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SSB Protein Limits RecOR Binding onto Single-stranded DNA*

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Michael D. Hobbs[1], **Akiko Sakai, and Michael M. Cox**[2]

From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706-1544

The RecO and RecR proteins form a complex that promotes the nucleation of RecA protein filaments onto SSB protein-coated single-stranded DNA (ssDNA). However, even when RecO and RecR proteins are provided at optimal concentrations, the loading of RecA protein is surprisingly slow, typically proceeding with a lag of 10 min or more. The rate-limiting step in RecOR-promoted RecA nucleation is the binding of RecOR protein to ssDNA, which is inhibited by SSB protein despite the documented interaction between RecO and SSB. Full activity of RecOR is seen only when RecOR is preincubated with ssDNA prior to the addition of SSB. The slow binding of RecOR to SSB-coated ssDNA involves the C terminus of SSB. When an SSB variant that lacks the C-terminal 8 amino acids is used, the capacity of RecOR to facilitate RecA loading onto the ssDNA is largely abolished. The results are used in an expanded model for RecOR action.

Recombination reactions catalyzed by the RecA protein form an integral part of DNA metabolism in *Escherichia coli*. The major role for recombination in bacteria is the repair of stalled replication forks (1–4). *E. coli* strains lacking intact recombination systems exhibit sensitivity to DNA-damaging agents, hypermutability and impaired growth rates (5).

RecA carries out recombination reactions as a helical nucleoprotein filament (6). Formation of the active filament requires ATP and Mg^{2+} ion. When bound to single-stranded DNA (ssDNA),[3] RecA hydrolyzes ATP ($k_{cat} = 30\ min^{-1}$). The active filament can promote the exchange of DNA strands between homologous DNA molecules *in vitro* (7). RecA filament formation occurs in at least two distinct phases (8). Filament nucleation occurs first and involves the binding of one or a few RecA protomers to DNA. Nucleation is the rate-limiting phase in filament formation and can, itself, be broken down into several discernible steps (8). Filament nucleation is followed by filament extension in the 5′ to 3′ direction along the DNA (9–12). Extension is rapid and occurs via cooperative addition of RecA monomers to the 3′ proximal end of the filament. RecA filament disassembly requires ATP hydrolysis and also occurs in the 5′ to 3′ direction (12). RecA filament growth in the 3′ to 5′ direction has not been observed under conditions in which RecA-mediated ATP hydrolysis occurs.

RecA activity is regulated at many levels. RecA transcription is controlled by the LexA repressor protein (13, 14). RecA protein function is also autoregulated by its 17 C-terminal amino acids (15–17). Finally, *E. coli* possesses many protein factors that directly inhibit or promote recombination (18, 19). Proteins that regulate RecA activity exert their control primarily by affecting the kinetics of filament formation and disassembly. Proteins that facilitate the formation of the filaments of RecA-class recombinases are called recombination mediator proteins (RMPs) (19), and they are as ubiquitous in living systems as the recombinases themselves (19–25). In *E. coli*, RMPs include the RecF, RecO, and RecR proteins (10, 26–28). Other proteins inhibit RecA filament formation (the RecX protein) or stabilize intact RecA filaments (the DinI protein) (29–32). In a few cases, RecA regulators alter the catalytic properties of the RecA filament itself. For example, the DinI protein inhibits the RecA-mediated autocatalytic cleavage of UmuD protein (33), a reaction required to activate DNA polymerase V (34). Similarly, the *Bacillus subtilis* RecU protein can inhibit the ATPase activity of that organism's RecA protein (35). RMPs and other regulators often interact and compete. The RecF protein, for example, antagonizes the activity of the RecX protein (26). These interactions provide a complex network for RecA filament control, the understanding of which is expanding as experiments progress (26, 36).

The SSB protein (18.2 kDa) affects RecA filaments in a complex way. During the extension phase of RecA filament formation, SSB protein has a positive effect by removing the DNA secondary structure that would otherwise limit filament extension (37). However, when SSB is prebound to ssDNA, it creates a significant kinetic barrier to RecA nucleation (27, 31). Overcoming this barrier is the function of the RMPs (18, 27, 31).

The *E. coli* RecF, RecO, and RecR proteins have all been implicated genetically in the nucleation of RecA filaments onto SSB-coated ssDNA. The phenotypes of mutations in the three genes are very similar, defining them as an epistatic group (5, 38). Mutations in all three genes are suppressed by *recA*441 and *recA*803 mutations (39). *In vitro*, the same RecA441 (previously tif; E38K, I298V) and RecA803 (V37M) proteins exhibit an enhanced capacity to displace SSB and bind ssDNA (40, 41). In addition, a gene in bacteriophage λ called *ninB* or *orf* (described further below) has been identified that can replace *recF*, *recO*, and *recR* functions in lambda recombination (42, 43). *In vivo*, mutant bacteria missing any of the *recFOR* functions exhibit a delayed activation of the SOS response that might reflect slow formation of the RecA filaments required to facilitate LexA

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[1] Recipient of NIH Graduate Training Grant T32 GM007215.
[2] To whom correspondence should be addressed: Dept. of Biochemistry, University of Wisconsin-Madison, 433 Babcock Dr., Madison, WI 53706-1544. Tel.: 608-262-1181; Fax: 608-265-2603; E-mail: cox@biochem.wisc.edu.
[3] The abbreviations used are: ssDNA, single-stranded DNA; RMPs, recombination mediator proteins; SSB, single-stranded DNA-binding (protein); DTT, dithiothreitol; RPA, replication protein A.

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cleavage (44, 45). *E. coli* strains in which SSB is overexpressed exhibit a *recFOR*-like phenotype (46), again suggesting that these proteins function together in overcoming the barrier to RecA filament nucleation represented by SSB. However, when utilized *in vitro*, the RecO and RecR proteins appear necessary and sufficient for the nucleation of RecA on SSB-coated ssDNA (10, 27). The addition of RecF protein has a neutral or inhibitory effect under most experimental conditions (10, 26, 27, 47). The one exception to this trend is a report of a RecF enhancement in reactions involving gapped DNAs (28). We have concentrated here on the function of the RecOR protein complex.

The *recO* gene was discovered in a genetic screen for genes involved in hfr recombination (48). The RecO protein (27 kDa) contains an oligonucleotide-binding fold (OB-fold) in its N-terminal domain and binds both single- and double-stranded DNA (49, 50). RecO catalyzes the annealing of complementary oligonucleotides and can also catalyze invasion of duplex DNA by a complementary ssDNA (50, 51).

The RecR protein has no known intrinsic enzymatic or DNA binding activities, although the RecR homologs in *Deinococcus radiodurans* and *B. subtilis* both bind to DNA (52, 53). *E. coli* RecR protein binds to both RecF and RecO proteins *in vitro* (27, 54, 55) but not at the same time (47). The C-terminal TOPRIM domain of RecR has been implicated as the interaction site with both RecO and RecF, suggesting that these proteins may compete for RecR binding (56). The competition between RecF and RecO for RecR protein binding has been documented *in vitro* (47). RecR increases the apparent affinity of both RecO and RecF for DNA (27, 54, 55).

The RecO and RecR proteins work together as RecA mediators. Stimulation of RecA binding to SSB-bound ssDNA does not occur in the presence of either RecO or RecR protein alone (10, 27, 28). The original model for RecOR-facilitated nucleation of RecA filaments onto SSB-coated ssDNA (RecAOR nucleation) calls for RecOR interaction with SSB and recruitment of RecA by the RecOR complex followed by filament nucleation (27). RecR protein (and probably RecO protein) remains bound to ssDNA after RecA nucleation *in vitro* and does not inhibit DNA strand exchange reactions (47). At least two types of mechanisms for RecAOR nucleation can be envisioned. RecOR might stimulate RecA nucleation by creating a suitable DNA binding site on SSB-coated ssDNA. The generation of such a site would permit RecA to nucleate onto the ssDNA through the steps employed when SSB is absent. Another possible mechanism for stimulation involves a RecOR-mediated activation of the intrinsic capacity of RecA to displace SSB and nucleate on ssDNA. This mechanism, which assigns more importance to a RecA-RecOR interaction, is suggested by the greatly enhanced capacity of the RecAΔC17 mutant protein to displace SSB in the absence of RecOR (17). These two pathways are not mutually exclusive.

Regardless of the mechanism of RecOR action, the rate at which RecA nucleates onto SSB-coated ssDNA in the presence of RecOR is surprisingly slow. Even though the effect of RecOR is dramatic, by decreasing the lag in RecA protein binding from 40+ min to ~10, the remaining 10-min lag for the functional RecOR complex appeared slow for an activity that must efficiently repair replication forks. The molecular basis of that 10-min lag is the focus of this report.

EXPERIMENTAL PROCEDURES

Enzymes and Reagents—The wild-type RecA protein was purified as described previously (16). The *E. coli* RecO (10), RecR (54) and SSB proteins (57) were purified using established protocols as was the wild-type *D. radiodurans* SSB protein (58). The *E. coli* RecF protein was overexpressed by transforming pBLW20 (54) into *E. coli* strain STL2669 (a generous gift from Susan T. Lovett) (16) and the resultant strain was cultured at 37 °C in LB medium with appropriate antibiotics to an OD of 1.0. Overexpression of RecF protein then was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside to 0.4 μM. The culture was incubated for 3 h before the cells were harvested. RecF protein was purified using the protocol established by Webb *et al.* (54). The concentration of each protein was determined by absorbance at 280 nm using their respective extinction coefficients: $\epsilon_{280} = 2.23 \times 10^4\ \text{M}^{-1}\ \text{cm}^{-1}$ for RecA and all RecA mutants (59); $\epsilon_{280} = 2.3 \times 10^4\ \text{M}^{-1}\ \text{cm}^{-1}$ for RecO (51); $\epsilon_{280} = 5.6 \times 10^3\ \text{M}^{-1}\ \text{cm}^{-1}$ for RecR (10), $\epsilon_{280} = 3.87 \times 10^4\ \text{M}^{-1}\ \text{cm}^{-1}$ for RecF (60); $\epsilon_{280} = 2.83 \times 10^4\ \text{M}^{-1}\ \text{cm}^{-1}$ for *E. coli* SSB and SSB mutants (61); and $\epsilon_{280} = 2.83 \times 10^4\ \text{M}^{-1}\ \text{cm}^{-1}$ for the *D. radiodurans* SSB (58). The RPA protein of *Saccharomyces cerevisiae* was a generous gift from Patrick Sung. Its concentration was determined by absorbance at 280 nm using the extinction coefficient $\epsilon_{280} = 8.8 \times 10^4\ \text{M}^{-1}\ \text{cm}^{-1}$ (62). All bacterial protein concentrations are reported in terms of monomers, including SSB. RPA concentrations are reported in terms of heterotrimers.

Unless otherwise noted, all reagents were purchased from Fisher and were of the highest grade available. Isopropyl 1-thio-β-D-galactopyranoside was purchased from Gold Bio Technology. DTT was obtained from Research Organics. Tris base was obtained from Research Products International. Phosphoenolpyruvate, pyruvate kinase, ATP, lactate dehydrogenase, potassium glutamate, β-mercaptoethanol, and NADH were purchased from Sigma.

Purification of SSBΔC8—The plasmid encoding the SSBΔC8 protein was prepared by PCR using wild-type *E. coli ssb* gene as a template. The SSBΔC8 mutant was created using a primer designed to replace amino acid 170 of the wild-type *ssb* gene with a stop codon and a primer complementary to the wild-type N terminus. The PCR product was cloned into pET21A to generate pEAW393 and transformed into BL21(DE3). SSBΔC8 protein expression was induced at 37 °C by the addition of 1.0 mM isopropyl 1-thio-β-D-galactopyranoside when cultures reached 1.0 A_{600}. After harvesting, cells were resuspended in lysis buffer (50 mM Tris-Cl (pH 8.3), 200 mM NaCl, 15 mM spermidine trichloride, 1 mM EDTA, 10% sucrose, 1 mM phenylmethylsulfonyl fluoride). All purification steps were carried out at 4 °C. The cells were lysed by adding lysozyme to 200 μg/ml for 30 min followed by sonication. Sodium deoxycholate was added dropwise to 0.05% and incubated for 30 min with slow stirring. SSBΔC8 was precipitated by the addition of ammonium sulfate to a final concentration of 150 mg/ml and further purified by heparin FF and S-100 chromatography.

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SSBΔC8 was stored in a storage buffer containing 20 mM Tris-Cl (pH 8.3), 500 mM NaCl, 50% glycerol, 1 mM DTT.

DNA Substrates—Bacteriophage ϕX174 circular single-stranded DNA (virion) was purchased from New England Biolabs. ϕX174 RF I supercoiled circular duplex DNA was purchased from Invitrogen. Full-length linear duplex DNA was generated by the digestion of ϕX174 RF I DNA (5386 bp) with the XhoI restriction endonuclease under the conditions suggested by the enzyme supplier. The digested DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) followed by ethanol precipitation. Circular ssDNA from bacteriophage M13mp8 (7229 nt) was prepared using previously described methods (63–65). The concentrations of M13 ssDNA were determined by absorbance at 260 nm, using 9.25 $mM^{-1} A_{260}{}^{-1}$ as the extinction coefficient. All DNA concentrations are given in μM nucleotides.

RecA ATP Hydrolysis Assay—The DNA-dependent ATP hydrolysis activity of RecA protein was observed via a coupled spectrophotometric enzyme assay (66, 67). Absorbance measurements were taken with a Varian Cary 300 dual-beam spectrophotometer equipped with a temperature controller and 12-position cell chamber. The cell path length and band pass were 0.5 cm and 2 nm, respectively. The regeneration of ATP from ADP and phosphoenolpyruvate driven by the oxidation of NADH can be followed by a decrease in absorbance. Although the absorbance maximum for NADH occurs at 340 nm, absorbance was measured at 380 nm to remain within the linear range of the spectrophotometer for the duration of the experiment. Concentrations of NADH were calculated using an extinction coefficient of $\epsilon_{380} = 1.21\ mM^{-1}\ cm^{-1}$ at 380 nm.

Rates of ssDNA-dependent ATP hydrolysis and lag times (τ) were measured at 37 °C in a reaction mixture (450 μl) containing 25 mM Tris-OAc (pH 7.5, 80% cation), 10 mM $Mg(OAc)_2$, 5% (w/v) glycerol, 3 mM ATP, and 1 mM DTT. An ATP regeneration system (3 mM phosphoenolpyruvate, 10 units/ml pyruvate kinase) and a coupling system (3 mM NADH, 10 units/ml lactate dehydrogenase, 3 mM potassium glutamate) was also included. The final pH after the addition of all reaction components was 7.6. Concentrations of DNA and proteins, as well as orders of addition, are reported in the text and in Figs. 1–5, 7, and 8. The figures indicate "RecOR" when these proteins were added to reactions as a mixture. When a protein was omitted from a reaction, the appropriate storage buffer was added to compensate for volume and storage buffer components. The storage buffer for wild-type RecA includes 20 mM Tris-Cl (80% cation), 0.1 mM EDTA, 10% (w/v) glycerol, and 1 mM DTT. RecF, RecO, and RecR proteins were stored in 20 mM Tris-Cl (pH 7.5), 0.1 mM EDTA, 100 mM NaCl, 60% (w/v) glycerol, and 1 mM DTT. The SSB protein was stored in 20 mM Tris-Cl (pH 8.3), 500 mM NaCl, 50% (w/v) glycerol, and 1 mM β-mercaptoethanol.

Lag Time Measurements—The lag times reported in this study were calculated using Microsoft Excel. The lag time (τ) corresponds to the time-intercept of a linear regression line fit to the steady state portion of data in ATP hydrolysis assays (Fig. 1*A*). In those cases in which a linear steady state was not achieved for at least 15 min by the end of an experiment, the apparent lag was taken as the tangent to the reaction progress curve at the latest time points, and the lag time is reported as a lower limit. In general, any measured lag time in excess of 30 min is reported as a lower limit, because lags of this duration or greater did not permit the establishment of a reliable final steady state. When lag times were computed in protein titration experiments, the lag time was plotted against the inverse of the concentration of the protein being titrated. These plots facilitate extrapolation to infinite protein concentration and can provide information about the mechanism by which the protein being titrated participates in the kinetically slow process (68).

DNA Three-strand Exchange Reactions Promoted by the Wild-type RecA Protein—Three-strand exchange reactions were carried out in 25 mM Tris-OAc (80% cation, pH 7.4), 1 mM DTT, 5% (w/v) glycerol, 3 mM potassium glutamate, and 10 mM $Mg(OAc)_2$. An ATP regeneration system (10 units/ml pyruvate kinase and 3 mM phosphoenolpyruvate) was also included. All incubations were carried out at 37 °C. The orders of protein addition is listed in the legend for Fig. 2. The wild-type RecA protein (6.7 μM) was preincubated with 20 μM ϕX174 circular ssDNA for 10 min. SSB protein (2 μM) and ATP (3 mM) were then added followed by another 10-min incubation. The reactions were initiated by the addition of ϕX174 linear double-stranded DNA to 20 μM. At various time points, 10-μl aliquots of the reactions were stopped with 5 μl of a solution containing 15% Ficoll, 0.25% bromphenol blue, 0.25% xylene cyanol, 72 mM EDTA, and 4% SDS. Samples were subjected to electrophoresis in 0.8% agarose gels with TAE buffer (40 mM Tris-OAc, 1 mM EDTA), stained with ethidium bromide, and exposed to UV light. Gel images were captured with a digital charge-coupled device camera utilizing GelExpert software (Nucleotech). When indicated, the intensity of DNA bands was quantitated with the software package TotalLab, v. 1.10, from Phoretix.

RESULTS

Experimental Rationale—The aim of this study was to characterize the kinetics of RecOR-mediated nucleation of RecA filaments onto SSB-coated ssDNA. Throughout, RecA-mediated hydrolysis of ATP is employed as an indirect measure of RecA protein binding to ssDNA. The rate of ATP hydrolysis by wild-type RecA protein correlates to the amount of RecA bound to ssDNA under most conditions (11, 67, 69). Using purified native proteins under controlled *in vitro* conditions we carried out two basic forms of experiments. Titrations of protein components (RecA, SSB, RecO, RecR) were carried out to define optimal reaction conditions and to determine the rate-limiting step in the loading of RecA onto SSB-coated ssDNA by RecOR. In a second class of experiments, the order and timing of additions were manipulated to study the effects of composition of putative protein complexes on the rate-limiting step. Two quantities were measured in most experiments: the steady state rate of RecA-mediated ATP hydrolysis and the lag time observed in achieving this rate. The lag time (τ_{obs}) was measured as the delay in reaction progress relative to a theoretically identical reaction with the slow-step eliminated. The *dashed line* in Fig. 1*A* illustrates linear regression based on the steady state rate of ATP hydrolysis for a given condition. The time intercept of this regression is reported as the lag time as described under "Experimental Procedures." Each experiment

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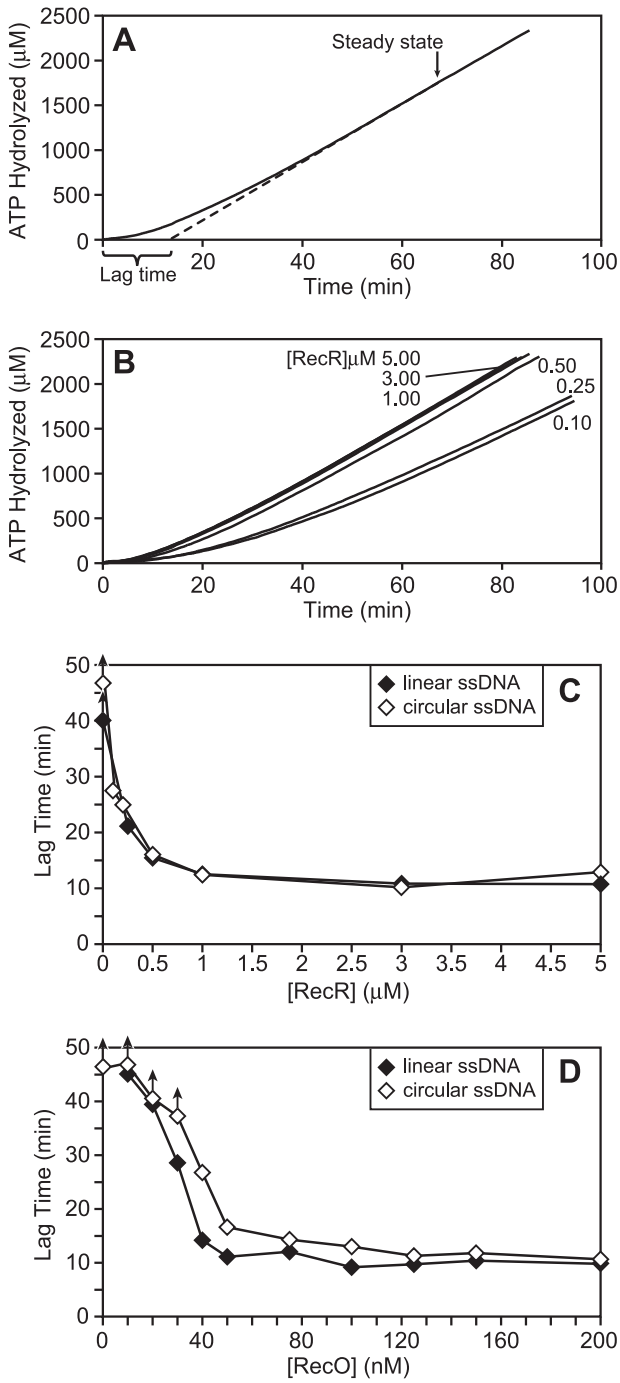


FIGURE 1. **Optimizing the concentrations of RecO and RecR proteins for the experiments of this study.** *A*, a typical reaction is illustrated, with a steady state of ATP hydrolysis evident after a 10–12-min lag. The steady state rate of ATP hydrolysis in this experiment is 31 μM min^{-1}, characteristic of RecA filaments that essentially saturate the available DNA. The reaction was carried out under standard reaction conditions (see "Experimental Procedures"). In this and the other experiments shown in this figure, the reactions contained 4 μM RecA protein, 3 μM M13mp8 ssDNA, 3 mM ATP, and 0.3 μM SSB. This experiment also contained 1 μM RecR protein and 0.1 μM RecO protein. *B*, the RecR protein concentration was optimized in this and similar titration experiments. Reactions were carried out under the conditions described in *A*, except that the RecR protein concentration was varied. The *numbers* correspond to RecR concentrations (in μM). *C*, the measured lag times are plotted against the concentration of RecR protein for the experiments described in *B* and similar trials. In *C* and *D*, *closed* and *open symbols* reflect trials carried out with linear and circular M13mp8 ssDNA, respectively, and *vertical arrows* above some symbols reflect lag times reported as lower limits. *D*, in this series, the RecR protein concentration was held constant at 2.0 μM, and the RecO concentration was varied.

was carried out a minimum of three times with consistent results. These experiments were complemented in a few cases by standard gel assays to measure DNA strand exchange. The RecF protein was added as a variant to many of the experiments shown. In all cases, the presence of the RecF protein was significantly inhibitory under the conditions of these experiments (data not shown). Reported lag times in excess of 35 min should be considered as the lower limits unless noted.

Optimizing RecO and RecR Requirements—An early exploration of RecOR function (31) employed relatively low levels of RecO and RecR protein (~100 nM) and suggested that the two proteins operate in a 1:1 complex (31). Subsequent studies often used somewhat higher concentrations of RecR protein, in excess of RecO by a factor of 3–5-fold (10, 28, 47). This suggests that system requirements for RecR are greater than for RecO. Because a titration can provide clues as to the composition of the overall complex or the pathway for its formation, we explored this issue in more detail. The standard conditions of these experiments include 3 μM circular ssDNA and either 0.3 or 0.5 μM SSB (The lower concentration is approximately stoichiometric to the ssDNA and represents the standard used in previous studies (10, 47); the larger concentration increases the barrier to RecA and RecOR to ssDNA binding and is used in some experiments.) We used a concentration of RecA protein (4 μM) that is in 4-fold excess relative to available DNA binding sites but, as experiments below demonstrate, is sufficient to saturate approximately the observed rate and extent of RecA loading onto the DNA in the presence of RecOR. The following experiments were preceded by an extensive set of preliminary trials that established appropriate concentration ranges for these experiments. In all of these experiments, the RecO and RecR proteins were added to SSB-coated ssDNA (0.3 μM SSB) and incubated for 10 min prior to addition of the RecA protein.

The RecR protein requirements were established first (Fig. 1, *B* and *C*). The RecO concentration employed in this experiment was 0.1 μM, a level found to be optimal for nucleation of RecA filaments on SSB-bound ssDNA in all previous studies (10, 28, 31, 47) and in our own preliminary trials. The RecR concentration was varied from 0 to 3 μM. The effect of the RecR protein increased with concentration up to 1.0 μM RecR (Fig. 1*B*). Similarly, the measured lag time in reaching a steady state decreased with RecR concentration. The shortest lag times (~10 min) were observed when RecR was present at a concentration of 1.0 μM or greater (Fig. 1*C*). The effect of RecR protein is thus maximized when RecR is present at ~10-fold excess relative to RecO protein under these conditions.

The requirements for RecO protein are established in Fig. 1*D* in the presence of a RecR protein concentration of 2.0 μM. The measured lag in the achievement of steady state leveled off at 40–50 nM RecO protein (Fig. 1*D*). Based on these results, the standard reaction conditions for the remaining experiments included 0.1 μM RecO protein and 1.0 μM RecR protein. In some of the trials, linearized M13mp8 ssDNA (M13mp8) replaced the circular ssDNA for further exploration of the enhancement in RecOR function at DNA ends observed in earlier work (47). This revealed a somewhat lower requirement for RecO protein (Fig. 1*D*). The conditions chosen for the present set of experiments reflect protein concentrations that are satu-

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rating with respect to both DNA substrates, and circular ssDNA was used in the rest of the study. These optima have been confirmed with three different preparations of RecO protein and two preparations of RecR protein isolated over a period of 2 years. The same optima were observed when higher SSB concentrations are used (data not shown). A major point to take away from these experiments is that a 10-min lag in RecA filament formation remained even after optimization of the RecOR proteins.

RecOR-facilitated RecA Complexes Are Active in DNA Strand Exchange—The RecO and RecR proteins persist in RecA filaments after their formation (27, 47). This leads to obvious questions about the function of the resulting RecAOR complexes. RecA filaments formed with RecOR protein have previously been shown to be active in strand exchange (31, 47), but we wished to determine more precisely the level of activity under the reaction conditions established in Fig. 1. We therefore tested the DNA strand exchange activity of these complexes, comparing it to the activity of RecA filaments made by adding RecA to the ssDNA prior to SSB; results are presented in Fig. 2.

When adding RecA to the ssDNA prior to SSB highly active RecA filaments formed quickly, hydrolyzing ATP at near the previously observed k_{cat} of 30 min^{-1} (Fig. 2*A*). The lag in ATP hydrolysis was reduced to ~1 min when RecA was added prior to SSB. We assume that any remnant lag that appears when RecA is added prior to SSB reflects the contribution of the extension rate of RecA filaments under these conditions. When SSB was added prior to the RecA protein, filaments were established much more slowly. When RecOR was used to load RecA onto the SSB-coated ssDNA, the final steady state rate of ATP hydrolysis by the resulting filaments was sometimes modestly reduced, possibly because of the persistent presence of RecOR in the complexes (10, 27).

For the strand exchange reaction, the orders of addition for the proteins were similar to those shown in Fig. 2*A*. RecAOR filaments promoted DNA strand exchange with nearly the same kinetics and efficiency as observed when RecA was present prior to SSB (Fig. 2, *B–D*). We conclude that the levels of RecOR proteins retained within RecA filaments under these conditions both are compatible with extensive DNA strand exchange and have only a small effect on the kinetics of DNA strand exchange.

SSB, but Not RecA Protein, Participates in the Rate-limiting Step of RecOR-mediated Nucleation of RecA Filaments—To determine the extent to which the RecA protein was affecting the slow step in RecOR-mediated nucleation of RecA filaments (RecAOR nucleation), the RecA protein was added in varying amounts to reactions containing circular ssDNA bound by SSB in the presence of the RecOR proteins. The final rate of RecA ATP hydrolysis approached the maximum rate of 30 min^{-1} with increasing RecA concentration (Fig. 3*A*). However, the lag times were not significantly affected (Fig. 3*B*). The results indicate that a step in which RecA protein binds to other components in the reaction is not rate-limiting. There are two other possible explanations. First, the rate-limiting process could involve only the RecOR proteins in a process occurring prior to RecA arrival. Second, the rate-limiting step could be first order with respect to RecA concentration, *e.g.* a slow conformation change involving RecA after RecA had joined the complex. Further experiments showed that the first of these explanations was the correct one.

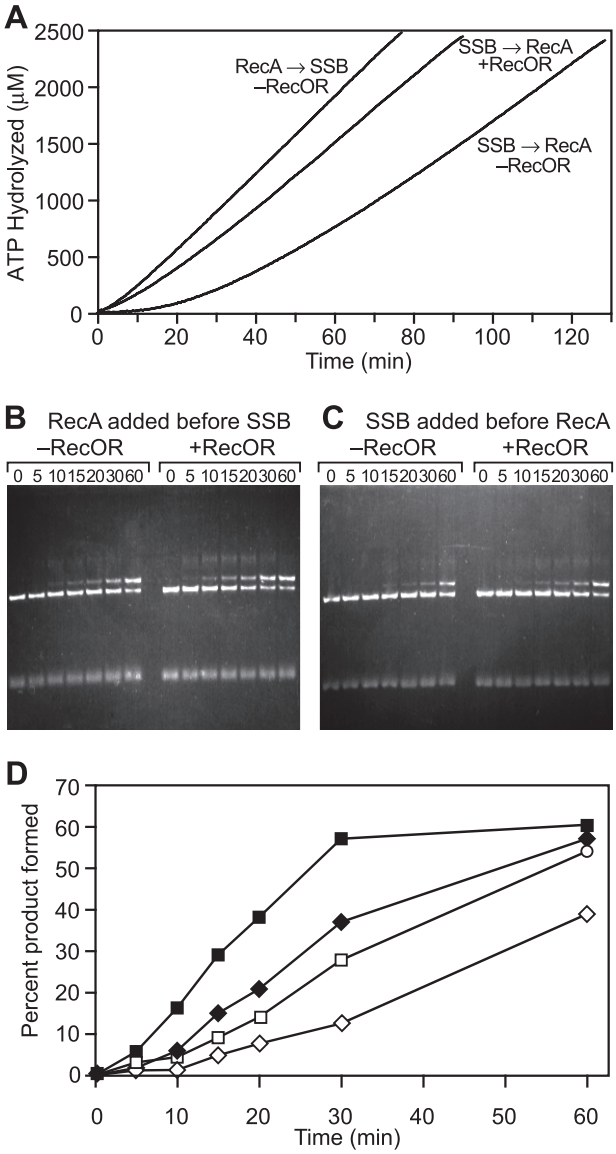


FIGURE 2. **RecAOR filaments promote DNA strand exchange.** Reactions carried out as described under "Experimental Procedures" contained 4 μM RecA protein, 3 μM circular M13mp8 ssDNA, 3 mM ATP, 0.3 μM SSB, and (where indicated) 1 μM RecR protein and 0.1 μM RecO protein. *A*, ATPase traces reflecting different orders of addition. Adding RecA protein prior to SSB essentially eliminates any lag in the achievement of steady state in the ATPase assay. Characteristic lags seen when SSB is added first are exacerbated if RecOR is not present. *B*, if RecA is added prior to SSB, DNA strand exchange is efficient in the presence or absence of RecOR. *C*, if RecA protein is added after SSB, DNA strand exchange is delayed, particularly so if RecOR is not present. *D*, quantitation of the data shown in *B* and C. *Filled markers*, RecA added before SSB either with (*square*) or without (*diamond*) RecOR. *Open markers*, SSB added before RecA either with (*square*) or without (*diamond*) RecOR.

To determine whether the SSB protein plays a significant role in RecOR activation, a similar RecA protein titration was carried out at a higher concentration of SSB (1.0 μM, Fig. 3*B*). As before, the measured lag time was not affected by RecA protein concentration. However, the additional SSB protein increased the lag time observed at all RecA protein concentrations by ~10 min. Thus, higher concentrations of SSB inhibit RecOR activa-

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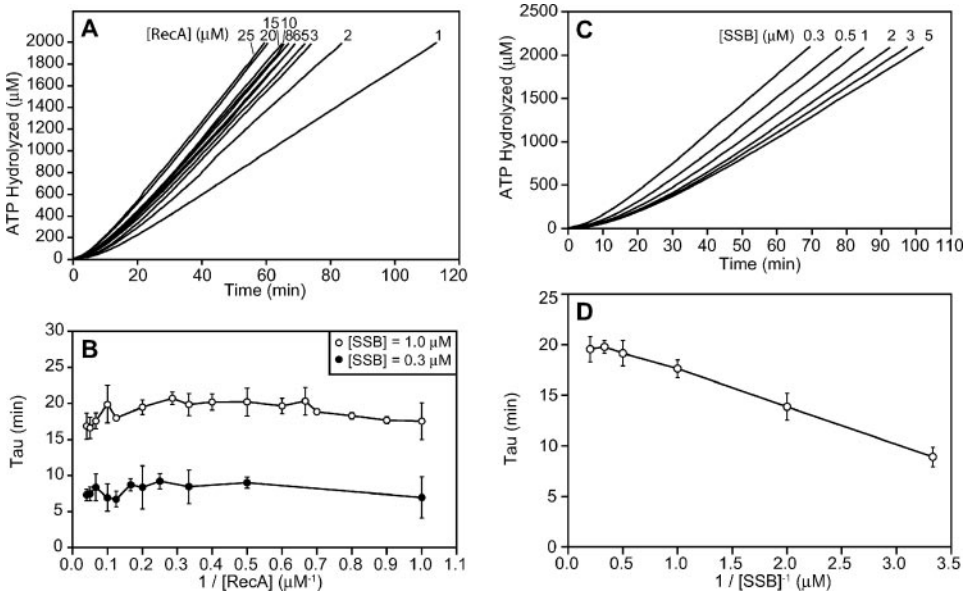


FIGURE 3. **SSB plays a role in the rate-limiting step in RecOR activation.** Reactions carried out as described under "Experimental Procedures" contained 3 μM circular M13mp8 ssDNA, 3 mM ATP, 1 μM RecR protein, and 0.1 μM RecO protein. *A*, RecA protein concentration effects. The concentration of SSB was 0.3 μM, and the RecA protein concentrations (in μM) are indicated by the *numbers* beside each line. *B*, the lag in RecA protein filament formation is shown as a function of RecA concentration, at two different SSB concentrations. All reactions were repeated three or more times. *C*, the effect of SSB concentration on the loading of RecA protein (4 μM) is shown. *D*, measured lags in the binding of RecA protein (4 μM) to ssDNA coated with the indicated concentration of SSB are shown. All reactions were repeated three or more times.

tion for nucleation of RecA filaments. The results indicate that SSB participates in the rate-limiting process in RecOR-mediated nucleation of RecA filaments.

Further evidence for a competition between SSB and RecOR proteins for binding to circular ssDNA as a barrier to RecOR activation was generated by observing the dependence of the lag time on SSB concentration (Fig. 3, *C* and *D*), expanding the SSB titration begun in Fig. 3*B*. An approximately linear and inverse relationship between the concentration of SSB and the lag time is observed, which is the expected result if SSB is inhibiting RecOR activation by competing for a requisite co-factor (circular ssDNA).

RecOR Binding to SSB-coated ssDNA Is Required for Optimal RecA Protein Loading but Is Slow—The kinetics of RecOR activation was measured. SSB-coated circular ssDNA was incubated with the RecOR proteins for various amounts of time before the addition of RecA protein, as illustrated in Fig. 4*A*. Fig. 4*B* shows the course of RecA-circular ssDNA binding reactions initiated after 1, 10, or 30 min of RecOR-SSB preincubation as measured using RecA-mediated ATP hydrolysis. In Fig. 4*C*, the relationship between the preincubation period and the lag time is illustrated. When SSB was bound to ssDNA prior to RecOR, the stimulation of RecA protein (as measured by reduction in the lag time for RecA filament assembly) continued to improve when RecOR was preincubated with SSB-coated ssDNA for longer times up to at least 30 min. This indicates that activation of RecOR occurs very slowly under these conditions and represents the kinetically slow step in RecA nucleation.

RecOR and SSB Proteins Compete for Binding to ssDNA—We carried out a series of order-of-addition experiments to better understand the nature of the slow activation process. When the SSB protein is prebound to circular ssDNA and the RecOR proteins are omitted, wild-type RecA protein nucleates onto the DNA very slowly as indicated by a lag time in excess of thirty min. The addition of the RecOR proteins before or with RecA resulted in a marked reduction of the lag time, as already seen, although a lag of about 10 min remained. Preincubation of RecOR with free RecA with or without ATP, or with ATP alone, had no effect on the observed lag time (data not shown). Thus, there is no evidence for a slow process involving RecOR interaction with RecA or ATP that occurs prior to the interaction of RecOR with SSB-coated ssDNA. A much more substantial decrease in lag time (to 1–2 min) was observed only when RecOR proteins were added prior to the SSB protein. In the experiment shown in Fig. 5, the RecOR proteins were added to the circular ssDNA either 10 min before SSB or 10 min after. The addition of RecOR to the circular ssDNA before adding SSB dramatically reduced the lag in RecOR activation compared with the situation in which SSB was prebound to the circular ssDNA (Fig. 5). If RecOR was present prior to SSB, the loading of RecA protein was comparable with that seen if RecA was added prior to SSB. In effect, RecOR prebound to ssDNA is a fully active RecA filament nucleation site. This result confirms that the slow step in the activation of RecOR for its RecA nucleation function involves simple binding to ssDNA, a step that is significantly inhibited by bound SSB. The results taken together also indicate that RecOR activation to promote RecA filament nucleation represents more than an interaction with SSB but must also entail a direct binding to ssDNA that can be impeded by SSB.

Binding of RecOR to SSB-coated ssDNA Involves the SSB C Terminus—The *E. coli* SSB protein features a C terminus in which the final 8 amino acid residues include multiple negatively charged groups (Fig. 6). This negatively charged C terminus is well conserved among bacterial SSB proteins. Many of the interactions of bacterial SSBs with other proteins involve the SSB C terminus (70–81). Mutations in the 6–8 C-terminal amino acids of the SSB protein can render a bacterial cell inviable (75).

We constructed an SSB variant in which 8 amino acid residues had been removed at the C terminus, designating this variant SSBΔC8. A similar C-terminal deletion mutant of SSB (10 residue deletion) binds to ssDNA normally but with somewhat greater affinity than does the wild-type SSB protein (75). The effects of this C-terminal deletion mutant are compared with

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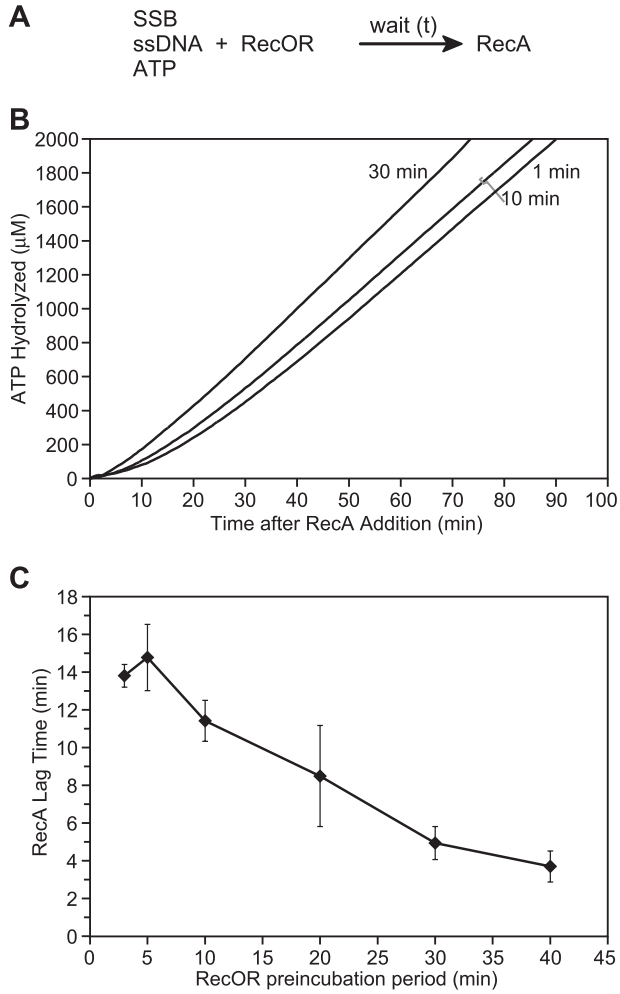


FIGURE 4. **Productive RecOR binding to SSB-coated ssDNA is slow.** Reactions carried out as described under "Experimental Procedures" contained 4 μM RecA protein, 3 μM circular M13mp8 ssDNA, 3 mM ATP, 0.3 μM SSB, 1 μM RecR protein, and 0.1 μM RecO protein. *A*, the reaction scheme, in which the time of incubation of RecOR proteins with SSB-coated ssDNA was varied, is shown. *B*, the effect of increasing the time in which RecOR is incubated with SSB-ssDNA is shown. Times reflect the RecOR incubation with SSB-ssDNA. *C*, the measured lag in RecA protein binding to SSB-coated ssDNA is shown as a function of SSB concentration.

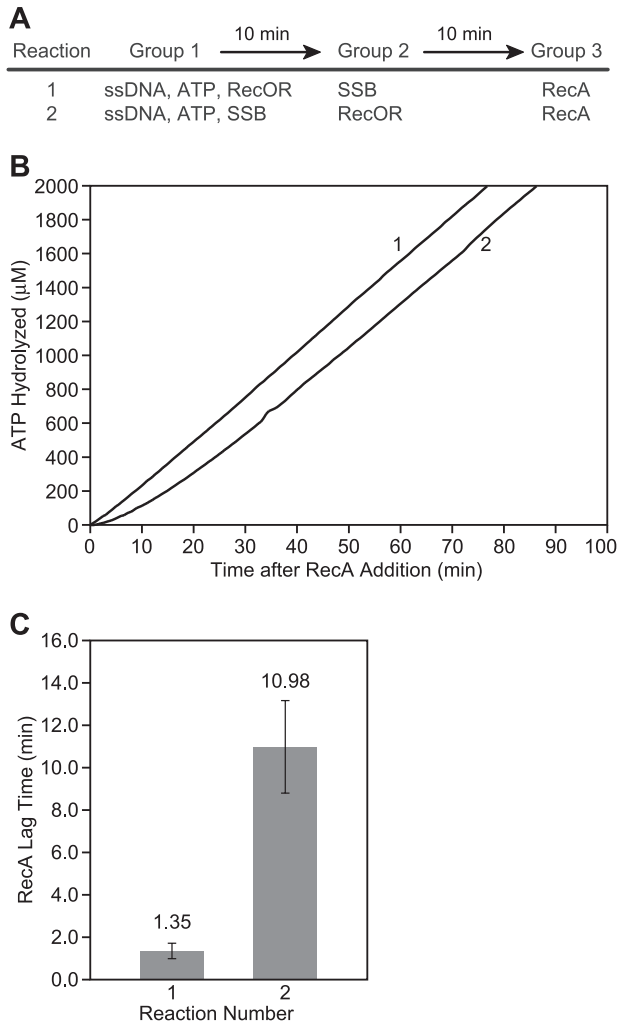


FIGURE 5. **Addition of RecOR to ssDNA prior to SSB abolishes the lag in RecA filament formation.** Reactions carried out as described under "Experimental Procedures" contained 4 μM RecA protein, 3 μM circular M13mp8 ssDNA, 3 mM ATP, 0.3 μM SSB, and (where indicated) 1 μM RecR protein and 0.1 μM RecO protein. *A*, the reaction scheme, showing the preincubation of either RecOR (reaction *1*) or SSB (reaction *2*) with the ssDNA before the addition of SSB or RecOR, respectively. RecA protein was added last. *B*, reaction time courses. *C*, measured lag times. Each reaction was repeated three times, with results averaged as shown.

those obtained for wild-type SSB in Fig. 7 using SSB at 0.5 μM to increase the barrier to RecA and RecOR.

The wild-type SSB is used in Fig. 7*A*. Without RecOR, we once again see the long lag in RecA protein binding seen in Figs. 1 and 2. The addition of RecOR greatly reduces the lag to about 10 min. When RecOR is added prior to the SSB, the lag is reduced to 1–2 min as seen in Fig. 5.

When the SSBΔC8 protein was substituted for wild-type SSB and used to precoat the ssDNA, the function of RecOR protein in stimulating the nucleation of RecA protein was greatly reduced (Fig. 7*B*). The observed lag for RecA protein binding to this SSBΔC8-coated ssDNA was minimally reduced by RecOR. This did not reflect an inability of RecA protein to displace the mutant SSB protein after nucleation, as the very short lag seen when RecA protein was added to the ssDNA prior to SSB was not affected when SSBΔC8 was employed (data not shown). A C-terminal RecA deletion mutant protein, RecΔC17, previously shown to nucleate on

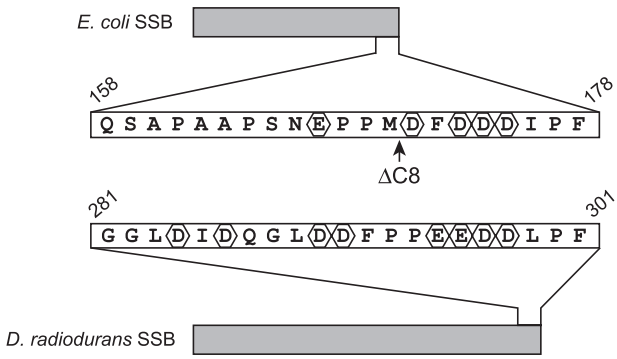


FIGURE 6. **The SSB C terminus.** The location of negatively charged residues in the conserved *E. coli* SSB C terminus is shown. The *arrow* indicates the position of the deletion in SSBΔC8. The C-terminal region of the *D. radiodurans* SSB, used in Fig. 8, is also shown.

SSB-coated ssDNA readily without RecOR (17), was equally robust in nucleating onto ssDNA coated with SSBΔC8 (data not shown). Thus, the result in Fig. 7*B* indicates that the SSB

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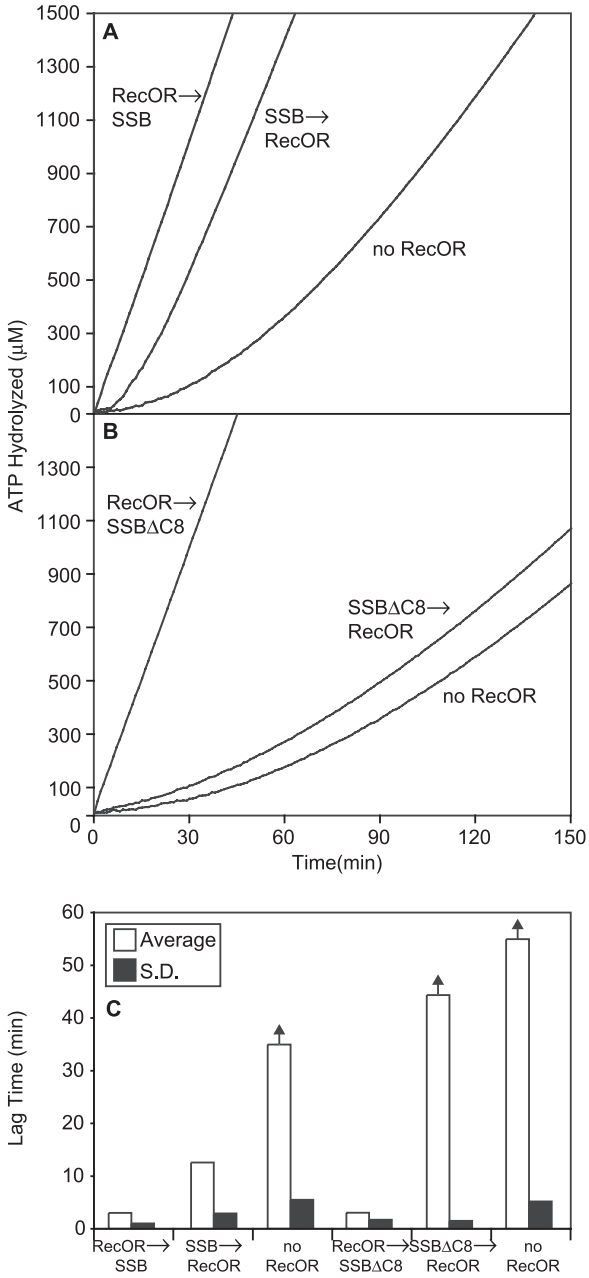


FIGURE 7. **RecOR function is decreased in the presence of SSBΔC8.** Reactions carried out as described under "Experimental Procedures" contained 4 μM RecA protein, 3 μM circular M13mp8 ssDNA, 3 mM ATP, 0.5 μM SSB or SSBΔC8, and (where indicated) 1 μM RecR protein and 0.1 μM RecO protein. *A*, wild-type SSB is used. RecOR or SSB was preincubated for 10 min prior to the addition of SSB or RecOR, respectively, to the ssDNA as indicated. A reaction without RecOR is shown for comparison. *B*, the same reactions are shown with SSBΔC8 replacing wild-type SSB. *C*, measured lag times are given for each situation. All reactions were repeated four times, except for the reaction with wild-type SSB, which was added prior to RecOR (repeated three times). The longer lag times (30+ min) did not reach a final steady state and are presented as lower limits for the lag times (indicated by the *vertical arrows*). The measurements shown represent the tangent to the progress curve taken at the last time point (150 min or later) that was common to all of the reactions averaged (prior to exhaustion of the ATP regeneration system).

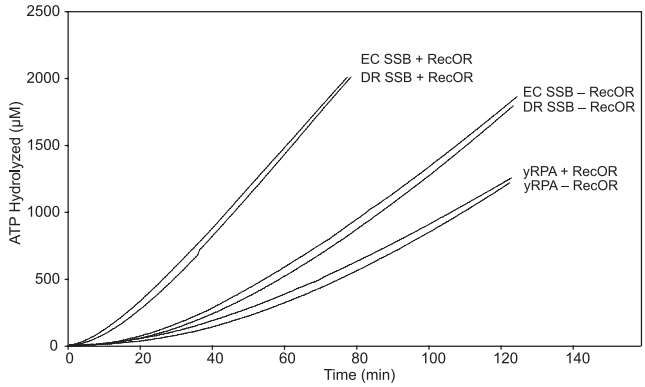


FIGURE 8. **RecOR function requires a bacterial SSB.** Reactions carried out as described under "Experimental Procedures" contained 4 μM RecA protein, 3 μM circular M13mp8 ssDNA, 3 mM ATP, and (where indicated) 1 μM RecR protein and 0.1 μM RecO protein (added 10 min after the SSB). Reactions also contained either 0.3 μM EcSSB, 0.6 μM DrSSB (which functions as a dimer rather than a tetramer), or 1.2 μM yeast RPA protein. The different concentrations make the concentration of functional SSB complexes (EcSSB tetramers, DrSSB dimers, or RPA heterotrimers) identical. RecA protein was added 10 min after the addition of RecOR or (where no RecOR was present) SSB.

C terminus plays a role in the process by which RecOR overcomes the SSB-mediated barrier to its binding to ssDNA in order to promote RecA filament nucleation.

The barrier to RecOR activation when SSBΔC8 is bound to ssDNA is simply the SSBΔC8. If RecOR was incubated with the ssDNA prior to the addition of SSBΔC8, the lag in RecA filament formation was again reduced to 1–2 min (Fig. 7, *B* and *C*). This demonstrates that RecA can displace the SSBΔC8 during the extension phase of filament formation. It also shows that the RecOR protein is potentially fully functional in the presence of the SSBΔC8 mutant protein and indicates that, once bound, the RecOR is not displaced by the mutant SSB protein.

A role for the acidic C terminus of SSB is further suggested by the results in Fig. 8. When the SSB of *D. radiodurans* was substituted for the *E. coli* SSB, the RecOR protein still functioned to decrease the lag in *E. coli* RecA protein filament formation. The *Deinococcus* SSB is twice the size of the *E. coli* SSB, has two OB folds per monomer, and functions as a dimer rather than a tetramer (58, 82). The protein is thus as distinct from *E. coli* SSB as any two bacterial SSBs known. However, both SSBs share the conserved acidic C terminus (Fig. 6). When yeast RPA protein was substituted for the SSB, RecOR no longer functioned to load RecA protein onto the ssDNA (Fig. 8). This result indicates that a bacterial SSB protein is needed for RecOR function, although the SSB does not need to be from the same bacterial species.

DISCUSSION

Our primary conclusion is that the slow step in RecOR-mediated loading of RecA protein onto SSB-coated ssDNA is simply the binding of RecOR to the ssDNA. Although there is an interaction between SSB and RecO, the SSB and RecOR proteins compete directly for binding to ssDNA. Overcoming the SSB barrier to RecOR function involves the acidic SSB C terminus.

An expanded model for RecOR action is provided in Fig. 9. On the basis of the documented interaction of SSB and RecO (27) and the results of the current study, we postulate that the first step is the formation of a complex involving RecOR and SSB and that the interaction involves the SSB C terminus. RecOR then binds directly to the ssDNA in the slow step of the overall process. Finally, RecA undergoes its nucleation and extension steps to form nucleoprotein filaments on the DNA. This model is distinct from the model of Umezu and Kolodner

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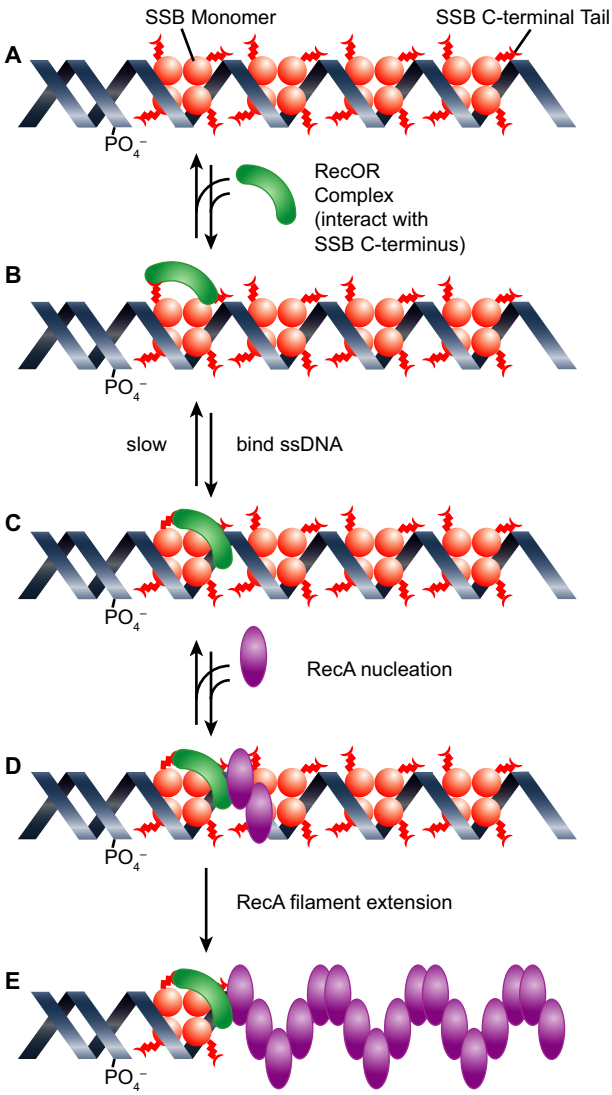


FIGURE 9. **A model for RecOR-mediated loading of RecA protein onto SSB-coated ssDNA.** RecOR activation is hypothesized to occur in two steps. The first involves and interaction with the SSB C terminus (shown as an irregular protrusion from each SSB subunit). The second is the rate-limiting binding to ssDNA. Once the RecOR is bound and activated, RecA filament nucleation and extension proceeds.

(27) in that the direct competition between SSB and RecOR for ssDNA binding implies that some SSB must be displaced from the DNA during the formation of an RecOR-ssDNA complex that is competent for RecA loading.

A direct interaction between RecOR (probably RecO) and the SSB C terminus represents the simplest explanation of the results we have obtained with SSBΔC8. However, we cannot eliminate the possibility that the SSB C terminus simply alters the interaction between SSB and ssDNA so as to permit the displacement of SSB by RecOR. Alternatively, the C-terminal deletion may alter the documented equilibrium between multiple SSB binding modes (57, 71), such that an SSB binding mode required by RecOR is rendered inaccessible at these reaction conditions. More work is needed to sort out these possibilities. More complex mechanisms, in which the SSB C terminus plays a role in mediating a RecOR conformation change or a complex alteration that activates RecOR, are eliminated by the results in Fig. 7 (where no SSB C-terminal peptide is present). If RecOR binds to ssDNA prior to the SSBΔC8, it functions normally in the nucleation of RecA protein in the complete absence of the SSB C terminus. The addition of an octapeptide with the sequence of the 8 C-terminal amino acid residues of SSB in *trans* to ssDNA coated with SSBΔC8 does not restore RecOR function if the RecOR is added after the SSB.[4]

The RecOR complex bound to ssDNA is fully functional as a RecA nucleation site. Once it is in place, RecA filaments are formed almost as rapidly as is observed if RecA is added before SSB. It is generally assumed that the normal substrate for RecA filament formation (and many other DNA transactions) in the cell is SSB-coated ssDNA. The presence of RecOR provides a major boost to RecA loading onto this substrate. However, the ~10 min lag that still remains even when RecOR is present provides a conundrum. 10 min is a short time for an experimenter, but it could represent a long time for a bacterial cell trying to reactivate its replication forks. There are at least three potential explanations. First, the delay could reflect a larger complex process in which multiple proteins are being recruited to the site where recombinational DNA repair is required. In this scenario, the RecOR might be slow but still not limit the overall progress of repair. Second, the delay may signal a cellular priority to have RecA filaments nucleated at specific locations dictated by a protein or proteins in addition to RecOR. For example, another protein could enhance RecOR activity at a particular location to effect filament formation there. The effect of RecF protein in stimulating RecOR function at the ends of DNA gaps could provide an example (28), although lags in RecA binding of 10 min or more are evident in that work even when RecF is assisting the loading. Third, and finally, a protein that functions with RecOR in the cell could remain to be discovered.

The role of RecF protein in RecA filament nucleation remains something of a mystery. When added to reactions in which RecA is nucleating onto SSB-coated ssDNA, RecF is generally inhibitory (10, 26, 27, 47). That is the result we consistently obtained in this work. RecF does enhance the function of RecOR in reactions in which gapped DNA is used, as reported by Morimatsu and Kowalczykowski (28). However, in addition to the requirement for gapped DNA, the enhancing effect of RecF has an unexplained requirement for SSB concentrations that are 8–10-fold higher than required to saturate the available ssDNA (28).[4] RecF does have the positive effect of antagonizing RecX protein function (26) and thus *should* have a positive effect on the extension phase of RecA filament formation. Additional work is required before RecF protein can be accommodated properly in the overall scheme for RecA regulation.

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