-strand Exchange Reactions Promoted by the Wild-type and Deletion Mutant Proteins—Three-strand exchange reactions were carried out in 25 mM Tris-OAc buffer (80% cation) or 25 mM MES buffer (33% anion) (reaction pH 7.3 and 6.0, respectively, after the addition of all reaction components), 1 mM dithiothreitol, 5% (w/v) glycerol, 3 mM potassium glutamate, and the indicated concentration of $Mg(OAc)_2$. Reactions also contained an ATP regeneration system of 10 units/ml pyruvate kinase and 3.1 mM phosphoenolpyruvate. All incubations are carried out at 37 °C. The wild-type RecA, RecAΔC6, RecAΔC13, or RecAΔC17 proteins (6.7 μM) were preincubated with 20 μM M13mp8 circular ssDNA for 10 min. SSB protein (2 μM) and the indicated amount of ATP were then added, followed by another 10-min incubation. The reactions were initiated by the addition of M13mp8 linear dsDNA to 20 μM. A 10-μl aliquot was removed to use as a zero time point, the reaction was incubated, and at the indicated time points 10-μl

aliquots were removed and the reaction was stopped by the addition of 5 μl of a solution containing 60 mM EDTA, 6% SDS, 25% (w/v) glycerol, and 0.2% bromphenol blue. Samples were subjected to electrophoresis at 10–20 mA in 0.8% agarose gels with 1× TAE buffer (40 mM Tris-OAc, 80% cation, and 1 mM EDTA), stained with ethidium bromide, and exposed to ultraviolet light. Gel images were captured with a digital CCD camera utilizing GelExpert software (Nucleotech). DNA bands were quantitated with the software package TotalLab version 1.10 from Phoretix.

RESULTS

Experimental Design—The purpose of this study was to examine the magnesium ion dependence of DNA strand exchange reactions promoted by RecA C-terminal deletion mutants. We focused on the RecAΔC6, RecAΔC13, and RecAΔC17 proteins described in the previous paper (23). Under the standard reaction conditions for DNA strand exchange promoted by wild-type RecA protein, the C-terminal deletion mutants promote a significantly slower reaction (23). We set out to find reaction conditions that improve the efficiency of the mutant protein-catalyzed DNA strand exchange in an attempt to understand the role of the negatively charged C terminus in the RecA-promoted DNA strand exchange reaction.

The RecA C-terminal Deletion Mutants Require Less Magnesium Ion for Optimal Strand Exchange than the Wild-type RecA Protein—In an attempt to find the optimal strand exchange conditions for the C-terminal deletion mutants, magnesium titrations were carried out. DNA strand exchange reactions were carried out in Tris-OAc buffer (reaction pH 7.3) at 3 mM ATP and magnesium ion concentrations from 0 to 40 mM (Fig. 2). For each protein, the final extent of strand exchange increases to an optimum and then decreases as the concentration of magnesium ion is increased. For the wild-type RecA protein, this optimum occurs at about 10 mM magnesium ion, in line with many results published over a period of two decades (4–6). Significantly, the deletion lacking 17 amino acids (RecAΔC17) exhibits optimal activity at magnesium ion concentrations that are now roughly equivalent to the ATP concentration. Higher magnesium ion concentrations are inhibitory. The optimal magnesium ion concentrations observed for the RecAΔC6 and RecAΔC13 mutants fall between the other two, so that the magnesium ion requirements for the reaction decline as more of the C terminus is removed. When reactions are compared at their respective optima, strand exchange reactions progress to similar extents for all of the wild-type and mutant RecA proteins.

Just as the magnesium ion concentration needed for an optimal reaction declines as the C terminus of RecA is truncated, the magnesium ion concentrations needed to see inhibition of the strand exchange reaction also decline. At magnesium ion concentrations above 15 mM, strand exchange products by the wild-type RecA protein include large DNA complexes that do not readily enter the gel. These large complexes are protein-dependent (data not shown), are not resolved in 120 min, and appear at successively lower magnesium ion concentrations for the C-terminal deletion mutants.

The yield of DNA strand exchange products produced by each protein after 60 min, at magnesium ion concentrations between 0 and 10 mM was quantitated (Fig. 3). The products in this experiment include the bands corresponding to the complex species in the well, joint molecules, and nicked circular products of DNA strand exchange. These were totaled and divided by the amount of all dsDNA (the above bands plus the linear dsDNA substrate) in the lane. The bands at the well were included in the quantitation because they are protein-dependent and because they are needed to account for all of the DNA in the lane. The magnesium ion concentration required for maximum conversion of substrate DNA into these products

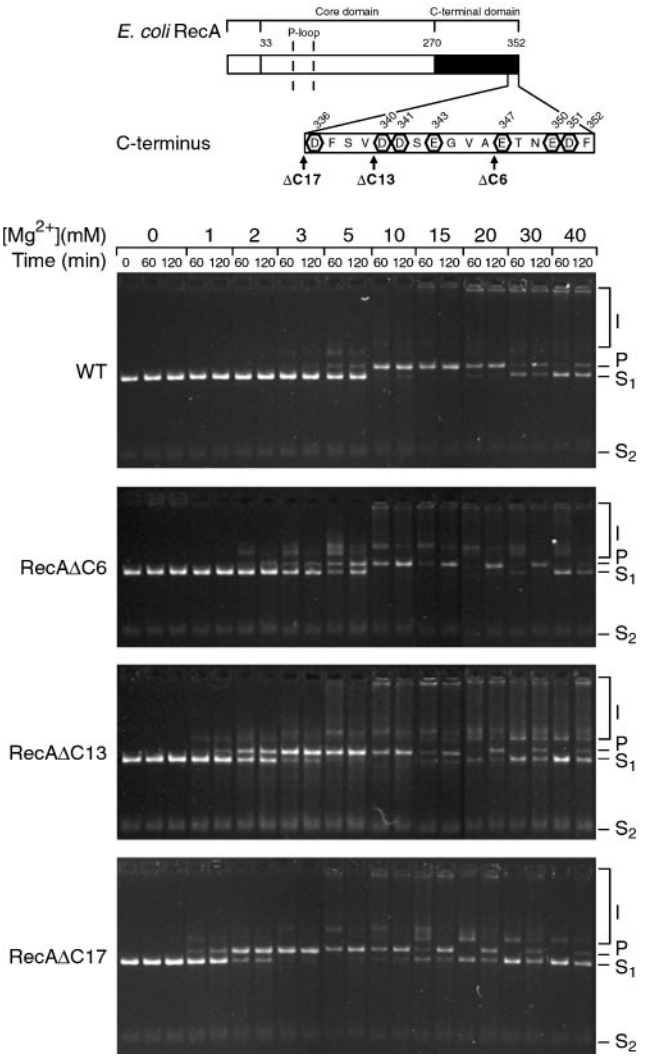

FIG. 2. **Magnesium ion effects on DNA strand exchange reactions promoted by the wild-type RecA, RecAΔC6, RecAΔC13 and RecAΔC17 proteins.** Shown at the *top* is a linear sequence representation that highlights the C-terminal region of RecA protein. The core domain, which includes the P-loop (ATP binding motif), is shown in *white*. The *shaded* and *black regions* of the sequence correspond to the N- 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: RecAΔC6, RecAΔC13, and RecAΔC17. Reactions were carried out as described under "Experimental Procedures" and contained RecA protein or RecA mutant protein (each at 6.7 μM) and 3 mM ATP. The substrates of the reaction are circular ssDNA (S_2, 20 μM) and linear duplex DNA (S_1, 20 μM). The intermediates of the reaction are joint molecules (*I*), and the final products are nicked circular duplex molecules (*P*). The reactions were carried out at pH 7.3 and with the concentration of $Mg(OAc)_2$ indicated.

is ~10 mM for wild-type RecA, as has been reported by several laboratories (4–6). As amino acid residues are removed from the carboxyl terminus, the mutant proteins promote an optimal DNA strand exchange reaction at progressively lower concentrations of magnesium ion (Fig. 3).

The RecAΔC17 Deletion Mutant Promotes an Optimal DNA Strand Exchange Reaction When the Magnesium Ion Concentration Is Approximately Equal to the ATP Concentration—In order to determine whether the RecAΔC17 deletion mutant's optimal strand exchange conditions were dependent on the ATP concentration, strand exchange reactions were carried out at several ATP concentrations (1, 3, and 6 mM), with the reaction extent examined as a function of magnesium acetate con-

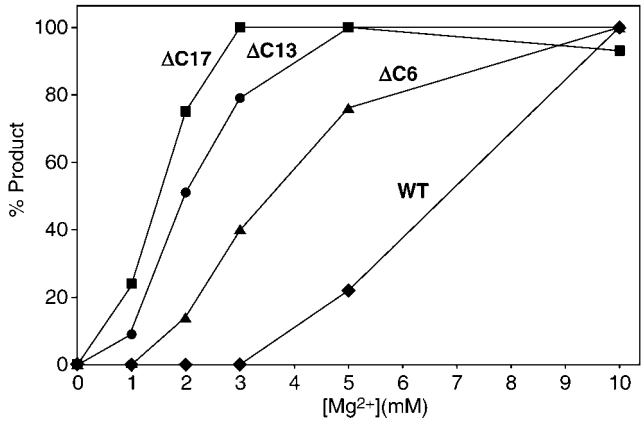

FIG. 3. **Magnesium dependence of DNA strand exchange reactions.** Reactions were carried out as described under "Experimental Procedures." DNA strand exchange products (both joint molecules and nicked circular duplex) generated by the wild-type RecA and the C-terminal deletion mutants are plotted as a function of $Mg(OAc)_2$ concentration. The quantitation was carried out as described under "Experimental Procedures."

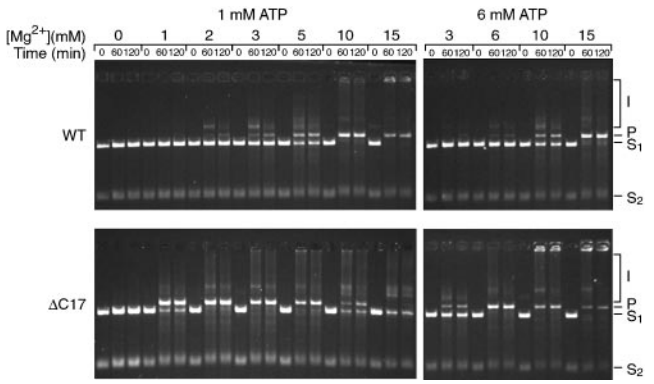

FIG. 4. **Magnesium dependence of DNA strand exchange reactions as a function of ATP concentration: Product visualization.** The results of reactions promoted by the wild-type RecA and RecAΔC17 proteins are shown, in the presence of 1 mM ATP (*left*) or 6 mM ATP (*right*). The labels on the gel are described in the legend to Fig. 2. The reactions were carried out at pH 7.3 and at the concentration of $Mg(OAc)_2$ indicated.

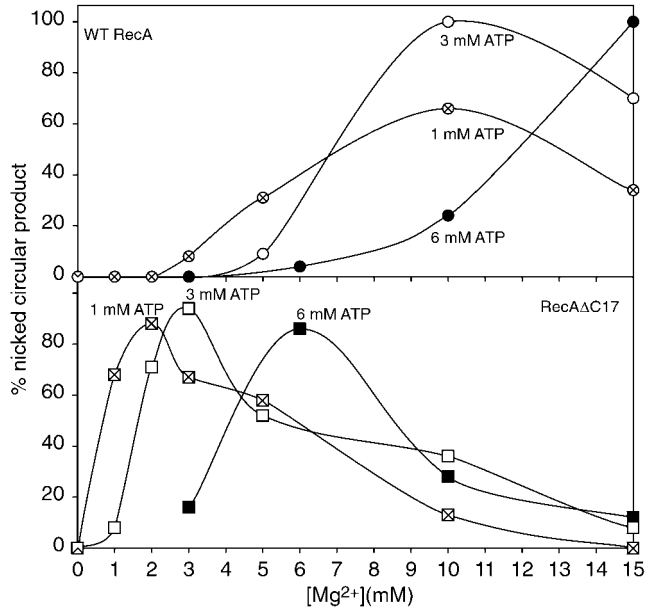

FIG. 5. **The effects of Mg^{2+} concentration on DNA strand exchange, as a function of ATP concentration: Quantitation.** Reactions promoted by wild-type RecA (*circles*, *top panel*) and the RecAΔC17 C-terminal deletion mutant (*squares*, *bottom panel*) are shown as a function of magnesium and ATP concentration. The quantitation was carried out as described under "Results." The percentage of duplex substrate converted to reaction product (nicked circular duplex) at 60 min is plotted *versus* magnesium ion concentration for the wild-type RecA and RecAΔC17 proteins when 1 mM ATP (*crossed symbols*; data from Fig. 4), 3 mM ATP (*open symbols*; data from Fig. 2), or 6 mM ATP (*closed symbols*; data from Fig. 4) is used in the DNA strand exchange reaction.

centration. At either 1 or 6 mM ATP (Fig. 4), the deletion mutant promotes an optimal strand exchange reaction at a magnesium level approximately equal to the ATP. Conversely, at 1 mM ATP, wild-type RecA protein still requires 10 mM magnesium ion and requires an even higher magnesium ion concentration when 6 mM ATP is present. The reactions were quantitated such that only the nicked circular final product of strand exchange was determined as a percentage of all the duplex DNA species present (Fig. 5). It is clear that the wild-type RecA protein requires excess magnesium, above what is needed to complex with ATP. As the ATP concentration increases, the concentration of magnesium ion needed for an optimal reaction remains at 6–8 mM above the ATP concentration. When the C-terminal 17 amino acids are removed from the protein, the optimal DNA strand exchange reaction is observed at a magnesium ion concentration that closely parallels the concentration of ATP.

A 3′ Overhang on the dsDNA Substrate Enhances the Wild-type RecA Protein-promoted DNA Strand Exchange Reaction at Low Magnesium Ion Concentrations—One of the results in Fig. 2 conflicts with a recently published result from our laboratory (29), in which the wild-type RecA protein promoted the formation of significant levels of nicked circular product in DNA strand exchange reactions throughout a range of 1–11 mM $Mg(OAc)_2$ (although the optimal reaction was still seen with 11 mM Mg^{2+}, the reaction was significant but much reduced at 1 mM). As in many of the current trials, the ATP concentration was 3 mM. This result appeared to contradict not only the results above, but also other published results indicating that wild-type RecA protein does not promote DNA strand exchange reactions at low magnesium ion concentrations (7). We investigated this apparent inconsistency in results. The DNA strand exchange reaction results in Fig. 6 address this issue. The DNA substrates utilized in the Rice *et al.* study were derived from ϕX174 bacteriophage, and the linear dsDNA substrate was generated by digestion of circular dsDNA with the *Pst*I endonuclease (29). This treatment generates 3′ overhangs at the DNA ends, which are distinct from the 5′ overhangs in the duplex DNA substrates used in Figs. 2–5. To determine whether the overhangs play a role in the reaction, we carried out DNA strand exchange reactions with M13mp8 linear dsDNA substrates generated by circular dsDNA digestion with the restriction enzyme *Pst*I, *Eco*RI, or *Sma*I to generate 3′ overhangs, 5′ overhangs, or blunt ends, respectively (Fig. 6). *Pst*I and *Eco*RI both leave 4-nucleotide overhangs. At 10 mM magnesium ion, the results of wild-type RecA reactions using the different linear dsDNA ends are virtually indistinguishable, whereas at 3 mM magnesium ion, appreciable amounts of the nicked circular final product of strand exchange can be seen only when 3′ overhangs are utilized. We note that the wild-type mediated reaction with the *Eco*RI-cleaved DNA (5′ overhangs) did result in more reaction intermediates than the blunt-ended DNA, although they were not converted to quantitatable final products. The 5′ overhangs would be complementary to the circular ssDNA, and thus enhance DNA pairing, at the distal end of the linear duplex. The RecAΔC17 protein appears to not require any particular end, regardless of the magnesium ion concentration.

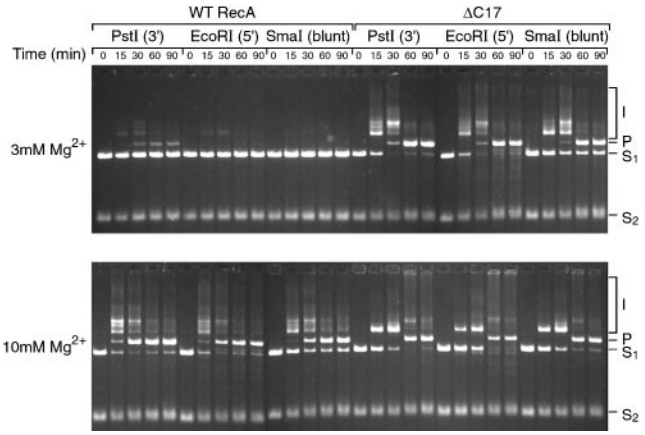

FIG. 6. **Effect of DNA end structure on the DNA strand exchange reaction promoted by the wild-type RecA and RecAΔC17 proteins.** Reactions were carried out at 3 and 10 mM magnesium and 3 mM ATP, as described under "Experimental Procedures." The linear dsDNA substrate was obtained by digesting circular duplex DNA with *Pst*I, *Eco*RI, or *Sma*I restriction endonucleases that generate 3′ overhangs, 5′ overhangs, or blunt ends, respectively. *Pst*I and *Eco*RI both leave 4-nucleotide overhangs. The labels on the gel are described in the legend to Fig. 2.

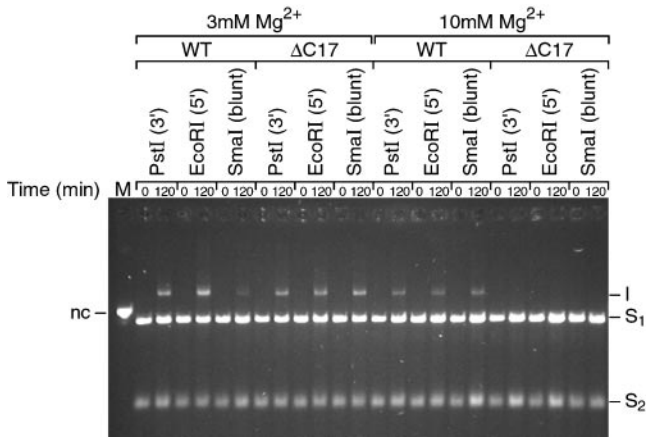

FIG. 7. **Effect of DNA end structure on the DNA pairing reaction in the absence of ATP hydrolysis.** Reactions were carried out with wild-type RecA and RecAΔC17 proteins at 3 and 10 mM magnesium and 3 mM ATPγS. The linear dsDNA substrates were generated as in Fig. 6. *Lane M*, nicked circular dsDNA marker.

The RecAΔC17 Deletion Mutant Requires Low Magnesium Ion for Joint Molecule Formation with ATPγS—DNA strand exchange (with long DNA substrates) generally does not proceed past the formation of joint molecules when ATP is not hydrolyzed. However, the initiation of DNA pairing in the presence of ATPγS should occur in a manner similar to the more extended DNA strand exchange. As is the case for the DNA strand exchange reaction with ATP, the optimal conditions for RecA protein-promoted formation of joint molecule intermediates in the presence of ATPγS include magnesium ion concentrations in significant excess relative to the ATPγS that is present (30). We examined the effect of DNA overhangs on both wild-type RecA and RecAΔC17-promoted DNA strand exchange reactions. The linear dsDNA substrates described above for Fig. 6 were used, with 3 mM ATPγS and either 3 or 10 mM $Mg(OAc)_2$ (Fig. 7). At a magnesium ion concentration of 10 mM, joint molecules formed by wild-type RecA, using the different linear dsDNA ends, are virtually indistinguishable. However, at 3 mM $Mg(OAc)_2$, appreciable amounts of joint molecule products can be seen only when 3′ or 5′ overhangs (not blunt ends) are utilized. The overall result is that a short single-stranded overhang is needed for reactions to be initiated with the wild-type protein in the absence of free magnesium ion. The reaction seen with 5′ overhangs is consistent with the absence of polarity in the initial (ATP hydrolysis-independent) pairing reactions promoted by RecA protein. Intermediates produced on the distal end of the linear duplex DNA (see Fig. 1) would be eliminated if ATP were hydrolyzed, as in Fig. 6.

In contrast, RecAΔC17 appears to not require any particular end at 3 mM magnesium, but it is unable to form joint molecules at 10 mM $Mg(OAc)_2$. The absence of observable joint molecules in this reaction at the higher concentration of magnesium ion, although a more complete DNA strand exchange reaction (albeit somewhat suboptimal) is seen under these conditions, is taken up under "Discussion."

RecAΔC17 Protein Is Unable to Promote Complete DNA Strand Exchange Reactions with ATP or Promote Joint Molecule Formation with ATPγS at pH 6, Even at Low Magnesium Ion Concentrations—In an accompanying paper (23) characterizing the various C-terminal deletion mutants of the RecA protein, we showed that the mutants were deficient in their ability to generate nicked circular products in DNA strand exchange reactions carried out at pH 6. In light of the results above showing that the optimal magnesium ion concentration for the RecAΔC17 protein-promoted reaction is ~3 mM, we tested the lower magnesium ion concentrations in DNA strand exchange reactions with ATP or ATPγS at pH 6 (Fig. 8). The RecAΔC17 protein is unable to promote a complete strand exchange reaction with ATP or promote joint molecule formation with ATPγS at pH 6, even at 3 mM magnesium ion. Thus, the effects of magnesium ion are distinct from the pH effects noted elsewhere (23), at least by this criterion. However, the wild-type RecA reaction at low magnesium is somewhat stimulated by the decreased pH.

The RecA E343K Point Mutant Requires Less Magnesium Ion for Optimal Strand Exchange than the Wild-type RecA Protein—To begin to determine whether the negatively charged residues of the RecA C terminus are contributing to the requirement for excess magnesium in wild-type RecA-mediated DNA strand exchange reactions, we constructed a mutant that replaces the acidic glutamate residue at position 343 with a basic lysine residue (E343K). Position 343 lies in the protein segment that is deleted in the RecAΔC13 mutant but is present in the RecAΔC6 truncation mutant (see Fig. 2). DNA strand exchange reactions were carried out with wild-type RecA and the RecA E343K mutant at 3 mM ATP and magnesium ion concentrations from 1 to 15 mM (Fig. 9). The E343K mutant promotes the strand exchange reaction at lower magnesium ion concentrations than the wild-type protein. Using the criterion described for the deletion mutants above, it appears that the optimal reaction occurs for the E343K mutant at about 5–8 mM Mg^{2+}, similar to that of RecAΔC6 protein. This experiment was carried out twice with consistent results. Converting one of the Glu residues in the C terminus to a positively charged Lys residue thus has an effect similar to the deletion of 6 residues from the C terminus.

DISCUSSION

The primary conclusion of this study is that the C terminus of the RecA protein modulates the protein's DNA strand exchange activity. The last 17 amino acid residues are responsible for the observed requirement for excess magnesium ion, above that necessary to form complexes with ATP, in a wild-type RecA protein-promoted DNA strand exchange reaction *in vitro* (31–34). Removal of these residues eliminates the measurable requirement for the excess Mg^{2+}. These results strongly suggest that the RecA C terminus has a regulatory role in RecA protein activity. At low magnesium ion concentrations, the C

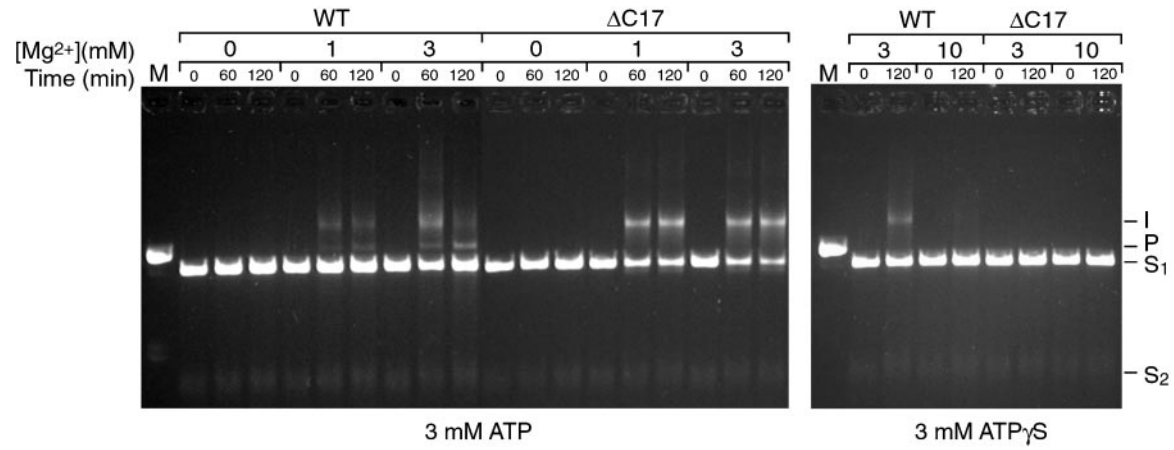

FIG. 8. **DNA strand exchange reactions promoted by the wild-type RecA and RecAΔC17 proteins at pH 6.0.** Reactions were carried out with 3 mM ATP (*left*) or 3 mM ATPγS (*right*) and the indicated concentrations of magnesium ion. Reactions included MES (33% anion) buffer. *Lane M*, nicked circular dsDNA marker.

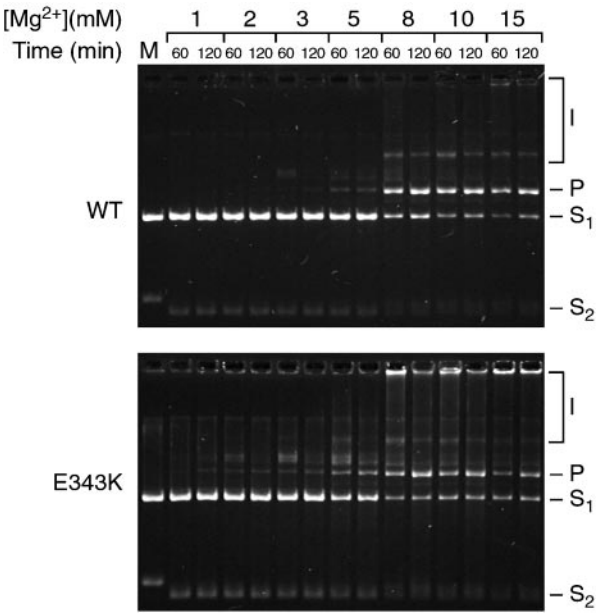

FIG. 9. **Magnesium ion effects on DNA strand exchange reactions promoted by the wild-type RecA and RecA E343K proteins.** Reactions were carried out as described under "Experimental Procedures" and contained RecA protein or RecA E343K mutant protein (each at 6.7 μM) and 3 mM ATP. DNA substrates, products, and intermediates are labeled as in Fig. 2. The reactions were carried out at pH 7.3 and with the concentration of $Mg(OAc)_2$ indicated.

terminus inhibits access of duplex DNA ends to the RecA nucleoprotein filament, although single strands (*i.e.* single strand extensions of duplex DNAs) can facilitate a limited DNA pairing reaction. At least *in vitro*, the protein undergoes a general activation for DNA strand exchange in the presence of excess magnesium ion. Since the excess Mg^{2+} is unnecessary for the RecAΔC17 deletion mutant, we infer that a conformational change involving the C terminus is a key part of the Mg^{2+}-mediated activation process.

The primary structure of the C terminus offers ample opportunities for interactions with magnesium ion. Contributions to the magnesium ion interaction could be made by (*a*) one or more of the residues Glu^{347}, Glu^{350}, and Asp^{351} (removed in RecAΔC6), (*b*) one or more of the residues Asp^{340}, Asp^{341}, and Glu^{343} (removed in RecAΔC13), and (*c*) residue Asp^{336} (removed in RecAΔC17). We note that the C-terminal 17 amino acid residues of RecA protein also include three serine and threonine residues. There is ample precedent that these could also be involved in magnesium ion coordination (35, 36). One of these is removed in each of the 6-, 13-, and 17-amino acid residue deletions. However, the key Mg^{2+} binding site need not be in the C terminus; the present data do not demonstrate Mg^{2+} binding at the C terminus, only that Mg^{2+} affects the conformation of the C terminus so as to bring about activation for DNA strand exchange. Egelman and colleagues have demonstrated that the identity of the nucleotide bound at the distant ATP binding site can have a large impact on the conformation of the C-terminal domain of RecA (37, 38). Similarly, the binding of Mg^{2+} to a site in the core or elsewhere in the C-terminal domain could cause a conformational shift that might affect the positioning of the C terminus. In an accompanying paper (39), we demonstrate that high Mg2+ levels have a significant effect on some activities of the RecAΔC17 protein.

The results are generally consistent with a C-terminal region that acts as a protein flap to modulate access of the duplex DNA substrate to the filament groove. This flap might act as a structural barrier, moved out of the way via the proposed conformational change mediated by the interaction of magnesium ion. Alternatively, the flap could act somewhat indirectly, mediating conformational changes in other parts of the protein that activate RecA. An allosteric effect of the C terminus on the conformation of the RecA protein core domain has previously been documented by Egelman and colleagues (22, 37). The wild-type RecA protein is deficient in homologous pairing of duplex DNA at low magnesium in the presence of ATP. Homologous pairing can be stimulated at low magnesium if either the last 17 residues of the protein are removed, as described above, or volume-occupying reagents are added to the reaction (12). Additionally, the initiation of DNA pairing can be stimulated, to a much lesser degree, if (*a*) ATPγS is used instead of ATP, (*b*) there is a single strand overhang present on the duplex DNA substrate, or (*c*) the pH is lowered to 6. Together, the results indicate that the C terminus is inhibitory to DNA pairing and strand exchange at low Mg^{2+} concentrations.

We propose that the state of RecA protein, bound to ssDNA at low Mg^{2+} (or Mg^{2+} levels commensurate with the ATP present) be designated Ac. The status of RecA on single-stranded DNA in the presence of ATP has historically been referred to as activated (40–42), hence the "A." The state present at low Mg^{2+} is relatively closed to interaction with incoming duplex DNA, hence the "c."

A change in state is needed to facilitate DNA pairing and strand exchange. In most studies carried out with the *E. coli* RecA protein, the change is brought about by adding Mg^{2+} in excess to the ATP concentration. We propose that the resulting

state be denoted Ao, using the "o" to denote that the protein is now open to DNA pairing with duplex DNA regardless of end structure. It is unlikely that these states reflect single, distinct conformations in the presence of ATP. Egelman and colleagues (22, 37, 38) have amply demonstrated that the hydrolysis of ATP can bring about a variety of conformational changes, particularly in the C-terminal domain. Each of the proposed states can hydrolyze ATP with nearly equal facility, as described below.

The noticeable enhancement of DNA pairing by short single-strand overhangs under the low Mg^{2+} conditions suggests that the barrier to DNA pairing is much reduced for ssDNA relative to duplex DNA. Even short single strands at the end of a duplex can provide sufficient stabilization of the initial pairing process to overcome the barrier and allow some DNA pairing to occur.

Magnesium ion could affect DNA strand exchange at many stages, but it is the stages after the formation of RecA-ssDNA nucleoprotein filaments that seem to be most affected. Previous data have suggested that the wild-type RecA protein does not require excess magnesium ion to bind to ssDNA. The rates of ssDNA-dependent ATP hydrolysis catalyzed by the wild-type RecA protein with 1 mM magnesium ion and 0.5 or 1 mM ATP and no SSB protein are close to the rates measured at 10 mM magnesium in the presence of SSB protein (43, 44). Given the similarities in ssDNA binding and ATP hydrolysis, the structural differences between the proposed Ac and Ao states may be subtle. The key functional distinction is that the low Mg^{2+} conditions do not support initiation of a robust DNA strand exchange reaction.

There is additional evidence in the literature that Mg^{2+} concentrations affect RecA conformation. RecA-ssDNA nucleoprotein filaments have been observed in the electron microscope in the presence of 1 mM magnesium. Notably, the contour lengths of these filaments were measured to be 116–120% relative to duplex DNA in the presence of 1 mM ATPγS (7) and 137% relative to duplex DNA in the presence of 1.3 mM ATP (45), which translates to less filament extension than the >150% extension observed when the filaments are formed with 10 mM magnesium ion (23, 46, 47). It is possible that this reduced filament extension seen at low Mg^{2+} concentrations is a result of an inhibitory conformation of the C terminus. Elevated Mg^{2+} levels also result in the formation of RecA filament bundles observable by electron microscopy (46, 48, 49). Finally, with elevated Mg^{2+} levels, RecA protein better resists displacement by SSB (50).

The RecAΔC17 protein exhibits a substantially altered pH reaction profile in DNA strand exchange reactions (23). We have determined that this pH dependence is not affected by magnesium, since, at pH 6, the RecAΔC17 protein is deficient in homologous pairing with ATPγS and in the formation of nicked circular product with ATP at low (Fig. 8) or high magnesium levels (23).

Combining the data from this study and the previous paper (23) allows us to refine the protein flap model for the function of the C-terminal domain of RecA protein. We propose that the C-terminal amino acid residues of wild-type RecA protein are inhibitory not only to the primary binding of RecA protein to establish a filament on dsDNA (as previously proposed) (17, 18) but also to DNA pairing of duplex DNA with a single strand bound within a RecA filament. These may be quite distinct processes, since the barrier to direct binding of RecA protein monomers to a duplex DNA should be different from the binding of a duplex DNA to a RecA filament already formed on a single strand. The barrier to direct binding of RecA protein to dsDNA is overcome at pH 6 with wild-type protein, presumably reflecting the protonation of one or more residues in the C terminus, but is not overcome by excess magnesium ion at neutral pH values. This again indicates that the pH- and magnesium ion-mediated changes in protein state are to some degree distinct, albeit both involve the C terminus in some way.

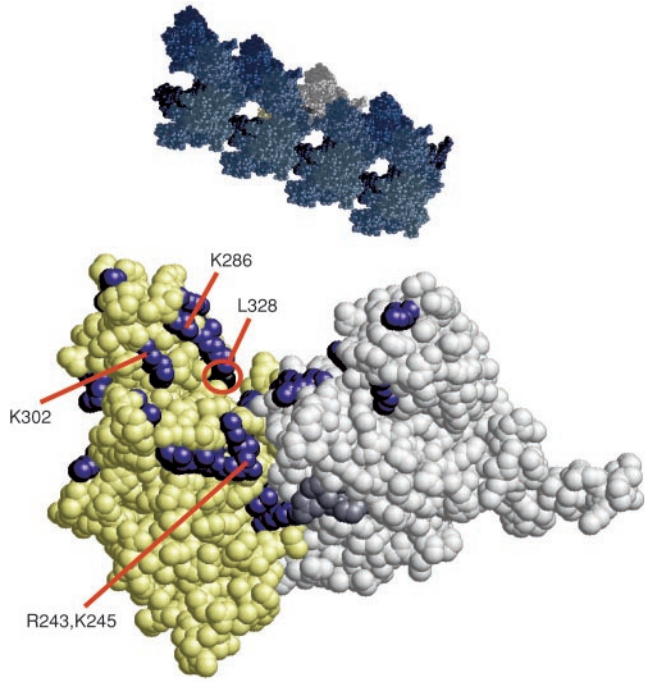

FIG. 10. **Space filling model of the RecA protein.** The figure is based on the published apoenzyme coordinates (Protein Data Bank accession code 2REB) (14). The *top model* represents a RecA filament made up of 24 monomers. The monomers shown in *light gray* and *yellow* (the latter is obscured by other monomers) in the filament are in the same orientation as the monomers *colored white* and *yellow* in the dimer model shown *below* the filament. In the dimer model, residues in *blue* are positively charged amino acids found in or near the filament cleft. The *black residue circled* in *red* is the last residue, Leu^{328}, ordered in this structure and is highlighted because there are 24 additional residues not seen in this model (the C terminus addressed in this study). Residues 243, 245, 286, and 302 are implicated in homologous pairing by previous studies as detailed under "Discussion." The nucleotide-binding motif (P-loop) is *colored gray* and lies near the filament axis, where ssDNA is thought to bind.

Kowalczykowski and colleagues (18) previously proposed that the C-terminal domain had a role in modulating DNA assimilation and strand exchange. However, their more substantial deletion led to an enhancement of DNA strand exchange (with no SSB present) under conditions similar to those in which the deletions studied here decrease the efficiency of strand exchange.

Shibata and colleagues (51) have put forth a model for homologous pairing in which the "gateway" for dsDNA binding to the presynaptic filament lies in the filament groove that is made up of, on one side, the C-terminal domain of the RecA protein. Mutations of some of the many basic residues in this cleft have been shown to abolish homologous pairing. The residues involved include Arg^{243}, Lys^{245} (52, 53), Lys^{286}, and Lys^{302} (51) (Fig. 10). These residues all lie within 20 Å of the last residue of the RecA protein seen in the apoenzyme crystal structures, Leu^{328} (14, 15). There are 24 C-terminal residues (including the seven negative charges that we have removed in the current study) that were disordered in those structures. It is possible that the negative charges of the C terminus can form salt bridges with basic residues in this cleft, thereby restricting the access of dsDNA to the presynaptic filament. These interactions may be part of a network of surface salt bridges. A network would help to explain the gradual reduction in the requirement for magnesium seen with the progressive removal

in the negative charges of the C terminus. Each salt bridge disruption could affect the strength of the next. Such a network would explain the effects of the RecA E343K mutant, which exhibits a behavior similar to RecAΔC6 although that mutation is not in the region removed with RecAΔC6. Magnesium could act by disrupting those salt bridges, enabling homologous pairing. In this way, the C-terminal tail of the RecA protein could form a flap that regulates accessibility to the nucleoprotein filament. The coupling of protein surface salt bridge disruption to DNA binding is a common mechanism used by DNA-binding proteins and has been reviewed recently (54).

As mentioned in the Introduction, the levels of free Mg^{2+} available in the cell are insufficient to bring about the activation seen *in vitro*. It is possible that the molecular crowding in the cell substitutes for the effects of high Mg^{2+} levels (12). Alternatively, another molecule may replace Mg^{2+} in the cell as an activating agent.

Acknowledgments—We thank Jim Keck, Ruth Saecker, and Tom Record for useful discussions.

REFERENCES

1. Lusetti, S. L., and Cox, M. M. (2002) *Annu. Rev. Biochem.* **71,** 71–100
2. Cox, M. M. (1999) *Prog. Nucleic Acids Res. Mol. Biol.* **63,** 310–366
3. Phillips, R. C., George, P., and Rutman, R. J. (1966) *J. Am. Chem. Soc.* **88,** 2631–2640
4. Cox, M. M., and Lehman, I. R. (1982) *J. Biol. Chem.* **257,** 8523–8532
5. Shibata, T., Das Gupta, C., Cunningham, R. P., and Radding, C. M. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 1638–1642
6. Roman, L. J., and Kowalczykowski, S. C. (1986) *Biochemistry* **25,** 7375–7385
7. Honigberg, S. M., Gonda, D. K., Flory, J., and Radding, C. M. (1985) *J. Biol. Chem.* **260,** 11845–11851
8. Kuhn, A., and Kellenberger, E. (1985) *J. Bacteriol.* **163,** 906–912
9. Kuhn, A. H., Jutte, H., and Kellenberger, E. (1983) *J. Virol.* **47,** 540–552
10. Lusk, J. E., Williams, R. J., and Kennedy, E. P. (1968) *J. Biol. Chem.* **243,** 2618–2624
11. Alatossava, T., Jutte, H., Kuhn, A., and Kellenberger, E. (1985) *J. Bacteriol.* **162,** 413–419
12. Lavery, P. E., and Kowalczykowski, S. C. (1992) *J. Biol. Chem.* **267,** 9307–9314
13. Kowalczykowski, S. C., and Eggleston, A. K. (1994) *Annu. Rev. Biochem.* **63,** 991–1043
14. Story, R. M., Weber, I. T., and Steitz, T. A. (1992) *Nature* **355,** 318–325
15. Story, R. M., and Steitz, T. A. (1992) *Nature* **355,** 374–376
16. Datta, S., Prabu, M. M., Vaze, M. B., Ganesh, N., Chandra, N. R., Muniyappa, K., and Vijayan, M. (2000) *Nucleic Acids Res.* **28,** 4964–4973
17. Tateishi, S., Horii, T., Ogawa, T., and Ogawa, H. (1992) *J. Mol. Biol.* **223,** 115–129
18. Benedict, R. C., and Kowalczykowski, S. C. (1988) *J. Biol. Chem.* **263,** 15513–15520
19. Rusche, J. R., Konigsberg, W., and Howard-Flanders, P. (1985) *J. Biol. Chem.* **260,** 949–955
20. Yarranton, G. T., and Sedgwick, S. G. (1982) *Mol. Gen. Genet.* **185,** 99–104
21. Larminat, F., and Defais, M. (1989) *Mol. Gen. Genet.* **216,** 106–112
22. Yu, X., and Egelman, E. H. (1991) *J. Struct. Biol.* **106,** 243–254
23. 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
24. Craig, N. L., and Roberts, J. W. (1981) *J. Biol. Chem.* **256,** 8039–8044
25. Lohman, T. M., and Overman, L. B. (1985) *J. Biol. Chem.* **260,** 3594–3603
26. Davis, R. W., Botstein, D., and Roth, J. R. (1980) *Advanced Bacterial Genetics*, pp. 70–82, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Messing, J. (1983) *Methods Enzymol.* **101,** 20–78
28. Neuendorf, S. K., and Cox, M. M. (1986) *J. Biol. Chem.* **261,** 8276–8282
29. Rice, K. P., Eggler, A. L., Sung, P., and Cox, M. M. (2001) *J. Biol. Chem.* **276,** 38570–38581
30. Menetski, J. P., Bear, D. G., and Kowalczykowski, S. C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 21–25
31. Cox, M. M., Soltis, D. A., Livneh, Z., and Lehman, I. R. (1983) *J. Biol. Chem.* **258,** 2577–2585
32. Flory, S. S., Tsang, J., Muniyappa, K., Bianchi, M., Gonda, D., Kahn, R., Azhderian, E., Egner, C., Shaner, S., and Radding, C. M. (1984) *Cold Spring Harbor Symp. Quant. Biol.* **49,** 513–523
33. Tsang, S. S., Chow, S. A., and Radding, C. M. (1985) *Biochemistry* **24,** 3226–3232
34. Morrical, S. W., Lee, J., and Cox, M. M. (1986) *Biochemistry* **25,** 1482–1494
35. Wedekind, J. E., Poyner, R. R., Reed, G. H., and Rayment, I. (1994) *Biochemistry* **33,** 9333–9342
36. Milburn, M. V., Tong, L., deVos, A. M., Brunger, A., Yamaizumi, Z., Nishimura, S., and Kim, S. H. (1990) *Science* **247,** 939–945
37. VanLoock, M. S., Yu, X., Yang, S., Lai, A. L., Low, C., Campbell, M. J., and Egelman, E. H. (2003) *Structure* **11,** 1–20
38. 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
39. Eggler, A. L., Lusetti, S. L., and Cox, M. M. (2003) *J. Biol. Chem.* **278,** 16389–16396
40. Roberts, J. W., Roberts, C. W., and Craig, N. L. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 4714–4718
41. Sassanfar, M., and Roberts, J. W. (1990) *J. Mol. Biol.* **212,** 79–96
42. Shinagawa, H. (1996) *EXS* **77,** 221–235
43. Kowalczykowski, S. C., Clow, J., Somani, R., and Varghese, A. (1987) *J. Mol. Biol.* **193,** 81–95
44. Morrical, S. W., and Cox, M. M. (1990) *Biochemistry* **29,** 837–843
45. Flory, J., Tsang, S. S., and Muniyappa, K. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 7026–7030
46. Egelman, E. H., and Stasiak, A. (1986) *J. Mol. Biol.* **191,** 677–697
47. Yu, X., and Egelman, E. H. (1992) *J. Mol. Biol.* **227,** 334–346
48. Egelman, E. H., and Stasiak, A. (1988) *J. Mol. Biol.* **200,** 329–349
49. Yu, X., and Egelman, E. H. (1992) *J. Mol. Biol.* **225,** 193–216
50. Kowalczykowski, S. C., and Krupp, R. A. (1987) *J. Mol. Biol.* **193,** 97–113
51. Kurumizaka, H., Aihara, H., Ikawa, S., Kashima, T., Bazemore, L. R., Kawasaki, K., Sarai, A., Radding, C. M., and Shibata, T. (1996) *J. Biol. Chem.* **271,** 33515–33524
52. Kurumizaka, H., Ikawa, S., Sarai, A., and Shibata, T. (1999) *Arch. Biochem. Biophys.* **365,** 83–91
53. Kurumizaka, H., Aihara, H., Ikawa, S., and Shibata, T. (2000) *FEBS Lett.* **477,** 129–134
54. Saecker, R. M., and Record, M. T. (2002) *Curr. Opin. Struct. Biol.* **12,** 311–319