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# A Single-stranded DNA-binding Protein Is Needed for Efficient Presynaptic Complex Formation by the *Saccharomyces cerevisiae* Rad51 Protein*

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**Protein-promoted DNA strand exchange requires formation of an active presynaptic complex between the DNA-pairing protein and single-stranded DNA (ssDNA). Formation of such a contiguous filament is stimulated by a ssDNA-binding protein. Here, the effects of replication protein A (RPA) on presynaptic complex formation and DNA strand exchange activities of Rad51 protein were examined. Presynaptic complex formation was assessed by measuring ATP hydrolysis. With $\phi$X174 ssDNA, the ATPase activity of Rad51 protein is stimulated ~1.4-fold by RPA, provided that Rad51 protein is in excess of the ssDNA concentration; otherwise, RPA inhibits ATPase activity. In contrast, with ssDNA devoid of secondary structure (poly(dT), poly(dA), poly(dI), and etheno-M13 DNA), RPA does not stimulate the already elevated ATPase activity of Rad51 protein, but inhibits activity at low Rad51 protein concentrations. These results suggest that Rad51 protein and RPA exclude one another from ssDNA by competing for the same binding sites and that RPA exerts its effect on presynaptic complex formation by eliminating secondary structure to which Rad51 protein is bound nonproductively. DNA strand exchange catalyzed by Rad51 protein is also greatly stimulated by RPA. The optimal stoichiometry for stimulation is ~20–30 nucleotides of ssDNA/RPA heterotrimer. The ssDNA-binding protein of *Escherichia coli* can substitute for RPA, showing that the role of RPA is not specific. We conclude that RPA affects both presynaptic complex formation and DNA strand exchange via changes in DNA structure, employing the same mechanism used by the ssDNA-binding protein to effect change in *E. coli* RecA protein activity.**

The *RAD51* gene of *Saccharomyces cerevisiae*, a member of the *RAD52* epistasis group, is required for mitotic and meiotic recombination (for a review, see Ref. 1). Cells deficient in *RAD51* function are sensitive to x-ray irradiation or DNA-alkylating agents, suggesting that this gene is required for repair of double-strand DNA breaks (2). Since formation of meiosis-specific double-strand DNA breaks is not inhibited in *rad51* cells, *RAD51* seems to function after formation of the break in meiotic recombination. The *RAD51* sequence is conserved in a wide variety of eucaryotic organisms, suggesting that it is important to cellular function in eucaryotes (3). Rad51 protein has homology to *Escherichia coli* RecA protein (2, 4, 5). Furthermore, image reconstruction from electron micrographs of complexes of Rad51 protein and double-stranded DNA (dsDNA)[1] confirmed this similarity and showed that the three-dimensional structure of the Rad51 protein-DNA filament is similar to the equivalent RecA protein-dsDNA complex (6). Finally, Rad51 protein from *S. cerevisiae* has single-stranded DNA (ssDNA)-dependent ATPase activity and promotes ATP-dependent DNA strand exchange (7, 8).

RecA protein plays a central role in genetic recombination in *E. coli* (for reviews, see Refs. 9–13). *In vitro* analyses have revealed that in the presence of ATP, RecA protein binds to ssDNA to form a nucleoprotein filament, referred to as the presynaptic complex, which is the active species in homologous DNA pairing. Pairing between the presynaptic filament and homologous dsDNA results in formation of the synaptic complex and is followed by DNA strand exchange, the process whereby one strand in the dsDNA is replaced by the homologous ssDNA (postsynaptic stage). The single-stranded DNA-binding protein (SSB protein) of *E. coli* stimulates RecA protein-mediated DNA strand exchange by acting at both the presynaptic and postsynaptic stages. At the presynaptic step, SSB protein disrupts secondary structure within the ssDNA to facilitate formation of a continuous RecA protein-ssDNA nucleoprotein filament. At the postsynaptic step, SSB protein facilitates DNA strand exchange by binding to the displaced ssDNA strand produced by DNA heteroduplex formation (14, 15). In both steps, physical interaction between RecA and SSB proteins is not required for stimulation (16–18).

Replication protein A (RPA), also referred to as replication factor A, is a single-stranded DNA-binding protein of *S. cerevisiae* (19–21). RPA is a heterotrimeric protein, consisting of polypeptides with molecular masses of 70.4, 29.9, and 13.8 kDa, and each subunit is essential for cell viability. RPA enhances DNA strand exchange promoted by Rad51 protein (7). Due to the superficial similarities of Rad51 and RPA to RecA and SSB proteins, respectively, it is reasonable to hypothesize that the role of RPA in DNA strand exchange catalyzed by Rad51 protein is similar to that of SSB protein in RecA protein-catalyzed DNA strand exchange. However, it is equally possible that RPA functions differently from SSB protein, to accommodate specific requirements of the eucaryotic recombination process. To address these issues, we measured presynaptic complex and DNA strand exchange activities of Rad51 protein and assessed the effects of both RPA and SSB protein on these reactions. While we find many parallels between the eucaryotic

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[1] The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; SSB protein, single-stranded DNA-binding protein; RPA, replication protein A.

and procaryotic systems, significant differences exist. In particular, active presynaptic complex formation by Rad51 protein has a more stringent requirement for a ssDNA-binding protein. We suggest that this requirement stems from the stable and nonproductive binding of Rad51 protein to regions of dsDNA (secondary structure) within native ssDNA.

## EXPERIMENTAL PROCEDURES

*DNA*—Both replicative form and viral (+)-strand $\phi$X174 DNAs were purchased from New England Biolabs Inc. For DNA strand exchange, the replicative form of $\phi$X174 DNA was linearized with *Pst*I. Etheno-M13 DNA was prepared as described (22). Poly(dT) and poly(dA) were purchased from Pharmacia Biotech Inc. Poly(dI) was purchased from P-L Biochemicals. The concentrations of $\phi$X174 dsDNA, $\phi$X174 ssDNA, poly(dT), and poly(dA) were determined using molar (nucleotide) extinction coefficients of 6500, 8125, 7300, and 8600 at 260 nm, respectively, and the concentration of poly(dI) was determined using a molar (nucleotide) extinction coefficient of 9400 at 246 nm. DNA was stored in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. All DNA concentrations are expressed in nucleotides unless otherwise noted.

*Proteins*—The three subunits of RPA, cloned on three separate plasmids (a generous gift from Dr. Richard Kolodner), were coinduced in yeast, and the RPA heterotrimer was prepared as described (23). Rad51 protein was overproduced in yeast using the plasmid and strain kindly provided by Dr. Patrick Sung and was purified as described (7), except that Cibacron blue column fractionation was used as the first step. SSB protein was overproduced and purified as described (24). The concentrations of RPA, Rad51 protein, and SSB protein were determined using extinction coefficients (determined, for Rad51 protein and RPA, from amino acid composition) of $8.8 \times 10^4$, $1.29 \times 10^4$, and $3.0 \times 10^4$ at 280 nm, respectively. Pyruvate kinase and lactate dehydrogenase were purchased from Sigma. The restriction endonuclease *Pst*I was purchased from New England Biolabs Inc.

*ATP Hydrolysis Assays*—ATPase activity was measured at 37 °C essentially as described (25). Rad51 protein and a single-stranded DNA-binding protein (when indicated) were added in the indicated order to ssDNA in 120 $\mu$l of buffer containing 2.5 mM ATP, 10 units/ml pyruvate kinase, 10 units/ml lactate dehydrogenase, 0.3 mM phosphoenolpyruvate, 256 $\mu$M NADH, 50 $\mu$g/ml bovine serum albumin, 1 mM dithiothreitol, 20 mM magnesium acetate, 50 mM KCl, and 30 mM Tris acetate (pH 7.5). The oxidation of NADH resulted in a decrease in absorbance at 340 nm, which was continuously monitored by a Hewlett-Packard Model 8452A diode array spectrophotometer. The rate of ATP hydrolysis was calculated from the rate of change in absorbance using the following formula: rate of $A_{340}$ decrease ($s^{-1}$) $\times$ 9880 = rate of ATP hydrolysis ($\mu$M/min).

*DNA Strand Exchange Assays*—DNA strand exchange was performed as follows (all concentrations are those in the final reaction mixtures). 33 $\mu$M (nucleotides) $\phi$X174 viral (+)-strand DNA was incubated with 9.8 $\mu$M Rad51 protein in a total volume of 8.5 $\mu$l of 2.5 mM ATP, 50 $\mu$g/ml bovine serum albumin, 1.0 mM dithiothreitol, 20 mM magnesium acetate, 50 mM KCl, and 30 mM Tris acetate (pH 7.5) at 37 °C. After 5 min, RPA or SSB protein was added to the indicated concentration, and incubation was continued for 30 min. *Pst*I-linearized $\phi$X174 dsDNA (1 $\mu$l) was added to a final concentration of 33 $\mu$M (base pairs) and incubated for an additional 90 min. Samples were deproteinized for 15 min by the addition of SDS and proteinase K to final concentrations of 0.5% and 1 mg/ml, respectively. Reaction products were separated by electrophoresis through a 1.0% agarose gel run in 40 mM Tris acetate and 2 mM EDTA (pH 8.5) at 40 V for 13 h and were visualized by staining afterward with ethidium bromide.

## RESULTS

*RPA Stimulates the ssDNA-dependent ATP Hydrolysis Activity of Rad51 Protein*—We first examined the effect of RPA on the ssDNA-dependent ATPase activity of Rad51 protein (Fig. 1). ATP hydrolysis was measured using a spectrophotometric assay that couples ADP production to oxidation of NADH, which results in a decrease in absorbance (25). In the presence of $\phi$X174 ssDNA, Rad51 protein displayed a linear rate of ATP hydrolysis. Subsequent addition of RPA increased the rate of ATP hydrolysis, showing that the ssDNA-dependent ATPase activity of Rad51 protein is stimulated by RPA. In the presence of $\phi$X174 dsDNA, ATP hydrolysis was also increased, but to only ~40% of the level achieved with ssDNA; RPA did not stimulate this activity (Fig. 2). In the absence of DNA, ATP hydrolysis by Rad51 protein was observed above the background level, but the rate was ~10–15-fold lower than in the presence of ssDNA. The addition of RPA had no effect on ATPase activity when ssDNA was absent. Also, the RPA preparation had no ATP hydrolysis activity in either the presence or absence of ssDNA (data not shown; Fig. 2).

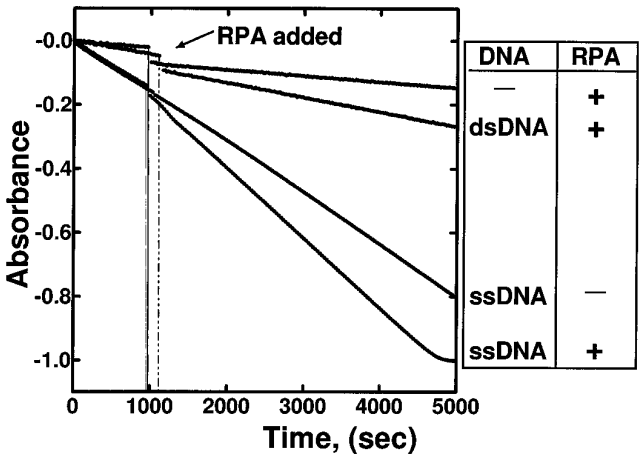


FIG. 1. **RPA stimulates the ssDNA-dependent ATPase activity of Rad51 protein.** ATP hydrolysis was measured in reactions containing Rad51 protein (5.0 $\mu$M) and $\phi$X174 ssDNA or dsDNA (10.3 $\mu$M; nucleotides and base pairs, respectively). Subsequently, either RPA was added to a final concentration of 554 nM, or an equal volume of buffer was added instead. The *arrow* indicates the time of RPA addition.

In Rad51 protein-catalyzed DNA strand exchange, optimal product formation occurs at ~3 nucleotides of ssDNA/Rad51 protein monomer; exceeding this amount of protein results in a strong inhibition (8). To determine the optimal ratio of Rad51 protein to ssDNA for ATPase activity, a Rad51 protein titration at a fixed concentration of $\phi$X174 ssDNA (6.87 $\mu$M) was performed (Fig. 2). The ATP hydrolysis rate reached a plateau value at 4–5 $\mu$M Rad51 protein (1.4–1.7 nucleotides of ssDNA/Rad51 protein monomer), irrespective of the presence or absence of RPA; a similar plateau was obtained with dsDNA. This optimal activity requires approximately twice the amount of protein that is required for optimal DNA strand exchange. RPA (222 nM) stimulated ssDNA-dependent, but not dsDNA-dependent, ATP hydrolysis at the higher Rad51 protein concentrations; interestingly, at the lower Rad51 protein concentrations (0.5 and 1.0 $\mu$M), RPA either had no effect or slightly decreased the ATP hydrolysis rate. Similar behavior was obtained previously for RecA and SSB proteins: when RecA protein was in excess over ssDNA, SSB protein stimulated formation of the RecA protein-ssDNA complex; but when ssDNA was in excess, SSB protein inhibited complex formation (18). Our findings suggest that a comparable relationship exists for Rad51 protein and RPA.

*RPA Inhibits ATPase Activity at Low Concentrations of Rad51 Protein*—To further quantify the effects of RPA on ATPase activity, reactions containing various concentrations of Rad51 protein and a fixed concentration of ssDNA (6.87 $\mu$M) were titrated with RPA. The ATP hydrolysis rate obtained in the presence of RPA relative to that in the absence of RPA was plotted against RPA concentration (Fig. 3). When the Rad51 protein concentration was equal to or higher than 2.5 $\mu$M ($\leq$2.75 nucleotides/Rad51 protein), RPA increased the ATP hydrolysis rate as expected. When the Rad51 protein concentration was 0.5 $\mu$M (13.7 nucleotides/Rad51 protein), ATPase activity was clearly decreased by RPA in a concentration-dependent manner. Therefore, RPA can have either a stimulatory or an inhibitory effect on the ssDNA-dependent ATPase activ-

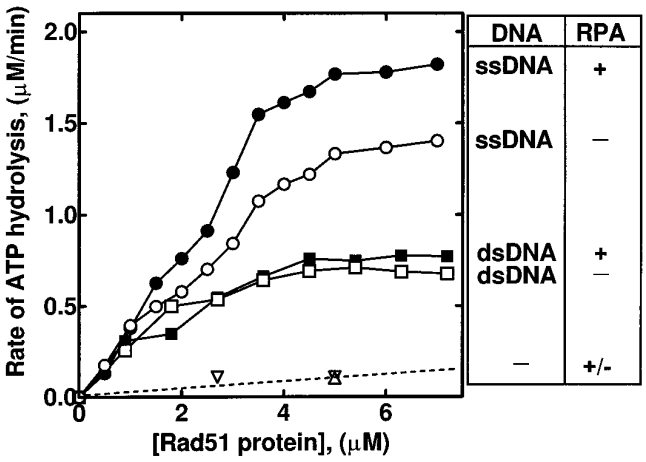


FIG. 2. **RPA stimulates the ssDNA-dependent, but not the dsDNA-dependent, ATPase activity of Rad51 protein.** ATP hydrolysis was measured in reactions containing 6.87 $\mu$M $\phi$X174 ssDNA or dsDNA (nucleotides and base pairs, respectively) and various concentrations of Rad51 protein either in the presence (●, ■) or absence (○, □) of 222 nM RPA. RPA was added after preincubation of Rad51 protein and ssDNA for 15–20 min. DNA-independent ATP hydrolysis by Rad51 protein in the presence (△) or absence (▽) of RPA is also shown.

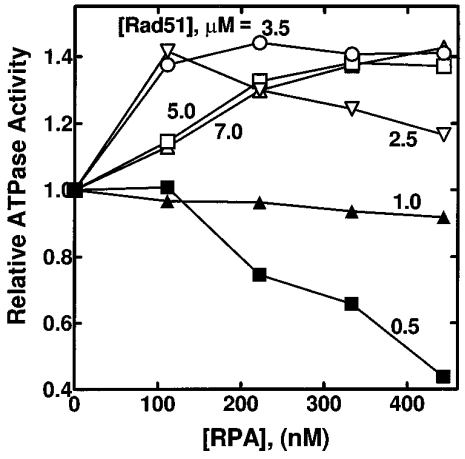


FIG. 3. **Effects of RPA on presynaptic complex formation are competitive with Rad51 protein concentration.** The ATP hydrolysis rates shown in Fig. 2 and other additional experiments were plotted as a function of RPA concentration. The ATP hydrolysis rates are shown relative to the rates obtained without RPA. All reactions contained a fixed amount of $\phi$X174 ssDNA (6.87 $\mu$M; nucleotides) and the following concentrations of Rad51 protein: 0.5 (■), 1.0 (▲), 2.5 (▽), 3.5 (○) 5.0 (□), and 7.0 (△) $\mu$M.

ity of Rad51 protein, depending on the Rad51 protein concentration. This behavior is strikingly similar to the effect of SSB protein on the ATPase activity of RecA protein (18) and suggests that, as for RecA and SSB proteins, Rad51 protein and RPA are competing for ssDNA-binding sites.

All the experiments reported above were performed by the addition of RPA to preformed presynaptic complexes of Rad51 protein and ssDNA. If, instead, Rad51 protein is added to the preformed complexes of RPA and ssDNA, then the ATP hydrolysis rate does not increase instantly, but rather displays a time-dependent increase before achieving a steady-state rate; at steady state, the ATP hydrolysis rate is similar to that obtained in the parallel reaction where RPA is added last.[2] This result further supports the idea that RPA and Rad51 protein bind ssDNA competitively and that Rad51 protein can displace RPA that is bound to ssDNA.

[2] T. Sugiyama and S. C. Kowalczykowski, unpublished observations.

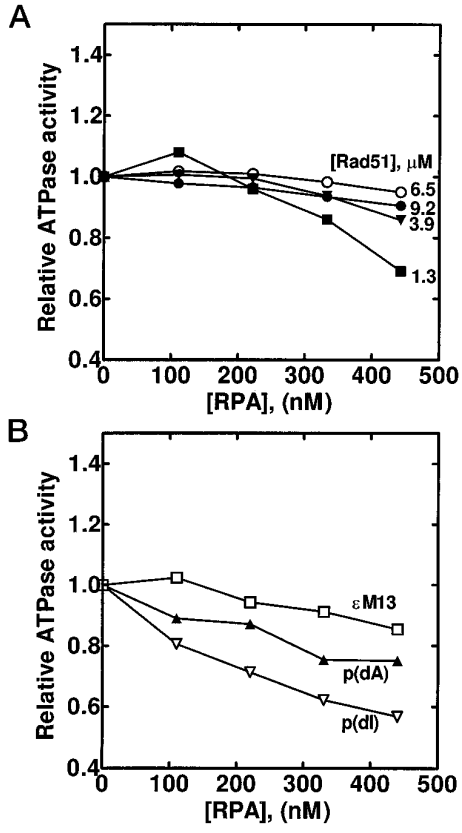


FIG. 4. **RPA does not stimulate the ATPase activity of Rad51 protein in the presence ssDNA devoid of secondary structure.** *A*, Rad51 protein (1.3 (■), 3.9 (▼), 6.5 (○), and 9.2 (●) $\mu$M) was incubated with 8.0 $\mu$M (nucleotides) poly(dT) in reaction buffer, followed by the addition of RPA. ATP hydrolysis was analyzed as described for Fig. 3. The absolute rates of ATP hydrolysis in the absence of RPA were 0.64, 2.10, 2.61, and 2.88 $\mu$M/min for 1.3, 3.9, 6.5, and 9.2 $\mu$M Rad51 protein, respectively. *B*, similar experiments were conducted using 2.5 $\mu$M Rad51 protein and 6.87 $\mu$M (nucleotides) etheno-M13 DNA (*εM13*; □), poly(dA) (*p(dA)*; ▲), and poly(dI) (*p(dI)*; ▽). The absolute rates of ATP hydrolysis in the absence of RPA were 1.45, 1.23, and 1.04 $\mu$M/min for etheno-M13 DNA-, poly(dA)-, and poly(dI)-stimulated reactions, respectively.

*In the Absence of DNA Secondary Structure, RPA Does Not Stimulate the ATPase Activity of Rad51 Protein*—In the *E. coli* system, SSB protein stimulates presynaptic complex formation by eliminating ssDNA secondary structure, which subsequently allows complete RecA protein filament formation (18). However, when ssDNA without secondary structure (*e.g.* poly(dT)) is used, SSB protein does not stimulate, but instead inhibits ATPase activity due to displacement of RecA protein from ssDNA. Therefore, it was of special interest to examine the effect of RPA on the ATPase activity of Rad51 protein stimulated by ssDNA devoid of secondary structure (Fig. 4, *A* and *B*). As expected from the analogy to the RecA and SSB protein system, RPA did not stimulate ATP hydrolysis at any Rad51 protein concentration tested when poly(dT) was used as a DNA cofactor (Fig. 4*A*). Furthermore, there was no stimulation when another ssDNA devoid of secondary structure (poly(dA), poly(dI), and etheno-M13 DNA) was used as the DNA cofactor (Fig. 4*B*). These results confirm the expectation that RPA acts in these reactions by eliminating secondary structure within ssDNA. In fact, RPA inhibited poly(dT)-dependent ATPase activity when the Rad51 protein concentration was low (1.3 $\mu$M; 6.15 nucleotides of ssDNA/Rad51 protein monomer), with less inhibition occurring at higher Rad51 protein concentrations (Fig. 4*A*). Similarly, RPA inhibited the reactions employing poly(dA), poly(dI), and etheno-M13 DNA

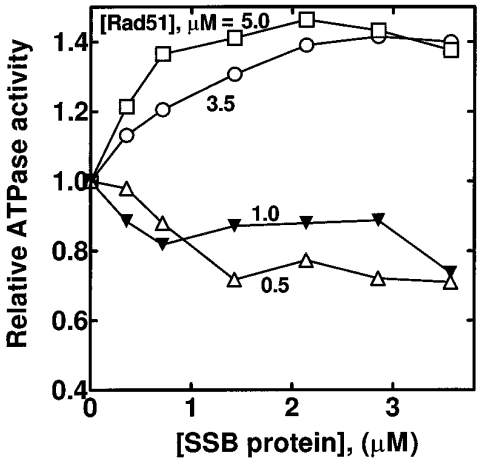


FIG. 5. ***E. coli* SSB protein can substitute for RPA in presynapsis.** Experiments were performed in a manner similar to that described for Fig. 3, except that SSB protein was added instead of RPA. SSB protein was added to reactions containing a fixed concentration of $\phi$X174 ssDNA (6.87 $\mu$M; nucleotides) and the following concentrations of Rad51 protein: 0.5 (△), 1.0 (▼), 3.5 (○), and 5.0 (□) $\mu$M, which were preincubated for 17–20 min.

(Fig. 4*B*). These results are somewhat different from the effect of SSB protein on RecA protein activity, where saturating amounts of SSB protein fully inhibit the ATPase activity of RecA protein irrespective of the RecA protein concentration (18), and suggest that the stability of the Rad51 protein-ssDNA filament relative to RPA is greater than that of the RecA protein filament relative to SSB protein.

*E. coli SSB Protein Can Stimulate the ATPase Activity of Rad51 Protein*—The results presented so far strongly suggest that RPA stimulates presynaptic complex formation of Rad51 protein by a mechanism that is quite similar to that developed for SSB and RecA proteins. Therefore, we examined whether SSB protein could substitute for RPA in these reactions. The addition of SSB protein to reactions containing Rad51 protein and $\phi$X174 ssDNA resulted in the changes shown in Fig. 5. SSB protein stimulated ATP hydrolysis when the Rad51 protein concentration was 3.5 and 5.0 $\mu$M (2.0 and 1.4 nucleotides/Rad51 protein, respectively) and inhibited it at 1.0 and 0.5 $\mu$M (6.9 and 13.7 nucleotides/Rad51 protein, respectively). These data are similar to those obtained for RPA shown in Fig. 3.

*RPA and SSB Protein Can Stimulate DNA Strand Exchange by Rad51 Protein*—Because SSB protein can substitute for RPA in presynaptic complex formation, we expected that SSB protein could substitute for RPA in DNA strand exchange mediated by Rad51 protein. Fig. 6*A* shows the effect of RPA on DNA strand exchange. RPA extensively stimulated formation of nicked circular dsDNA (*OC*; the final product of the DNA strand exchange reaction) and the slower migrating joint molecules (*JM*; intermediates of the exchange reaction), as reported (7, 8). Often we did not detect any product in the absence of RPA; but in other experiments, we could sometimes observe a trace amount (data not shown). This is in contrast to RecA protein-mediated DNA strand exchange, where products can be found in the absence of SSB protein (26). Quantitative analysis of the gel (Fig. 6*C*) shows that stimulation by RPA reached its optimum (~30% of the total DNA) at a ratio of ~20–30 nucleotides of starting ssDNA/RPA heterotrimer.

When SSB protein was used in place of RPA in otherwise identical reactions (Fig. 6*B*), SSB protein increased both joint molecule and nicked circular dsDNA formation. Stimulation by SSB protein reached its optimal level (~20% of the total DNA) at a ratio of ~7–10 nucleotides of starting ssDNA/SSB protein monomer (Fig. 6*C*), which is similar to that required for the

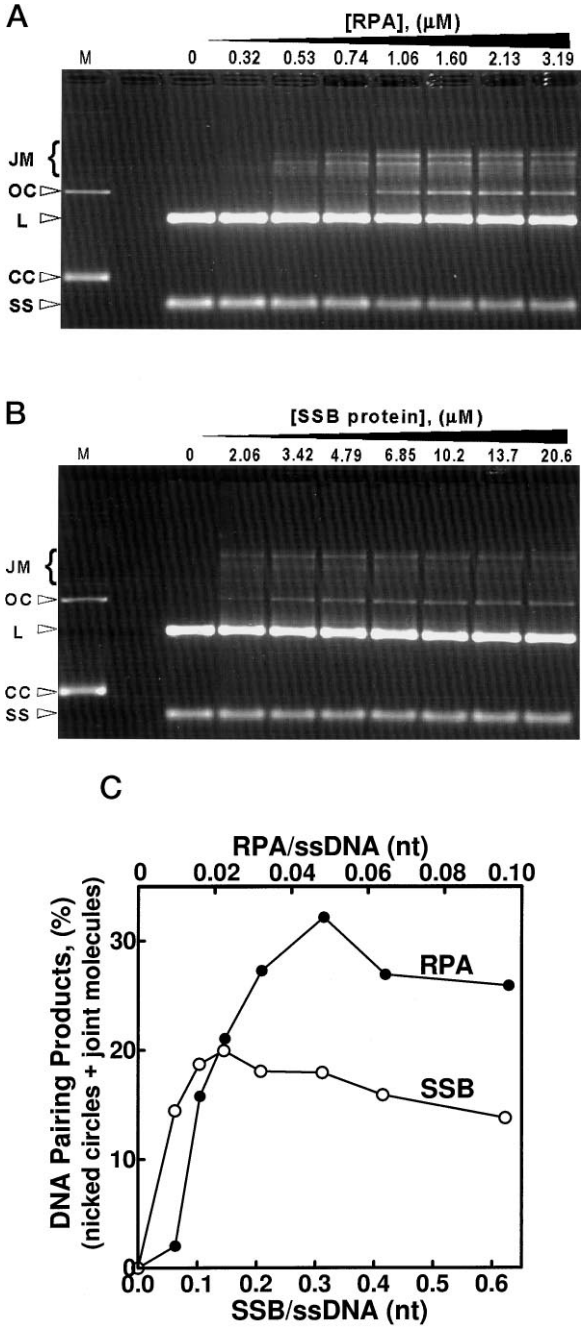


FIG. 6. **RPA and SSB protein stimulate DNA strand exchange promoted by Rad51 protein.** The DNA strand exchange reaction was performed in the presence of RPA (*A*) or SSB protein (*B*). A mixture of covalently closed circular and nicked circular $\phi$X174 ssDNAs in the *first lane* serves as a marker (*M*). The positions of single-stranded (*SS*), covalently closed circular (*CC*), linear double-stranded (*L*), and nicked circular (*OC*) forms of $\phi$X174 DNA as well as the homologously paired joint molecules (*JM*) are indicated. *C* shows the percentage of DNA-pairing products (the sum of nicked circular form and joint molecules) measured from each gel in *A* and *B*. These values are plotted against the molar ratio of the DNA-binding protein to the starting concentration of $\phi$X174 ssDNA. *nt*, nucleotides.

RecA protein-mediated reaction (26). These results indicate that SSB protein can substitute for some of the functions of RPA required for DNA strand exchange mediated by Rad51 protein. They also suggest that no specific protein-protein interaction between Rad51 protein and RPA is required for stimulation of DNA strand exchange catalyzed by Rad51 protein alone. Although the maximum yield of the product formation supported by SSB protein appears to be lower than that sup-

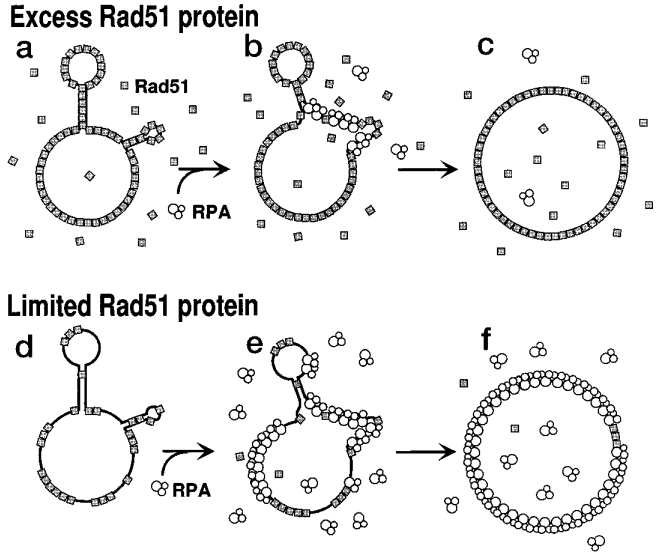


FIG. 7. **Model for the actions of Rad51 protein and RPA in presynaptic complex formation.** Under conditions of excess Rad51 protein (*a–c*), Rad51 protein is shown bound to both single- and double-stranded regions of native ssDNA (*a*). The addition of RPA (*b*) results in some transient displacement of Rad51 protein from DNA and destabilization of duplex regions. With excess Rad51 protein, subsequent displacement of RPA from DNA results in formation of a contiguous presynaptic filament. Under conditions of limiting Rad51 protein (*d–f*), Rad51 protein partitions between single- and double-stranded regions of the DNA (*d*). RPA binds to protein-free regions (*e*); at higher concentrations of RPA, displacement of Rad51 occurs (*f*).

ported by RPA, replicate experiments established that this difference was not significant (data not shown).

## DISCUSSION

As reported by Sung (7), DNA strand exchange catalyzed by Rad51 protein is stimulated by RPA. Here, we show that presynaptic complex formation, as measured by ssDNA-dependent ATP hydrolysis, is also stimulated by RPA. Stimulation of ATPase activity was observed only when the concentration of Rad51 protein exceeded one-fifth of the ssDNA nucleotide concentration and only when the ssDNA contained secondary structure. Based on the assumption that the ATPase activity of Rad51 protein is proportional to the amount of Rad51 protein bound to ssDNA, this suggests that RPA functions by eliminating DNA secondary structure, allowing more Rad51 protein to bind to ssDNA (Fig. 7, *a–c*). In the presence of a limited amount of Rad51 protein, RPA inhibited rather than stimulated ATPase activity, suggesting that RPA and Rad51 protein compete for the same binding sites on ssDNA (Fig. 7, *d–f*); this interpretation is also consistent with more direct physical assays of ssDNA occupancy.[3] The same conclusion was used to explain the effects of SSB protein on the activities of RecA protein (18). In agreement, SSB protein substitutes for the function of RPA in both the DNA strand exchange and ATPase activities of Rad51 protein. Therefore, we conclude that the mechanism of stimulation of Rad51 protein activity by RPA is very similar to that of RecA protein activity by SSB protein.

The finding that SSB protein can substitute for some of the functions of RPA that are required for DNA strand exchange mediated by Rad51 protein argues against the need for a specific protein-protein interaction between Rad51 protein and RPA that is essential to this reaction. Although the genetic evidence for an important role for RPA is compelling (27, 28), these *in vivo* studies most strongly implicate Rad52 protein as the potential target for interaction with RPA. Thus, by virtue of an absence of a specific need for RPA in the reactions described here with Rad51 protein alone, our studies both support the conclusion and suggest further that RPA acts primarily via interaction with a different protein(s) of the *RAD52* group, minimally, Rad52 protein (28).[4]

For all the similarities, there exist some notable differences between the two systems. One obvious difference is the overall rate of reaction. In our experiments, the $k_{cat}$ for $\phi$X174 ssDNA-dependent ATP hydrolysis by Rad51 protein is 0.40–0.44 $min^{-1}$ under optimal conditions and ~0.5 $min^{-1}$ for poly(dT). This value is ~50-fold lower than for RecA protein (18). Also, DNA strand exchange promoted by Rad51 protein is 20–30-fold slower than for RecA protein, and the yield is reduced (26). This difference may simply reflect an intrinsically slower rate for eucaryotic recombination relative to procaryotic recombination; alternatively, it may reflect the need for other stimulatory factor(s) in presynaptic complex formation and/or a later step of DNA strand exchange.

Another difference is the rather stringent requirement for a ssDNA-binding protein in the DNA strand exchange reaction. Rad51 protein catalyzes DNA strand exchange very poorly when a ssDNA-binding protein is absent. In contrast, RecA protein catalyzes the same reaction in the absence of SSB protein at approximately half the efficiency of the reaction in the presence of SSB protein (26). A possible explanation for this severe requirement for a ssDNA-binding protein may stem from the fact that Rad51 protein can bind to dsDNA as well as to ssDNA (2).[5] In the absence of a ssDNA-binding protein, Rad51 protein will bind to both the single- and double-stranded regions of native ssDNA (Fig. 7*a*). The Rad51 protein filament formed on the base-paired regions of the DNA might inhibit DNA strand exchange. Therefore, removal of Rad51 protein from the double-stranded regions by RPA and its replacement by a uniform Rad51 protein-ssDNA complex might be critical for efficient DNA strand exchange *in vitro*. Thus, the nonproductive binding of Rad51 protein to these regions of DNA secondary structure, the stabilization of these dsDNA regions, or an instability of the resultant discontinuous presynaptic filament could explain the poor DNA strand exchange activity in the absence of a ssDNA-binding protein.

Our results also suggest a potential second function for RPA. Maximal ATPase activity (reflecting optimal presynaptic complex formation) required approximately one RPA heterotrimer/70 nucleotides of ssDNA; however, optimal DNA strand exchange requires twice as much RPA (one RPA heterotrimer/~20–30 nucleotides of ssDNA). This suggests that there is another role of RPA in DNA strand exchange other than to enhance formation of the presynaptic complex. This proposed additional function is unknown, but it might be a postsynaptic role similar to that established for SSB protein in RecA protein-promoted DNA strand exchange (14, 15). In this second capacity, SSB protein binds to and prevents reinvasion of the displaced DNA strand in RecA protein-catalyzed DNA strand exchange.

Alani *et al.* (23) reported that the DNA binding stoichiometry of RPA to ssDNA (or "site size") is 80–100 nucleotides/RPA heterotrimer. Based on this value, more RPA than is needed to saturate the ssDNA is required for optimal stimulation of both ATPase and DNA strand exchange activities. We also analyzed the ssDNA binding characteristics of our RPA preparation by fluorescence quenching, under the conditions of both our DNA

[3] T. Sugiyama, N. Kantake, and S. C. Kowalczykowski, unpublished observations.

[4] A. Shinohara, M. Shinohara, and T. Ogawa, submitted for publication.

[5] E. M. Zaitseva and S. C. Kowalczykowski, unpublished observations.

strand exchange reactions and the previously reported RPA-ssDNA binding reaction (23). Under both conditions, the site size of our RPA preparation was 20–30 nucleotides, and the magnitude of the quenching indicated that at least 70% of our RPA was active in DNA binding.[2] We cannot specify the reason for the different observed site size of RPA; post-translational modification related to phosphorylation of RPA subunits could provide an explanation (29).

Finally, the facile binding of Rad51 protein to dsDNA differentiates it from RecA protein and offers an explanation for its relatively poor DNA strand exchange activity. Optimal ATPase activity occurs at ~1.5 nucleotides of ssDNA/Rad51 protein monomer; this ratio is essentially the same at different RPA concentrations (Fig. 2).[2] However, optimal DNA strand exchange occurred at a lower Rad51 protein concentration, corresponding to ~3 nucleotides of ssDNA/protein monomer (8).[2] At the ratio of Rad51 protein to ssDNA optimal for ATP hydrolysis, DNA strand exchange is strongly inhibited. One way to reconcile these different optima is to propose that because of the relatively strong binding of Rad51 protein to dsDNA, Rad51 protein partitions between both the ssDNA and dsDNA present in DNA strand exchange reactions. Consequently, optimal DNA strand exchange is a compromise between the inhibitory consequences of incomplete presynaptic complex formation relative to inhibition due to dsDNA binding. Incomplete presynaptic complex formation would also explain why product formation in the DNA strand exchange reaction is limited to only 30% of starting DNA. An alternative explanation for the differing optimal ratios is to suggest that the active complex for DNA strand exchange has a structure different from that needed for ATP hydrolysis. Further analysis of this system and of the effects of other Rad proteins should help elucidate the precise function of these proteins in recombination.

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REFERENCES

1. Petes, T. D., Malone, R. E., and Symington, L. S. (1991) in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis, and Energetics* (Broach, J. R., Jones, E., and Pringle, J., eds) Vol. I, pp. 407, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
2. Shinohara, A., Ogawa, H., and Ogawa, T. (1992) *Cell* **69,** 457–470
3. Heyer, W.-D. (1994) *Experientia* (*Basel*) **50,** 223–233
4. Aboussekhra, A., Chanet, R., Adjiri, A., and Fabre, F. (1992) *Mol. Cell. Biol.* **12,** 3224–3234
5. Basile, G., Aker, M., and Mortimer, R. K. (1992) *Mol. Cell. Biol.* **12,** 3235–3246
6. Ogawa, T., Yu, X., Shinohara, A., and Egelman, E. H. (1993) *Science* **259,** 1896–1899
7. Sung, P. (1994) *Science* **265,** 1241–1243
8. Sung, P., and Robberson, D. L. (1995) *Cell* **82,** 453–461
9. Radding, C. M. (1988) in *Genetic Recombination* (Kucherlapati, R., and Smith, G. R., eds), pp. 193–229, American Society for Microbiology, Washington, D. C.
10. West, S. C. (1992) *Annu. Rev. Biochem.* **61,** 603–640
11. Cox, M. M. (1993) *Bioessays* **15,** 617–623
12. Kowalczykowski, S. C., Dixon, D. A., Eggleston, A. K., Lauder, S. D., and Rehrauer, W. M. (1994) *Microbiol. Rev.* **58,** 401–465
13. Kowalczykowski, S. C., and Eggleston, A. K. (1994) *Annu. Rev. Biochem.* **63,** 991–1043
14. Chow, S. A., Rao, B. J., and Radding, C. M. (1988) *J. Biol. Chem.* **263,** 200–209
15. Lavery, P. E., and Kowalczykowski, S. C. (1992) *J. Biol. Chem.* **267,** 9315–9320
16. Muniyappa, K., Shaner, S. L., Tsang, S. S., and Radding, C. M. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 2757–2761
17. Kowalczykowski, S. C., Clow, J. C., Somani, R., and Varghese, A. (1987) *J. Mol. Biol.* **193,** 81–95
18. Kowalczykowski, S. C., and Krupp, R. A. (1987) *J. Mol. Biol.* **193,** 97–113
19. Brill, S. J., and Stillman, B. (1989) *Nature* **342,** 92–95
20. Heyer, W.-D., Rao, M. R. S., Erdile, L. F., Kelly, T. J., and Kolodner, R. D. (1990) *EMBO J.* **9,** 2321–2329
21. Brill, S. J., and Stillman, B. (1991) *Genes Dev.* **5,** 1589–1600
22. Menetski, J. P., and Kowalczykowski, S. C. (1985) *J. Mol. Biol.* **181,** 281–295
23. Alani, E., Thresher, R., Griffith, J. D., and Kolodner, R. D. (1992) *J. Mol. Biol.* **227,** 54–71
24. LeBowitz, J. (1985) *Biochemical Mechanism of Strand Initiation in Bacteriophage Lambda DNA Replication.* Ph.D. Thesis, Johns Hopkins University, Baltimore, MD
25. Kreuzer, K. N., and Jongeneel, C. V. (1983) *Methods Enzymol.* **100,** 144–160
26. Cox, M. M., and Lehman, I. R. (1982) *J. Biol. Chem.* **257,** 8523–8532
27. Firmenich, A. A., Elias-Arnanz, M., and Berg, P. (1995) *Mol. Cell. Biol.* **15,** 1620–1631
28. Park, M. S., Ludwig, D. L., Stigger, E., and Lee, S.-H. (1996) *J. Biol. Chem.* **271,** 18996–19000
29. Din, S., Brill, S. J., Fairman, M. P., and Stillman, B. (1990) *Genes Dev.* **4,** 968–977