

The Rad51-dependent Pairing of Long DNA Substrates Is Stabilized by Replication Protein A*

Received for publication, May 2, 2002, and in revised form, August 5, 2002
Published, JBC Papers in Press, August 6, 2002, DOI 10.1074/jbc.M204328200

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Rad51 protein forms nucleoprotein filaments on single-stranded DNA (ssDNA) and then pairs that DNA with the complementary strand of incoming duplex DNA. In apparent contrast with published results, we demonstrate that Rad51 protein promotes an extensive pairing of long homologous DNAs in the absence of replication protein A. This pairing exists only within the Rad51 filament; it was previously undetected because it is lost upon deproteinization. We further demonstrate that RPA has a critical postsynaptic role in DNA strand exchange, stabilizing the DNA pairing initiated by Rad51 protein. Stabilization of the Rad51-generated DNA pairing intermediates can occur either by binding the displaced strand with RPA or by degrading the same DNA strand using exonuclease VII. The optimal conditions for Rad51-mediated DNA strand exchange used here minimize the secondary structure in single-stranded DNA, minimizing the established presynaptic role of RPA in facilitating Rad51 filament formation. We verify that RPA has little effect on Rad51 filament formation under these conditions, assigning the dramatic stimulation of strand exchange nevertheless afforded by RPA to its postsynaptic function of removing the displaced DNA strand from Rad51 filaments.

Homologous genetic recombination is an integral feature of DNA metabolism in all organisms. Functions include the repair of replication forks halted at DNA barriers such as DNA lesions and breaks and the repair of double-strand breaks in DNA arising from nonreplication sources. Central steps in these processes are carried out by recombinases such as the bacterial RecA protein or the eukaryotic Rad51 protein. These proteins bind first to single-stranded DNA within a gap or a DNA terminal extension, forming an extended nucleoprotein filament. The filament then initiates a search for homologous double-stranded DNA (dsDNA)¹ and pairs the bound single-stranded DNA with the complementary strand of the incoming duplex in a process known as DNA strand exchange. Synapsis is the point at which homologous alignment of the two DNAs is

achieved, and reactions are often divided into presynaptic (nucleoprotein filament formation) and postsynaptic (extension of the paired DNA segment) phases. The Rad51 protein of *Saccharomyces cerevisiae* promotes a very efficient DNA strand exchange reaction under the right reaction conditions (1, 2).

Single-stranded DNA-binding proteins, generally referred to as SSBs, have a multitude of roles in DNA metabolism. As part of their role in genetic recombination, SSBs stimulate recombinase-mediated *in vitro* DNA strand exchange reactions. The SSB of *S. cerevisiae* is replication protein A, or RPA. RPA has been implicated in the recombination pathway genetically (3–7) and through its physical interaction with recombination protein Rad52 (8). Although RPA stimulates most *in vitro* DNA strand exchange reactions promoted by the Rad51 protein, RPA is not required for Rad51-mediated DNA strand exchange in reactions with oligonucleotides (2, 9). When much longer DNA derived from bacteriophages is used, formation of exchanged products of any kind is strongly dependent on RPA (10–12).

The importance of both presynaptic and postsynaptic roles for an SSB in recombinase-mediated strand exchange is illustrated by work on *Escherichia coli* recombination proteins. The SSB of *E. coli*, when prebound to ssDNA, inhibits the nucleation stage of RecA protein filament formation (13). However, once successful nucleation of RecA occurs on the DNA (via binding to a region vacated by SSB, the action of the RecOR mediator proteins, or simply adding RecA before SSB), the role of SSB becomes that of a presynaptic facilitator (14). SSB binds to and helps denature regions of secondary structure in the ssDNA that would otherwise hinder RecA filament extension and is then displaced by the growing RecA filament. SSB thus permits the formation of a contiguous extended filament on the DNA. Furthermore, upon the initiation of DNA strand exchange, the *E. coli* SSB facilitates the postsynaptic phase of the reaction by binding to the displaced DNA strand (15). In the model proposed by Lavery and Kowalczykowski (15), the binding of SSB to the displaced strand can prevent a reverse branch migration reaction and prevent reinitiation of exchange by the displaced strand. Kodadek was the first to propose a postsynaptic role for an SSB during the strand exchange reaction, focusing on the protein homologues in the T4 bacteriophage system (16). Recent work with the *Streptococcus pneumoniae* RecA and its cognate SSB demonstrates that SSB simultaneously inhibits presynaptic filament formation and strongly stimulates strand exchange, suggesting that, for this system at least, the postsynaptic role is a major one (17).

In the case of RPA, work published to date has focused on presynaptic roles in which RPA facilitates the formation of complete Rad51 filaments on ssDNA through removal of DNA secondary structure (18–20). Kowalczykowski and co-workers demonstrated that RPA stimulates Rad51 binding to ssDNA unless the ssDNA is devoid of secondary structure (10). Addi-

* This work was supported by National Institutes of Health Grants GM32335 (to M. M. C.) and GM14711–34 (to R. B. I.). 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.

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¹ The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; SSBs, single-stranded DNA-binding proteins; RPA, replication protein A; cssDNA, circular single-stranded DNA; ldsDNA, linear double-stranded DNA; AMT, amino-4,5',8-trimethylporalen; ExoVII, exonuclease VII; MOPS, 4-morpholinepropanesulfonic acid.

tionally, they noted that optimal strand exchange in their system requires twice as much RPA as that required to stimulate maximal ATPase activity and suggested that RPA may be acting postsynaptically.

The alignment of homologous DNAs during DNA strand exchange does not always result in the formation of exchanged products that survive deproteinization. A pairing structure that is completely dependent upon the continued presence of recombinase, referred to here as an enforced pairing, has been described previously. The *E. coli* RecA protein can mediate the formation of paranemic joints, or joints that are formed away from a DNA end, that extend for thousands of base pairs (21, 22). Because a free end is not available to allow interwinding of the paired strands, the paranemic joints are not stable when deproteinized. Human Rad51 can also form paranemic joints, but this reaction has only been demonstrated with oligonucleotides with heterology at the ends (23). A second type of enforced pairing has been demonstrated for human Rad51 using GC-rich oligonucleotides. Rad51 will align oligonucleotide substrates that have a 40% GC content but will not complete the exchange reaction (23).

In this work we demonstrate that the Rad51 protein does efficiently and extensively pair ϕ X174 substrates without the assistance of RPA. This pairing, however, is unstable once Rad51 protein is removed. The pairing survives deproteinization if RPA is present. The RPA-independent but Rad51-enforced pairing can alternatively be stabilized via degradation of the strand displaced during strand exchange by a single-strand exonuclease. This indicates that RPA stabilizes Rad51-mediated pairing of long substrates through sequestration of the displaced strand. The significance of this postsynaptic role is demonstrated by performing reactions under conditions that include a low magnesium concentration, which coincidentally are the optimal reaction conditions for Rad51-mediated DNA strand exchange. These conditions minimize the necessity of secondary structure removal by RPA and, thus, the presynaptic role of RPA. Under conditions where RPA addition does little to stimulate presynaptic filament formation, RPA still strongly stimulates the formation of stably paired DNA molecules. The work indicates that RPA has a critical postsynaptic role in Rad51-mediated DNA strand exchange.

EXPERIMENTAL PROCEDURES

Enzymes and Biochemicals—The yeast Rad51 protein was purified from the yeast strain LP2749-9B harboring plasmid pR51.3, which was provided by Patrick Sung (University of Texas Health Sciences Center, San Antonio, Texas (1)). Briefly, an extract from LP2749-9B was centrifuged at 45,000 rpm for 90 min. The supernatant was fractionated by ammonium sulfate precipitation to give fraction II. Fraction II was then subjected to chromatographic fractionation using Q Fast Flow, hydroxyapatite, and Mono Q columns to yield purified Rad51 protein. The final preparations were judged to be greater than 98% pure by Coomassie Blue staining of a 10% denaturing polyacrylamide gel. Rad51 protein was stored in 25 mM Tris-HCl at pH 7.5 containing 0.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 400 mM KCl. RPA was expressed in *E. coli* strain BL21 (DE3) transformed with plasmid JM126, which simultaneously expresses all three subunits of this factor (24). The purification procedure employed has been described (11), except the ssDNA cellulose column was replaced with a Source Q column. RPA was stored in 25 mM Tris-HCl at pH 7.5 containing 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 200 mM KCl. The concentrations of each protein were determined by UV absorption at 280 nm using the extinction coefficients of $1.29 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for Rad51 and $8.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for RPA (10).

The Q fast flow, Mono Q, and Source Q columns were from Amersham Biosciences. The Macro-Prep ceramic hydroxyapatite column and the Affi-Gel blue column were from Bio-Rad. Exonuclease VII (ExoVII) was purchased from U. S. Biochemical Corp. The *Pst*I, *Not*I, and *Stu*I endonucleases and the buffers used with *Stu*I and *Not*I were from New England Biolabs, and Buffer H used in the reaction with *Pst*I was purchased from Promega. T4 polynucleotide kinase and its buffer were

purchased from New England Biolabs, and [γ - 32 P]ATP was purchased from Amersham Biosciences. Proteinase K, lactic dehydrogenase, pyruvate kinase, phosphoenolpyruvate, nicotinamide adenine dinucleotide (reduced form), amino-4,5',8-trimethylpsoralen (AMT), ATP, MOPS, HEPES, glycerol, spermidine trihydrochloride, and bromophenol blue were purchased from Sigma. Proteinase K was incubated for 30 min at 37 °C before use to remove nucleases. Uranyl acetate was from Ladd Co. EDTA, ammonium acetate, magnesium chloride, SDS, and light mineral oil were purchased from Fisher. Light mineral oil was extracted with MilliQ purified water before use. Dithiothreitol was purchased from Research Organics Inc. Xylene cyanol FF was purchased from Eastman Kodak Co.

DNA Substrates—The following oligonucleotides were purchased from Operon Technologies in polyacrylamide gel electrophoresis-purified form: A, AGTAGACTCAGCGAACTCACTGATCCAGTCTTAGCATCAGTCACGATACCTCGAGATACATACGGACGTA; B, TGATCCAGTCTTAGCATCAGTCACGATACCTCGAGATAC; C, GTATCTCGAGGTATCGTGACTGATGCTAAGACTGGATCA. Lyophilized oligonucleotides were resuspended in TE (10 mM Tris-Cl (80% cation), 1 mM EDTA (pH 8.0)) and stored at -20 °C. Concentrations of single-stranded oligonucleotides were determined by UV absorption at 260 nm using the extinction coefficients of $691.9 \text{ mM}^{-1} \text{ cm}^{-1}$ for A, $375.4 \text{ mM}^{-1} \text{ cm}^{-1}$ for B, and $385.3 \text{ mM}^{-1} \text{ cm}^{-1}$ for C. The 39-mer oligonucleotides were 5'- 32 P-labeled and annealed as previously described without further gel purification (2).

Circular single-stranded ϕ X174 virion DNA (5386 bases in length) was purchased from New England Biolabs. M13mp8 cssDNA was prepared as described (25). Concentrations of ssDNAs were determined by UV absorption at 260 nm using the extinction coefficient, $9.03 \text{ mM}^{-1} \text{ cm}^{-1}$. Heterologous DNA for strand exchange controls was generated using the plasmid pBII2, which is the pBluescript II vector (Stratagene) modified to include a 1,427-bp insert in the *Eco*RI site to yield a DNA that is 4388 bp in length. The insert contains the sequence of the *Caenorhabditis remanei tra2* gene (GenBank™ number AF187965) from the 2123 *Eco*RI site to the 3550 *Eco*RI site (26). The pBII2 plasmid DNA was linearized using *Not*I. Nicked circular ϕ X174 DNA was purchased from New England Biolabs. Linear double-stranded ϕ X174 DNA was generated from a restriction digestion of ϕ X174 replicative form I DNA (from Invitrogen) with either *Pst*I (producing four nucleotide 3' overhangs) or *Stu*I (producing blunt ends). Double-stranded DNA concentrations were determined by UV absorption at 260 nm using the extinction coefficient, $6.50 \text{ mM}^{-1} \text{ cm}^{-1}$. All DNA concentrations are given in terms of total nucleotides.

Rad51-facilitated Three-strand DNA Exchange of ϕ X174 DNA Substrates—Standard three-strand DNA exchange reactions with ϕ X174 DNA substrates were carried out in 35 mM K-MOPS buffer (pH 7.2) with 2 mM ATP, 2.4 mM MgCl_2 , and 1 mM dithiothreitol at 38 °C. Reactions included 20.8 μM cssDNA substrate, 6.8 μM Rad51 protein, 0.6 μM RPA, 20.8 μM *Pst*I-cut lds ϕ X174 DNA, and 4 mM spermidine, all final concentrations. A preincubation mixture was assembled, including the cssDNA and Rad51 protein with the buffer and ATP in a volume of 9 μl . After 5 min at 38 °C, the RPA was added in a 1.5 μl aliquot. RPA storage buffer is substituted for RPA where noted. After another 10 min, the lds ϕ X174 DNA and spermidine were introduced to initiate strand exchange, bringing the final reaction volume to 12.5 μl . Reactions were scaled up as required. For the strand exchange time courses, 12.5- μl aliquots of larger reaction mixtures were removed at the indicated times and stopped with the addition of proteinase K to 0.4 mg/ml, SDS to 1%, and EDTA to 7 mM in a final volume of 15.6 μl . Deproteinization was carried out for 30 min at 38 °C.

Reaction samples were subjected to electrophoresis on 1% agarose gels in $1 \times$ TAE buffer (40 mM Tris acetate (80% cation), 1 mM EDTA (pH 8.0)) at 27 V, constant voltage, for ~16 h. The substrate, product, and joint molecule bands were distinguishable after staining with ethidium bromide and exposure to ultraviolet light. Images were captured with a digital CCD camera using the GelExpert software (Nucleotech). Reaction progress was calculated either as the DNA present as joint molecules plus nicked-circular DNA product or, in some cases, as the nicked circular DNA product alone as a percentage of the total duplex DNA present in a gel lane. Band intensities were quantitated with the TotalLab software (Phoretix).

Rad51-promoted Three-strand DNA Exchange of Oligonucleotide Substrates—Reactions with oligonucleotides were carried out under the same solution conditions as the basic reaction described above. The reactions contained 2 mM ATP, 2.4 mM MgCl_2 , 37.3 μM 70-mer ssDNA substrate, 13.6 μM Rad51 protein, 0.6 μM RPA, and 20.8 μM 32 P-labeled 39-mer dsDNA substrate (in terms of molecular concentration, the single-stranded oligonucleotides are in 2-fold excess to the duplex sub-

strate). In controls the appropriate storage buffer was substituted for Rad51 or RPA proteins. Mixtures containing buffer, ATP, MgCl₂, Rad51 protein, and the 70-mer oligonucleotide were preincubated at 38 °C for 5 min. RPA was then added followed by a second incubation for 5 min. The reaction was then initiated by the addition of the duplex 39-mer substrate and spermidine to 4 mM. Aliquots of 9 μ l were removed at the indicated time points and stopped with the addition of EDTA and SDS to final concentrations of 28 mM and 1.4%, respectively, in a final volume of 14 μ l. A gel-loading buffer (2.5% Ficoll (type 400), 0.08% bromophenol blue, 0.08% xylene cyanol FF, all final concentrations) was then added to each stopped aliquot followed by electrophoresis on 10% acrylamide, 1 \times Tris/boric acid/EDTA gels. Bands were visualized by exposing PhosphorImager screens (Molecular Dynamics) for 30 min and scanning with a Molecular Dynamics PhosphorImager (model 425E).

ATP Hydrolysis Assays—ATP hydrolysis activity was measured using a coupled enzyme assay described previously (27, 28). To have smaller reaction volumes, the protocol was modified to use cuvettes that held 65 μ l, with a 1-cm path length, and light mineral oil was added to prevent evaporation of the sample during the assay. The reaction was the same as the basic reaction described for the three-strand exchange reaction with ϕ X174 DNA except that each included 8.4 units/ μ l pyruvate kinase, 8.4 units/ μ l lactate dehydrogenase, 1.5 mM phosphoenolpyruvate, and 1.5 mM NADH to couple ATP hydrolysis to the oxidation of NADH. Reactions were initiated in a test tube by preincubating Rad51 with either ϕ X174 or M13mp8 *css*DNA. After 5 min at 38 °C, either RPA or RPA storage buffer was added. The reactions were then transferred to pre-warmed cuvettes and covered with a layer of mineral oil, and absorbance data were collected at 38 °C at 380 nm. A background reading was obtained by substituting Tris-EDTA for DNA, and this background was subtracted from the raw data to obtain the final data set. An NADH extinction coefficient of 1.21 mM cm⁻¹ was used to determine the rate of loss of NADH by conversion to NAD, which is equivalent to the conversion of ATP to ADP.

Electron Microscopy of Rad51 Protein-ssDNA Filaments—Reaction conditions were the same as described for the basic three-strand exchange reaction with ϕ X174 DNA, except that the incubations with RPA or RPA storage buffer were extended to 20 min to ensure stable filament formation. After the incubation with RPA or RPA storage buffer, reaction mixtures were diluted 200 times with 41.7 mM K-MOPS (pH 7.2), 2.9 mM MgCl₂, 67 mM KCl, and 2.4 mM ATP and then immediately adsorbed to a glow discharge activated carbon film attached to an electron microscope grid. The grid was then touched to the surface of a drop of the same solution for 1 min. The sample was washed by touching the grid to the surface of a drop containing 20 mM ammonium acetate, 1 mM HEPES (pH 7.0) and 5% (w/v) glycerol followed by floating the grid for 1 min on a drop of the same solution. The sample was then stained by touching and floating on drops containing 5% uranyl acetate and 5% glycerol for 30 s. Finally the carbon surface was washed by floating on a drop of 5% glycerol for 5 s. The grid was then picked up with cleaned forceps and sequentially submerged in two beakers of water and one beaker of ethanol and finally dried under a heat lamp for 5 min.

This protocol is designed for visualization of complete reaction mixtures, and no attempt was made to remove unreacted material. Although this approach should yield results that give a true insight into reaction components, it does lead to samples with a high background of unreacted proteins.

Rad51 filament lengths were measured by a procedure described previously for RecA filaments (29). Circular filaments formed in the absence of RPA were measured in two ways, by including the length of the protrusions (nibs) by measuring up and down the nib and by ignoring the nibs. Circular filaments formed in the presence of RPA usually possessed gaps. For these structures, only regions that were obviously filamented were included in the measurement.

Cross-linking Strand Exchange Reactions with ϕ X174 DNA Substrates—The reaction conditions were the same as for the basic reaction described for the three-strand exchange reaction with ϕ X174 DNA, with reactions scaled up as needed for the number of samples planned. Where indicated, heterologous pBIIIt2 linear dsDNA (ldsDNA) was substituted for homologous ldsDNA. At the indicated times, two 12.5- μ l aliquots were removed. One aliquot was deproteinized immediately with proteinase K, SDS, and EDTA as described above for the basic reaction with ϕ X174 DNA. The other aliquot was subjected to cross-linking of the DNA essentially as described (22). Briefly, AMT was added to the aliquot in 1 μ l to yield a final concentration of 23 μ g/ml. The sample was then irradiated under ultraviolet light at an intensity of \sim 6 mW cm⁻² for 5 min and then deproteinized as described above. Samples were analyzed by agarose gel electrophoresis, and the reac-

tions were quantified as described for the basic three-strand exchange reaction with ϕ X174 DNA.

Some samples were also analyzed by electron microscopy. These were treated as above through the deproteinization step, then dialyzed against 20 mM NaCl and 5 mM EDTA for 5 h at room temperature on Millipore type VM (0.05 mm) filters (29). They were then spread for electron microscopy as described previously (30). Photography and measurement of the DNA molecules were performed as described previously (31). The length of hybrid DNA generated for a representative sample of intermediates was estimated. Because of the large numbers of samples, obtaining accurate measurements of significant numbers of intermediates in all of them was impractical; we have therefore estimated the degree of exchange as described earlier (29). As an internal standard, the entire length of the double-stranded DNA region was judged. The standard deviation resulting from these measurements was low (average length = 5.4 \pm 0.1 kilobase pairs), as shown by the data denoted by the *open bars* of Fig. 5D.

The level of cross-linking used was determined to result in one cross-link approximately every 150 base pairs on average. This determination was made by cross-linking ϕ X174 ldsDNA at a concentration of 29 μ M with various concentrations of AMT and denaturing the cross-linked molecules as described previously (32). The number of cross-links per molecule was counted and plotted *versus* the AMT concentration to generate an equation for calculating the number of cross-links per molecule at a given AMT concentration and degree of exposure.

Stimulation of DNA Exchange of ϕ X174 DNA Substrates by Exonuclease VII—The reaction conditions were the same as those used in the basic reaction described for three-strand exchange with ϕ X174 DNA, with the following changes. The preincubation mixtures contained the Rad51 protein (or Rad51 storage buffer in control reactions lacking protein) plus *css*DNA. After a 10-min incubation at 38 °C, reactions were initiated by simultaneous addition of spermidine, ϕ X174 *Pst*I-cut ldsDNA, either RPA or RPA storage buffer, and either ExoVII to 0.08 units/ μ l or ExoVII storage buffer. Incubations were continued at 38 °C to allow strand exchange. At the indicated time points, reactions were deproteinized, analyzed by agarose gel electrophoresis, and quantitated as described for the basic reaction. Alternatively, some samples were examined by electron microscopy as described above for the cross-linking experiments.

RESULTS

Experimental Rationale—The goal of these experiments was to evaluate the postsynaptic role of RPA in Rad51 protein-mediated DNA strand exchange reactions. To this end, the experiments were performed at a low magnesium concentration, as developed by the Sung laboratory during optimization of the Rad51-mediated strand exchange reaction with ϕ X174 DNA substrates.² Free magnesium ion in excess of the ATP concentration was only 0.4 mM. Little secondary structure may form in ssDNA under these conditions, so that RPA might not be needed to facilitate presynaptic Rad51 filament formation. This would allow us to distinguish between the effects of RPA on the presynaptic and postsynaptic phases of Rad51-mediated DNA strand exchange. In addition, the ratio of ssDNA to Rad51 protein is kept to three nucleotides/monomer to minimize the possibility that any observed effects of RPA could instead reflect an activity of excess Rad51 protein.

RPA Strongly Stimulates the DNA Strand Exchange Reaction with ϕ X174 Substrates—The *in vitro* strand exchange reaction illustrated in Fig. 1A has been used extensively to monitor the recombinase activity of proteins such as the RecA protein from *E. coli* and the Rad51 protein from *S. cerevisiae*. The initial pairing of the circular ssDNA with the linear dsDNA (ldsDNA) results in a reaction intermediate called a joint molecule. Complete exchange throughout the length of the ldsDNA leads to formation of a nicked circular DNA and a linear ssDNA product. The reactions are analyzed by agarose gel electrophoresis after deproteinization of the DNA. When long substrates such as ϕ X174 DNA (5386 nucleotides/base

² P. Sung, personal communication.

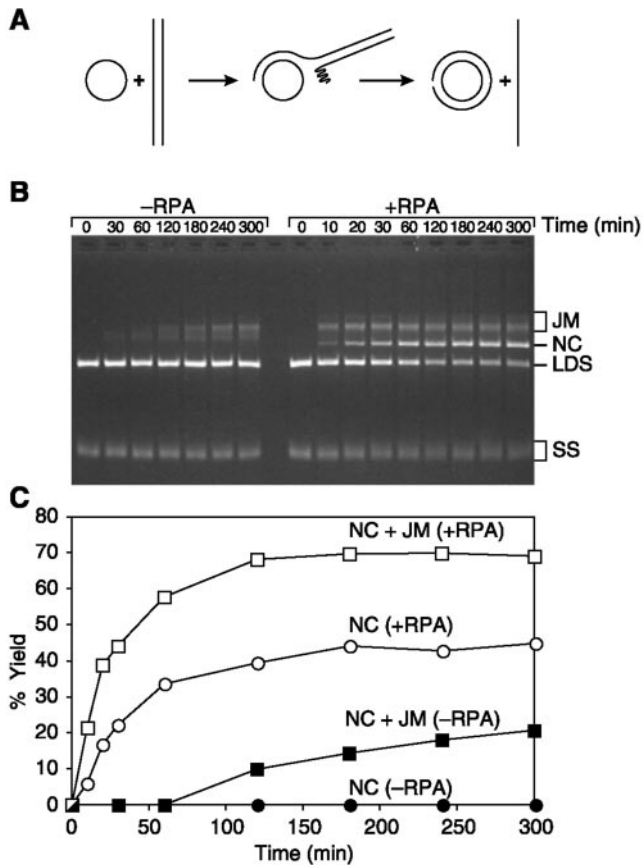


FIG. 1. RPA strongly stimulates the DNA strand exchange reaction with ϕ X174 substrates. *A*, an illustration of the *in vitro* three-strand exchange reaction. *B*, time courses of reactions in which either RPA ($0.6 \mu\text{M}$) or RPA storage buffer was added after preincubation of Rad51 protein with cssDNA. Reactions contained $6.8 \mu\text{M}$ Rad51 protein, $20.8 \mu\text{M}$ cssDNA, and $20.8 \mu\text{M}$ ldsDNA so that the ssDNA was in 2 to 1 molecular excess relative to the dsDNA. The labels are: *NC*, nicked circular DNA (products); *JM*, joint molecules (intermediates); *LDS*, linear double-stranded DNA (substrate); *SS* (circular and linear single-stranded DNA). The generation of total reacted product (*JM* and *NC*, squares) and fully completed product only (*NC*, circles) for these reactions are plotted in *panel C*. *Open* and *closed* symbols represent reactions with and without added RPA, respectively.

pairs in length) are used, the reaction proceeds quite efficiently, but only if RPA is included (Fig. 1*B*). A very low level of joint molecule formation can be detected when RPA is omitted, but only after 2 or more h of reaction. Almost no final product is detected even after 5 h.

DNA Strand Exchange with Oligonucleotide Substrates Is Slightly Stimulated by RPA—A second type of *in vitro* strand exchange reaction used to monitor recombinase activity is carried out with oligonucleotide substrates. Fig. 2*A* illustrates a typical reaction, with a 70-mer single-strand oligonucleotide and a 39-mer duplex substrate. Because both RecA and Rad51 catalyze this reaction efficiently in the absence of an SSB, we investigated what effect RPA would have on the Rad51 reaction. RPA modestly stimulates the already robust oligonucleotide reaction (Fig. 2, *B* and *C*).

RPA Does Not Significantly Increase the Length of Rad51 Nucleoprotein Filaments Formed on ssDNA—To evaluate how successful we were in minimizing RPA presynaptic contributions under our conditions, we examined the effect of RPA addition to Rad51-ssDNA filaments. Because Rad51 is a DNA-dependent ATPase, the rate of hydrolysis is an indirect indicator of the amount of Rad51 bound. ATP hydrolysis was measured using a spectrophotometric assay that couples the

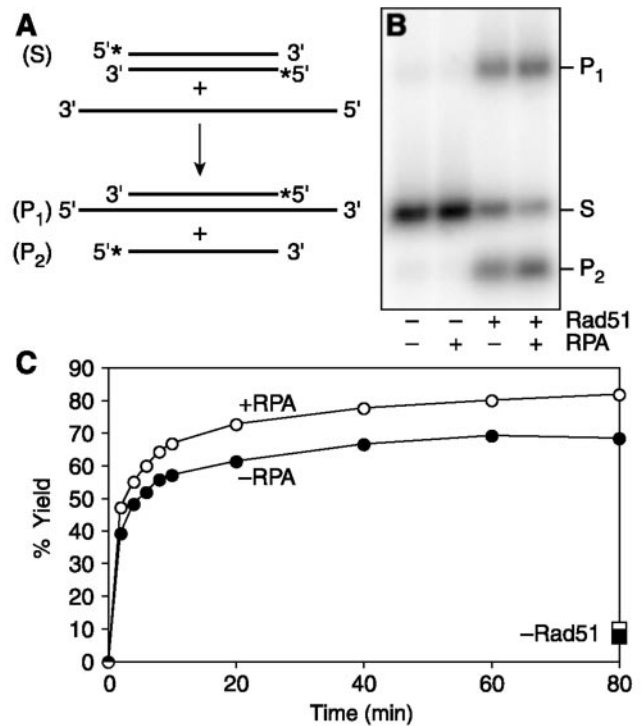


FIG. 2. RPA slightly stimulates DNA strand exchange with oligonucleotide substrates. *A*, the strand exchange reaction promoted by the Rad51 protein with oligonucleotide substrates. The initiating double-strand is a labeled 39-mer, and the initiating single-strand is an unlabeled 70-mer. *S*, substrate; *P*₁ and *P*₂, products. *B*, representative oligonucleotide reactions. RPA is added subsequently to the Rad51-ssDNA preincubation mix, and where indicated, Rad51 or RPA storage buffers are substituted for Rad51 or RPA protein. The control reactions (–Rad51) were allowed 80 min for exchange, whereas the reactions with Rad51 protein were allowed 60 min for exchange. Reactions contained, where indicated, $13.6 \mu\text{M}$ Rad51 protein, $37.3 \mu\text{M}$ ssDNA 70-mer, $0.6 \mu\text{M}$ RPA, and $20.8 \mu\text{M}$ dsDNA 39-mer. The ssDNA is, thus, present at a 2:1 excess relative to the dsDNA. An extensive time course of the same reaction is shown in *panel C*.

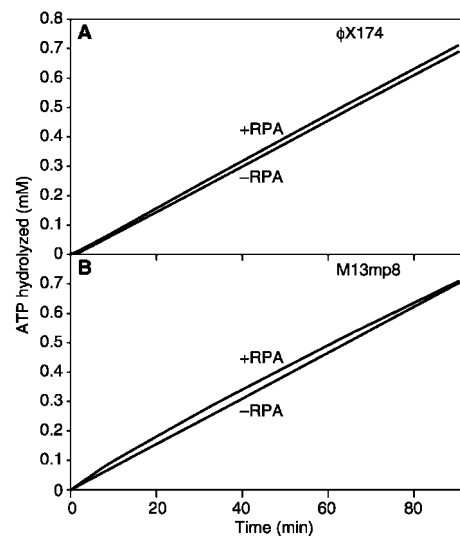


FIG. 3. RPA does not stimulate the rate of ATP hydrolysis by Rad51 protein on circular ssDNA under standard strand exchange conditions. *A*, the hydrolysis of ATP by Rad51 protein on ϕ X174 cssDNA in the presence of RPA or RPA storage buffer added subsequently to the Rad51-ssDNA preincubation. Protein and DNA concentrations are as in Fig. 1. *B*, the same experiment as shown in *A*, substituting M13mp8 cssDNA for the ϕ X174 DNA.

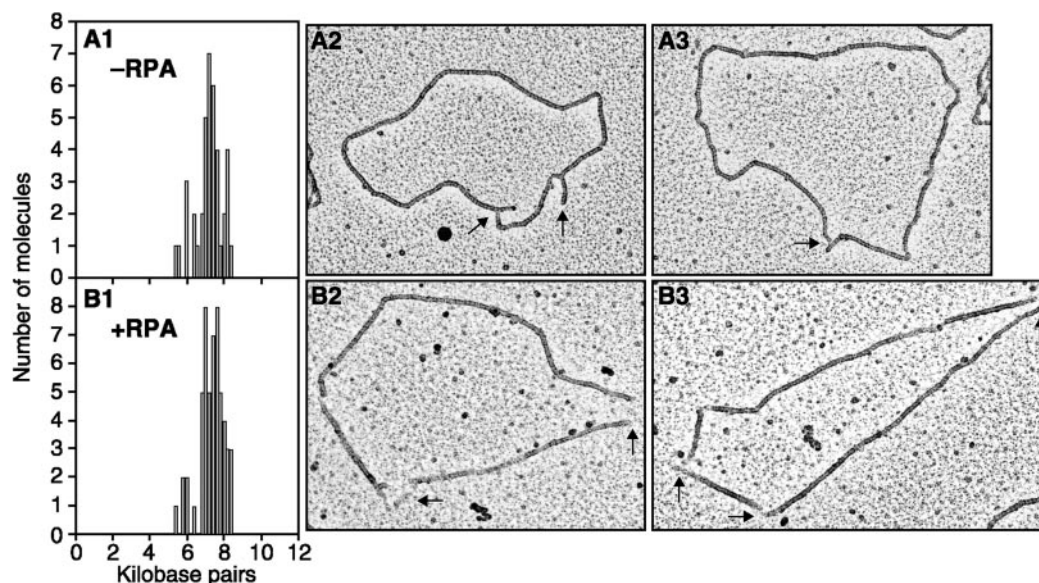


FIG. 4. RPA does not significantly increase the measured contour length of Rad51 protein bound to circular single-stranded ϕ X174 DNA under standard strand exchange conditions given in Fig. 1. *A1*, *A2*, and *A3*, Rad51 filaments on cssDNA were formed by an addition of RPA storage buffer to the Rad51-cssDNA preincubation mixture. The measured lengths of such filaments are represented in *A1*. *A2* and *A3* are electron micrographs of representative molecules. The *arrows* indicate structures referred to as nibs. These were usually short regions. *B1*, *B2*, and *B3*, these *panels* indicate the same information described for *A1*, *A2*, and *A3*, except that RPA was substituted for RPA storage buffer. The *arrows* in *B2* and *B3* indicate gaps in the striated region of the filament.

hydrolysis of ATP to the oxidation of NADH, which is monitored as the reduction in the absorbance at 380 nm. Under the same conditions used in Fig. 1, we found that RPA addition causes no detectable increase in the rate of ATP hydrolysis by the Rad51 protein bound to ϕ X174 ssDNA (Fig. 3A). We note that the DNA strand exchange reaction was almost completely dependent on RPA under these same conditions. To ensure that this result was not specific to ϕ X174 cssDNA, the experiment was repeated with M13mp8 cssDNA, and similar results were obtained (Fig. 3B).

To further evaluate RPA presynaptic contribution to strand exchange under our conditions, we examined these presynaptic filaments directly by electron microscopy to determine whether there were any gaps or other discontinuities in the nucleoprotein filaments formed in the absence of RPA that would represent obvious impediments to DNA pairing (keeping in mind that some impediments may not be evident at electron microscopy resolution). Filaments were compared before and after the addition of RPA. Representative filaments from each of these samples are shown in Fig. 4. In the absence of RPA, extensive filamentation was observed all along the length of the ssDNA (Fig. 4A, 2 and 3). Although no filament discontinuities were observed, small protrusions of the filament were found. These are designated by *arrows* as shown in Fig. 4A, 2 and 3, and we term the protrusions “nibs.” The measurements were performed as if the nibs were looped regions, but the resulting length (average = 7.1 ± 0.7 kilobase pairs, Fig. 4A, 1) does not vary greatly if the nibs are ignored (average = 6.8 ± 0.7 kilobase pairs, data not shown). We do not know the molecular composition of the nibs or whether the nibs may prevent the completion of DNA strand exchange by blocking the extension of paired complexes. Upon the addition of RPA, a few small gaps in the filament were observed in the typical filament (generally at DNA turns), the nibs are no longer present, and the filaments appear more linear and stiff (Fig. 4B, 2 and 3). The contour lengths of these filaments (average = 7.2 ± 0.7 kilobase pairs, Fig. 4B, 1) are equivalent within error to those formed in the absence of RPA. There may be a small increase in

contour length upon RPA addition, but we were unable to detect a statistically significant increase.

Rad51 Protein Extensively Pairs Long DNA Substrates in the Absence of RPA—We considered the apparent paradox in the literature in which Rad51 protein promotes an efficient DNA strand exchange with short substrates in the absence of RPA, whereas the formation of visible products of any kind with long DNA substrates is virtually RPA dependent. It seemed possible that Rad51 paired the long substrates as well as the short substrates in the absence of RPA but that this pairing was unstable when deproteinized (Fig. 1). Deproteinization is required when the reaction is visualized on agarose gels. To test this hypothesis, strand exchange reactions were carried out under the same conditions described for Fig. 1 in the absence of RPA. After 30 min of reaction, samples were treated with AMT to cross-link the DNA. Using this technique, efficient Rad51-mediated pairing of ϕ X174 DNA substrates was observed (Fig. 5, A and B). This observed pairing is dependent upon Rad51 protein, homologous ldsDNA, and cross-linking and increased in a time-dependent manner. Surprisingly, the lengths of paired DNA generated in these experiments in the absence of RPA were quite extensive, with the paired segments incorporating up to half of the DNA substrates (~ 2500 bp; Fig. 5, C and D). The data in Fig. 5D include estimates of the length of the entire double-stranded region of the paired molecules, providing an internal standard by which to validate the measurement technique. Almost all paired molecules contained very short tails relative to the length of exchanged DNA (Fig. 5C); otherwise, the displaced strand was not visible. This type of pairing is completely dependent on the presence of a four-nucleotide overhang, complementary to the cssDNA, on the end of the ldsDNA, as no cross-linked product was formed in reactions lacking RPA that substituted blunt-ended ldsDNA or nicked circular DNA for the *Pst*I-cut ldsDNA (data not shown). Thus, Rad51 is able to extensively pair long DNA substrates in the absence of RPA, but this pairing is unstable when the reactions are deproteinized.

In the Absence of RPA, Rad51 Protein-promoted Pairing Can

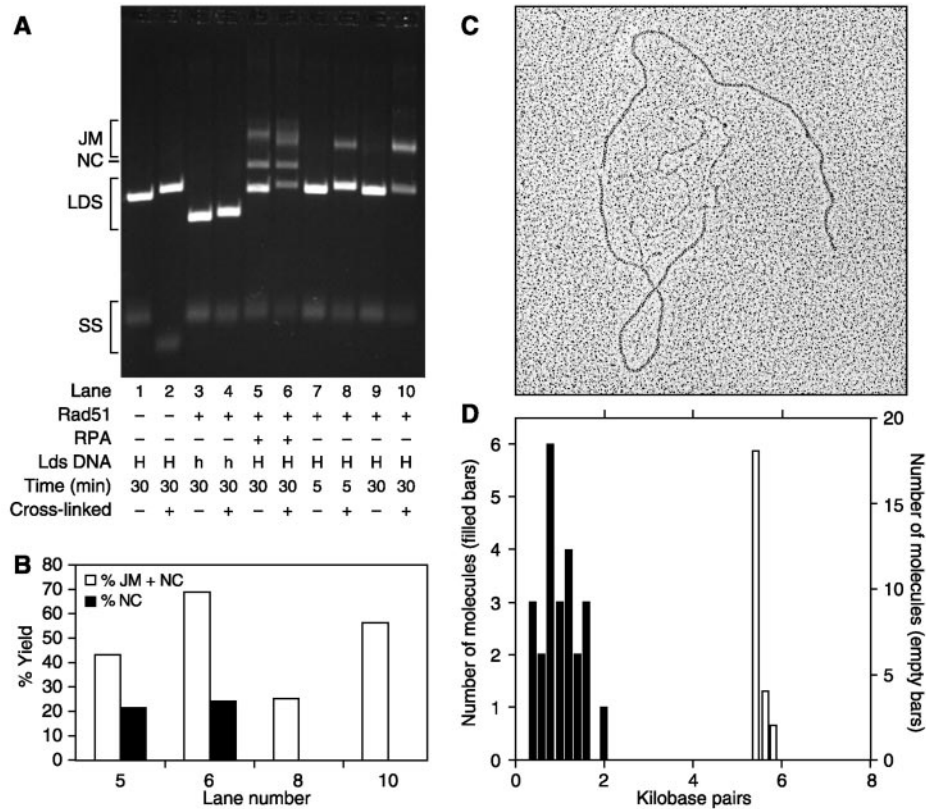


FIG. 5. DNA cross-linking stabilizes Rad51 protein-mediated DNA pairing intermediates in the absence of RPA under standard strand exchange conditions. *A*, reactions were carried out under the same conditions as described in Fig. 1, with the following exceptions. Where noted in the figure, homologous ldsDNA was replaced with heterologous DNA from the plasmid pBII2. The time shown indicates the reaction time after initiation by the addition of ldsDNA and spermidine. Noncross-linked samples were processed by immediate deproteinization. Cross-linked samples were treated with AMT and exposed to UV light before deproteinization. Cross-linking the DNA changes its mobility in the gel slightly even in the absence of protein, as observed when lanes 1 and 2 are compared. Labels are: *NC*, nicked circular DNA (products); *JM*, joint molecules (intermediates); *LDS*, linear double-stranded DNA (substrate); *SS*, single-stranded DNA; *H*, homologous; *h*, heterologous. *B*, the yields of the reactions in lanes 5, 6, 8, and 10 were quantitated as either total reacted product (*JM+NC*) or completed product (*NC*). *C*, electron micrograph showing a typical cross-linked joint molecule of the reaction in lane 10. *D*, from the reaction shown in lane 10, a plot of the distribution of lengths that were exchanged in molecules where pairing had occurred (filled bars). A plot of the total length of the double-stranded region in the same molecules is also presented as a control to validate the measurements (empty bars).

Be Stabilized by Nucleolytic Degradation of the Displaced Strand—The results above indicate that the presynaptic role of RPA is modest under these reaction conditions and that long segments of DNA can be paired without RPA. The simplest explanation for the dramatic effect of RPA in standard reactions is that it has a critical postsynaptic role. RPA could sequester the noncomplementary strand of the duplex DNA and render it unable to pair again with the other strand of duplex, effectively stabilizing the joint molecule intermediate. Without the RPA, the paired complexes are evidently enforced by Rad51 and are not stable when Rad51 is removed unless cross-linked.

To determine whether sequestration of the noncomplementary strand would stabilize Rad51-mediated pairing formed in the absence of RPA, we added a single-strand-specific exonuclease, ExoVII, to these reactions. This bi-directional exonuclease could degrade the noncomplementary strand and prevent it from re-associating with its complement in the substrate DNA. An exonuclease has been demonstrated previously to facilitate RecA-mediated DNA strand exchange by degrading the noncomplementary strand (33). In the absence of RPA, the addition of the nuclease efficiently stabilized Rad51-mediated pairing as observed by gel electrophoresis (Fig. 6A). The percentage of the substrates that were paired to some extent in the presence of exonuclease was comparable with the percentage paired in the presence of RPA and was much greater than that achieved in the absence of RPA (Fig. 6B). Extensive pairing was achieved

in the absence of RPA when exonuclease was added, encompassing up to about half of the length of the circle (Fig. 6, C and D). In some cases, a small single-strand tail of the displaced strand was observed (Fig. 6C, bottom). As a control, the experiment was repeated in the absence of Rad51 but in the presence of ExoVII. No degradation of the DNA was observed by electron microscopy, indicating that strand exchange is not taking place through a mechanism that would involve resection of the ldsDNA to produce a single-strand region that could pair with the cssDNA (data not shown).

DISCUSSION

We conclude that Rad51 protein mediates extensive and efficient pairing of long DNA substrates in the absence of RPA, that this pairing intermediate completely reverts to substrates upon removal of Rad51, and that RPA stabilizes this pairing intermediate to deproteinization through sequestration of the displaced strand. The binding and sequestration of the displaced strand by RPA is a critical part of the strand exchange process with long DNA substrates. This conclusion is based on three observations. First, the large effects of RPA that are seen in DNA strand exchange with long substrates do not reflect an absence of DNA pairing when RPA is omitted. The pairing occurs, and it can encompass thousands of base pairs (Fig. 5). However, the paired species that is produced is unstable after removal of Rad51 and readily reverts to substrate form unless the DNA is first cross-linked. We term such protein-dependent

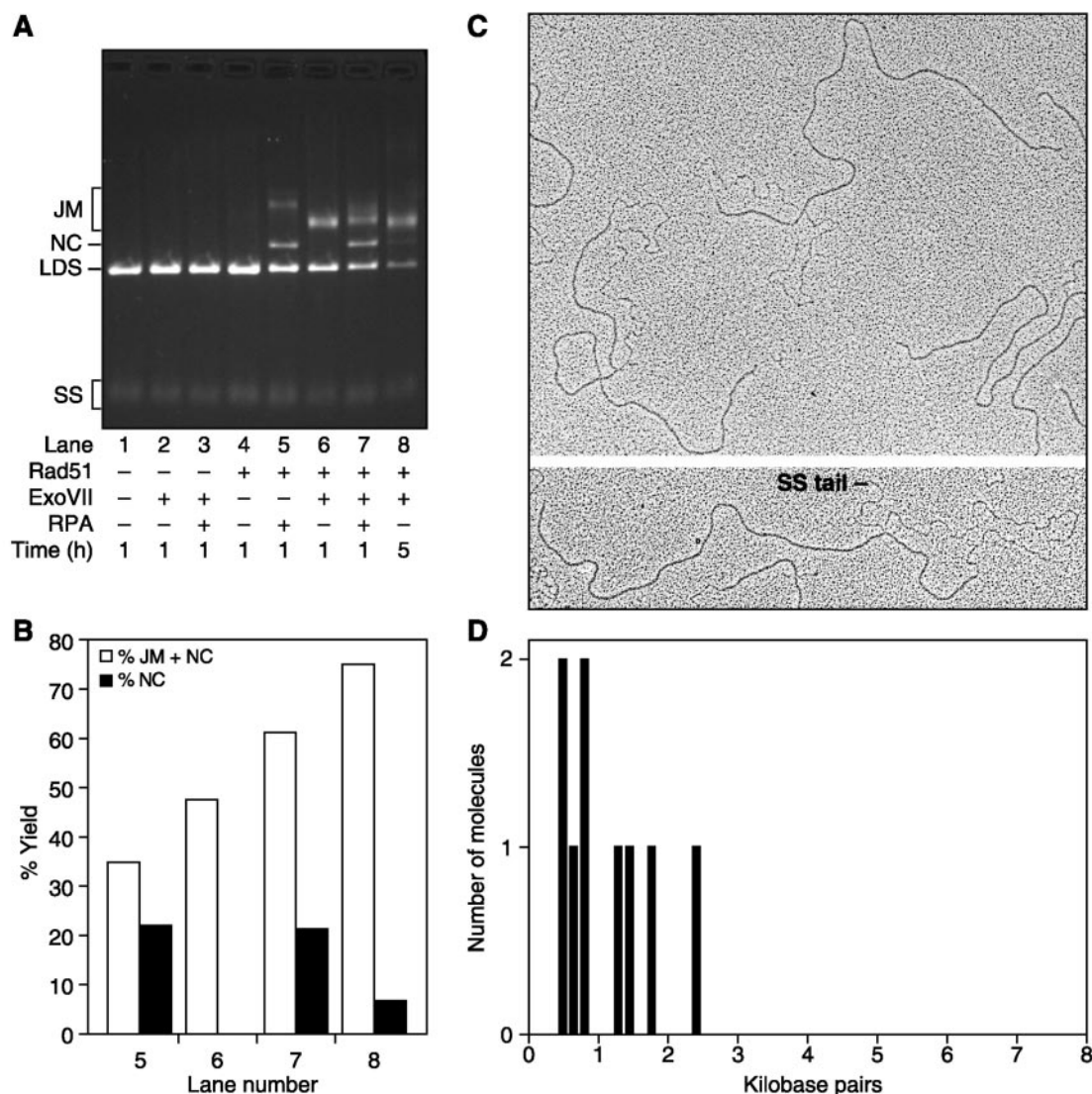


FIG. 6. In the absence of RPA, Rad51 protein-promoted pairing is stabilized by degradation of the displaced strand. *A*, the reactions were carried out under the same conditions as described in Fig. 1, with the following changes. Rad51 protein was preincubated with cssDNA. Then either RPA, 0.08 units of ExoVII/ μ l, or both were added with the ldsDNA. *NC*, nicked circular DNA (products); *JM*, joint molecules (intermediates); *LDS*, linear double-stranded DNA (substrate); *SS*, single-stranded DNA. *B*, the yields of the reactions in lanes 5–8 were quantitated as either total reacted product (*JM+NC*) or fully completed product (*NC*). *C*, electron micrographs of the reaction in lane 6. *Top*, products are shown in which pairing has taken place, and no displaced strand is visible. *Bottom*, a product is shown in which at least a portion of the displaced strand is evident. *D*, a plot of the distribution of lengths that were exchanged in molecules where pairing had occurred.

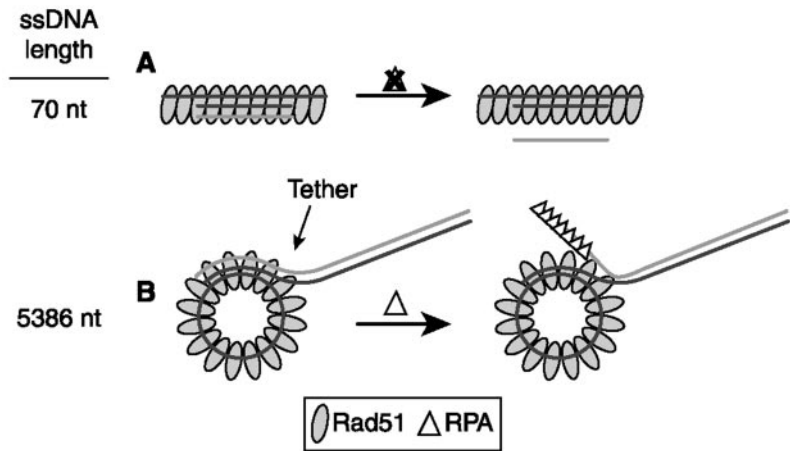
pairing “enforced pairing.” Thus, the Rad51 filaments that form in the absence of RPA are functional. The facile but unstable pairing suggests that to generate stably exchanged DNA products, RPA must make a contribution subsequent to the pairing reaction, *i.e.* a postsynaptic contribution. The second observation demonstrates that postsynaptic sequestration of the displaced DNA strand provides the needed stabilizing effect, since removal of this strand by nucleolytic degradation is essentially as effective as RPA in generating paired products that are stable enough to be seen in an agarose gel. Third, the presynaptic role of RPA in removal of secondary structure from the ssDNA was minimized by performing experiments at a low magnesium concentration. Even though RPA strongly stimulates the exchange of long DNA substrates under these conditions (Fig. 1), RPA does not significantly increase the amount of Rad51 bound to the ssDNA (Figs. 3 and 4). Thus, the postsynaptic role was isolated and found to be of critical importance.

The postsynaptic role for RPA in the Rad51 reactions is consistent with similar roles observed for other SSB proteins in

strand exchange reactions and provides a new system with which to investigate the mechanism of SSB action in these reactions. In the original model, proposed by Kodadek for the T4 bacteriophage system, the gene 32 protein binds to the displaced strand and stabilizes the product (16). A similar role has been proposed for SSB in RecA protein-mediated DNA strand exchange (15). The net effect in both cases is to prevent a reversal of strand exchange via reverse branch migration. We propose a refinement of this model for Rad51-promoted strand exchange of long substrates based on our observations of extensive pairing of DNA substrates in the absence of RPA and the observed complete reversal of the reaction to substrate form upon removal of Rad51. Instead of RPA simply binding to the outgoing strand after it has been expelled from the recombinase filament, we propose that strand exchange initially results in a complex in which the outgoing strand remains within the filament and that RPA plays a direct role in the stable removal of the outgoing DNA strand from the filament (Fig. 7B).

We speculate that without RPA, the three DNA strands are

FIG. 7. Model comparing Rad51-promoted strand exchange of oligonucleotides and ϕ X174-length substrates. *A*, oligonucleotides can exchange throughout the entire length of the duplex region without a factor present to sequester the displaced strand. This exchange throughout the duplex results in formation of the final product and release of the noncomplementary strand. Thus, RPA is not required for formation of products stable to Rad51 removal. *B*, incomplete exchange along the length of long substrates in the absence of RPA results in a tether that would obstruct release of the noncomplementary strand and maintain the intimate association of the three strands. Formation of products stable to removal of Rad51 protein will occur if the noncomplementary strand is bound and sequestered by RPA. *nt*, nucleotides.



retained within the Rad51 filament in locations that permit a rapid and complete formation of the original duplex and single strand, whereas RPA removes the outgoing strand from the filament and, thus, stabilizes the DNA pairing intermediate. There are at least two possible scenarios for how Rad51-mediated enforced pairing could extend over thousands of base pairs and yet still readily and completely revert to substrate form upon removal of protein. First, it is possible that strand transfer occurs with the displaced strand remaining bound in an accessible site within the filament that allows for rapid and complete re-formation of the substrate duplex upon removal of the protein. Alternatively, the pairing we see after cross-linking may reflect an incomplete strand transfer. Radding and co-workers (23) propose that the search for homology and subsequent pairing by a recombinase occurs by flipping bases of the dsDNA (A and T) out to sample complementarity with the bases of the ssDNA, and key predictions of this proposal have been verified. The structure of the DNA within Rad51 and RecA filaments has been determined by NMR, and it demonstrates that an interconversion in the sugar pucker could allow for rotation of the base to be sampled for homology (35). The base-flipping mechanism could allow for ready reversal of a Rad51 protein-dependent association of homologous strands.

The generation of DNA strand exchange products that will survive protein removal is much less dependent upon RPA when long DNA substrates are replaced by short oligonucleotides (2, 9–12) (Fig. 2, *B* and *C*). This apparent paradox is partially resolved by the observation of extensive enforced DNA pairing with long DNA substrates (Fig. 5). The strand exchange seen with the oligonucleotides could be stabilized by release of the displaced strand (Fig. 7*A*), but a complete strand exchange leading to release of the displaced strand (36) (or a proposed shift of the displaced DNA strand within the filament (37)) may be blocked with the longer DNA substrates. Unreacted dsDNA in an incomplete exchange would act as a tether that could facilitate a conversion back to substrates (Fig. 7*B*). The failure to complete long DNA strand exchange reactions in the absence of RPA could reflect the presence of some Rad51 filament discontinuities (a remnant requirement for presynaptic RPA involvement), inefficient release of the displaced strand from the filament over longer segments (the postsynaptic role), or both.

The effects of RPA are observed only if RPA is present when the DNA pairing takes place. The Rad51-enforced pairing that occurs in the absence of RPA appears to progress rapidly to a form that cannot be subsequently acted on by RPA. In the robust reaction seen in Fig. 1, RPA is added to the reaction 5 min after Rad51 and ssDNA and 10 min before dsDNA is

added. If RPA is added either with the dsDNA or subsequent to it, the yields of DNA pairing intermediates decrease as the length of time increases between dsDNA addition and RPA addition (data not shown). This is the cause of the lower yields in Fig. 6 for RPA-stimulated exchange (35 versus 60%), as RPA is added with the dsDNA in this experiment. For RPA to function optimally in its postsynaptic role, it must be present when the dsDNA pairs with the filament. The mechanisms by which RPA facilitates the postsynaptic phase of strand exchange and the events that render a paired complex refractive to the late addition of RPA need additional investigation. We note that SSBs from other organisms are able to stimulate the Rad51-mediated reaction with ϕ X174 substrates as efficiently as RPA under many conditions (10, 18, 38), suggesting that species-specific complexes between RPA and Rad51 do not play critical roles in the effects observed *in vitro*.

Sequestration of the displaced strand appears to be integral to the mechanism of Rad51-mediated strand exchange of long DNA substrates. Few joint molecule products are detected in these reactions unless RPA or an exonuclease is present or unless the intermediates are cross-linked before protein removal. In contrast, *E. coli* RecA protein-mediated DNA strand exchange with long DNA substrates appears to be less dependent on SSB (15, 39, 40), although some studies have featured excess RecA protein that could itself sequester the displaced DNA strand (15, 39). It seems likely that the RPA-independent pairing seen at long time points in Rad51 reactions could be due to the binding of Rad51 protein itself to the displaced strand. A postsynaptic role for RPA in DNA strand exchange may serve to prevent the release of unbound and nuclease-vulnerable DNA as a reaction product *in vivo*.

Acknowledgments—We thank Patrick Sung for plasmids and strains for Rad51 protein and RPA production and Stephen Van Komen for useful discussions. We also thank Patrick Sung and Charles Radding for reading and providing comments on an early draft of the manuscript. We thank Maria Schnös and Sindhu Chitteni Pattu for assistance with the electron microscopy experiments.

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