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A Novel Function of Rad54 Protein

STABILIZATION OF THE Rad51 NUCLEOPROTEIN FILAMENT*

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Homologous recombination is important for the repair of double-stranded DNA breaks in all organisms. Rad51 and Rad54 proteins are two key components of the homologous recombination machinery in eukaryotes. *In vitro*, Rad51 protein assembles with single-stranded DNA to form the helical nucleoprotein filament that promotes DNA strand exchange, a basic step of homologous recombination. Rad54 protein interacts with this Rad51 nucleoprotein filament and stimulates its DNA pairing activity, suggesting that Rad54 protein is a component of the nucleoprotein complex involved in the DNA homology search. Here, using physical criteria, we demonstrate directly the formation of Rad54-Rad51-DNA nucleoprotein co-complexes that contain equimolar amounts of each protein. The binding of Rad54 protein significantly stabilizes the Rad51 nucleoprotein filament formed on either single-stranded DNA or double-stranded DNA. The Rad54-stabilized nucleoprotein filament is more competent in DNA strand exchange and acts over a broader range of solution conditions. Thus, the co-assembly of an interacting partner with the Rad51 nucleoprotein filament represents a novel means of stabilizing the biochemical entity central to homologous recombination, and reveals a new function of Rad54 protein.

Homologous recombination plays an essential role in repair of DNA double-stranded breaks, a lethal type of DNA damage. The mechanisms of double-stranded break repair by recombination have been extensively investigated at the genetic and biochemical levels (1–6). In yeast, genetic analysis reveals a set of genes, called the *RAD52* epistasis group, that are directly involved in double-stranded break repair (7). This group includes the *RAD50*, *RAD58/MRE11*, *XRS2*, *RPA1*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, and *RAD59* genes. Among the proteins encoded by these genes, the Rad51 protein is the most evolutionarily conserved; its homologues are spread from bacteria to mammals (8). The Rad51 protein family members possess a unique property; they form helical nucleoprotein filaments with DNA (9–11) and promote DNA pairing and strand transfer, a basic step of homologous recombination (11–16).

More recently, it was demonstrated that Rad52 protein, Rad54 protein, and the Rad55/Rad57 heterodimer stimulate DNA pairing promoted by Rad51 protein. The mechanistic studies revealed that stimulation by Rad52 protein and the Rad55/Rad57 proteins is at the early (presynaptic) stage of DNA strand exchange. Rad52 protein enhances the ability of Rad51 protein to displace RPA from ssDNA[1] (17–20), as do the Rad55/Rad57 proteins, but by a different mechanism (21).

The mechanism by which Rad54 protein stimulates the DNA pairing activity of Rad51 protein is different (22–28). It was found that, in contrast to other proteins that stimulate the DNA pairing activity of Rad51 protein, Rad54 protein acts at a later (synaptic) stage of DNA strand exchange (24, 29). Rad54 protein belongs to the Snf2/Swi2 group of proteins, which are involved in ATP-dependent remodeling of protein complexes, including mainly chromatin complexes (30). Rad54 protein was discovered to have a dsDNA topology-modifying activity, and this activity was implicated in the mechanism of stimulation of DNA strand exchange (23, 27). Previously, we inferred from the analysis of joint molecule formation by Rad51 protein that Rad54 protein binds to the assembled Rad51-ssDNA nucleoprotein filament (24, 31). We suggested that Rad54 protein is a component of a nucleoprotein filament that is involved in the search for DNA homology, and, in that capacity, Rad54 protein modifies the topology of dsDNA-target, thereby making it more readily accessible for DNA pairing.

Here, in experiments with DNA attached to polystyrene beads, we provide direct evidence for a stoichiometric complex of Rad54 protein bound to the Rad51-ssDNA nucleoprotein filament. We discovered that the binding of Rad54 protein to the filament increased the stability of the Rad51 nucleoprotein complex substantially. We suggest that this stabilization of the filament represents a novel presynaptic and, possibly, synaptic function of Rad54 protein that, in addition to its dsDNA unwinding activity, contributes to its stimulation of DNA strand exchange.

EXPERIMENTAL PROCEDURES

Proteins and DNA—Saccharomyces cerevisiae Rad51 protein, RPA, and Rad54 protein were purified as described (18, 31, 32). The Rad54 protein is a glutathione *S*-transferase fusion protein that is fully active both *in vivo* (31) and *in vitro* (24). The oligonucleotides used in this study were as follows: number 2, 63-mer (TCCTTTTGATAAGAGGTCATTTTTGCGGATGGCTTAGAGCTTAATTGCTGAATCTGGTGCTGT); 5, 32-mer (CCATCCGCAAAAATGACCTCTTATCAAAAGGA); 6, 32-mer (TCCTTTTGATAAGAGGTCATTTTTGCGGATGG); 26, 48-mer (TCCT TTTGATAAGAGGTCATTTTTGCGGATGGCTTAGAGCTTAATTGC); 48, 48-mer (GCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCT TATCAAAAGGA-biotin); 77, 100-mer (biotin-GGGCTACGTCTTGCTG GCGTTCGCGACGCGAGGCTGGTGGCCTTCCCCATTATG-

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; STMP, salt titration midpoint.

ATTCTTC TCGCTTCCGGCGGCATCGGGATGCCCGCGTTGCAGGCCA); 78, 100-mer (TGGCCTGCAACGCGGGCATCCCGATGCCGCCGGAAGCG AGAAGAATCATAATGGGGAAGGCCACCAGCCTCGCGTCGCGAAC GCCAGCAAGACGTAGCCC); 109, 63-mer (GTCGACGACGTCTGAGTACTCATCTAGTGTGACATCATCGCATCGAGAACAGCACCAGATT CA); and 111, 63-mer (TGAATCTGGTGCTGTTCTCGATGCGATGAT GTCACACTAGATGAGTACTCAGACGTCGTCGAC).

All oligonucleotides were synthesized using standard phosphoroamidite chemistry and were purified by electrophoresis in a polyacrylamide gel containing 50% urea (33). The concentrations of the oligonucleotides were determined spectrophotometrically using the extinction coefficient ϵ_{260} = 9833 $M^{-1}\cdot cm^{-1}$ (1 A_{260} = 33 μg/ml). Oligonucleotide dsDNA preparations were as described (34). Oligonucleotides were stored at −20 °C. Covalently closed circular pUC19 DNA was purified using alkaline lysis, followed by precipitation with LiCl and by CsCl-ethidium bromide equilibrium centrifugation (34). Relaxed circular DNA was produced by treating pUC19 supercoiled DNA (50 μg/ml) with topoisomerase I from wheat germ (0.75 units/ml) for 30 min at 37 °C, followed by phenol deproteinization and repeated ethanol precipitations. All DNA concentrations are expressed as molar in nucleotide concentration. Single-stranded oligonucleotides were labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP in the buffer containing 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 5 mM dithiothreitol at 37 °C for 1 h. To inactivate the T4 polynucleotide kinase, the reaction mixture was heated for 10 min at 75 °C. Labeled oligonucleotides were stored at −20 °C.

Immobilization of Biotinylated DNA on Polystyrene Streptavidin Beads—A tube containing a 1% (w/v) stock of beads (0.95-μm mean diameter; Bang Laboratories, Inc.) was stirred vigorously for 2 min. Aliquots of 20–40 μl of a bead suspension were withdrawn from the stock and pelleted by centrifugation for 4 min using a bench top centrifuge (2000 × *g*). The pellet was resuspended (2 min of intense stirring) in an original volume of 0.1 M $NaHCO_3$ and mixed with ^{32}P-labeled biotinylated DNA. Prior to the immobilization, the radioactivity (cpm) of the biotinylated DNA was determined using a scintillation counter LS6500 (Beckman) and its specific activity (cpm per mg) was calculated. The immobilization was carried out for 15 min at 37 °C. Non-immobilized DNA was removed by centrifugation for 4 min. After immobilization, the beads with attached DNA were pelleted by centrifugation, and the pellets were washed in the original volume of $NaHCO_3$ and pelleted again by centrifugation. The washing procedure was repeated twice. The amount of DNA attached to beads was determined by measuring the radioactivity of the bead, supernatant, and wash fractions and by calculating it as a fraction relative to total DNA with known specific activity.

Protein Binding to Bead-immobilized ds- or ssDNA—Fig. 1 illustrates our procedure for measuring the binding of Rad51 and Rad54 proteins to the DNA-beads. Rad51 and Rad54 proteins were bound to DNA-beads in standard binding buffer containing 33 mM HEPES (pH 7.0), 20 mM (or 25 mM) magnesium acetate, and 2 mM ATP. Previously, we established that these conditions are optimal for DNA strand exchange promoted by the yeast Rad51 protein (32, 35). Typically, the reaction volumes were in multiples of 10 μl; 10 μl represented the smallest volume unit of the reaction mixture. Where indicated, the mixture was supplemented with an ATP regeneration system containing either 3 mM phosphoenolpyruvate and 20 units/ml pyruvate kinase or 20 mM phosphocreatine and 30 units/ml phosphocreatine kinase. Rad51 protein was incubated with beads for 15 min at 37 °C. Rad54 protein was incubated with dsDNA-beads or Rad51-dsDNA-beads for 10 min at 37 °C, unless indicated otherwise. When longer periods of incubation were applied, the Rad51-dsDNA filaments were formed at 37 °C and then cooled to room temperature (23 °C) prior to the addition of Rad54 protein. After completion of protein binding, 10-μl aliquots were withdrawn from reaction mixtures, and beads were pelleted by centrifugation for 4 min. Where indicated, pellets were resuspended in 10 μl of standard buffer and pelleted again. Finally, beads were resuspended in 10 μl of SDS-polyacrylamide gel running buffer and mixed with 2 μl of 6× SDS loading buffer (350 mM Tris-HCl, pH 6.8, 1% SDS, 6% 2-mercaptoethanol, 36% glycerol, and 0.1% bromphenol blue) and incubated at 95 °C for 3 min. Samples were then analyzed by electrophoresis in 8% SDS-polyacrylamide gels. Protein bands were visualized by staining with Coomassie Brilliant Blue (R-250). Typically, we analyzed both the bound and free protein fractions and determined the relative amounts of proteins in these fractions using high resolution cooled CCD digital camera and GelPro 3.0 software.

To determine the salt titration midpoint (STMP) of the Rad51-DNA complexes, the preformed Rad51-DNA or Rad54-Rad51-DNA nucleoprotein complexes were incubated in reaction mixtures of 11.9 μl in the presence of various concentrations (0–880 mM) of NaCl for 5 min. The beads were pelleted by centrifugation, and the amounts of DNA-bound proteins were determined as described above. Salt that was added along with Rad54 or Rad54 K341R protein, whose stocks contained 1 M NaCl, was taken into account in all STMP calculations.

Restriction Endonuclease Protection Assay—To form Rad51 nucleoprotein filaments, Rad51 protein (0.575 or 0.85 μM) was incubated with dsDNA (3.45 μM, nucleotides) (63 base pairs, oligonucleotides 109–111) in buffer containing 33 mM HEPES (pH 7.0), 10 mM magnesium acetate, 2 mM dithiothreitol, 2 mM ATP, 100 μg/ml bovine serum albumin at 37 °C for 15 min. For ATP regeneration, 15 mM phosphocreatine and 20 units/ml of phosphocreatine kinase were included in the reaction. Aliquots of 9 μl containing the Rad51-dsDNA complexes were withdrawn from the reaction mixture and mixed with 1 μl of Rad54 protein at various concentrations. DNA cleavage was initiated by the addition of 1 μl of *Rsa*I (10 units) endonuclease. The reactions were carried out for 15 min at 37 °C. DNA products were deproteinized by the addition of EDTA to 50 mM, SDS to 1%, and proteinase K to 700 mg/ml, incubated for 5 min at 37 °C, and analyzed in 10% polyacrylamide gels.

DNA Strand Exchange—To form Rad51 nucleoprotein filaments, Rad51 protein (1 μM) was incubated with ssDNA (63-mer, oligonucleotide 2) (3 μM) in buffer containing 33 mM HEPES (pH 7.0), 10 mM magnesium acetate, 2 mM dithiothreitol, 2 mM ATP, 100 μg/ml bovine serum albumin at 37 °C for 15 min. The reaction temperature was decreased to 30 °C, and, where indicated, Rad54 protein (0.125 μM) was added to the reaction. Aliquots (8 μl) were mixed with the indicated amounts of NaCl (1 μl), and pairing reactions were initiated immediately by the addition of 1 μl of dsDNA (32 base pairs, oligonucleotides 5 and 6) (3 μM, nucleotides) and continued for 15 min. DNA products were deproteinized by the addition of EDTA to 50 mM, SDS to 1%, and proteinase K to 500 μg/ml followed by incubation for 5 min at 37 °C. Samples were mixed with one-tenth volume of loading buffer (20% Ficoll, 0.1% bromphenol blue) and analyzed by electrophoresis in a 10% polyacrylamide gel in TBE buffer (90 mM Tris borate, pH 8.3, and 0.5 mM EDTA). The extent of DNA strand exchange was quantified using a Storm 840 PhosphorImager (Amersham Biosciences).

RESULTS

Rad54 Protein Forms Stoichiometric Complexes with Rad51-DNA Filaments—Previously, we found that a stoichiometric amount (1:1) of Rad54 protein relative to Rad51 protein is required for maximal stimulation of joint molecule formation by the Rad51 nucleoprotein filament (24). This finding implied that a co-complex with the nucleoprotein filament, containing equimolar amounts of Rad54 and Rad51 proteins, is the functional species. To obtain physical evidence for this complex, we quantified the binding of Rad54 protein to Rad51 nucleoprotein filaments assembled on biotinylated DNA, which was attached to streptavidin-coated polystyrene beads (Fig. 1).

First, we confirmed the stoichiometry of Rad51 protein binding to ssDNA (48-mer). For this purpose, we incubated a fixed amount of ssDNA-beads with increasing amounts of Rad51 protein and then analyzed the amount of Rad51 protein bound to the DNA-beads by SDS-gel electrophoresis. As expected, Rad51 protein binds to the ssDNA with a stoichiometry that is ~1 protein monomer per 3 nucleotides of ssDNA, based on the amount of Rad51 protein (~0.6 μM) bound to DNA (1.93 μM, total concentration) at saturation (Fig. 2, *A* and *B*). Then, in a similar experiment, we examined the binding of Rad51 protein to dsDNA (100 base pairs) immobilized on beads at two different concentrations. As with ssDNA, the stoichiometry of Rad51 protein binding to the dsDNA is ~1 protein monomer per 3 base pairs of dsDNA at two different DNA concentrations (Fig. 2*C*). In these experiments, the level of nonspecific Rad51 protein binding to the polystyrene beads lacking DNA never exceeded 10% of Rad51 protein binding to either dsDNA-beads or ssDNA-beads (Fig. 2). Therefore, Rad51 protein binds to ssDNA and dsDNA that is immobilized on beads with the same binding stoichiometry that was observed previously (10, 36, 37).

Next, we determined the stoichiometry of Rad54 protein binding to a Rad51 nucleoprotein filament that was assembled

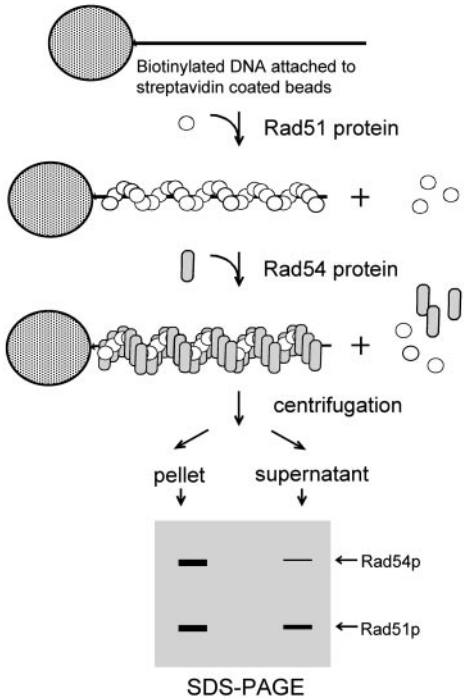

FIG. 1. **Experimental design.** Biotinylated DNA that was immobilized on streptavidin-coated beads was used as a binding substrate for Rad51 and Rad54 proteins. Nucleoprotein complexes were separated from unbound proteins by centrifugation, and proteins bound and those remaining free were analyzed by SDS-polyacrylamide gel electrophoresis (the image of the gel is simply an illustration of the expected results).

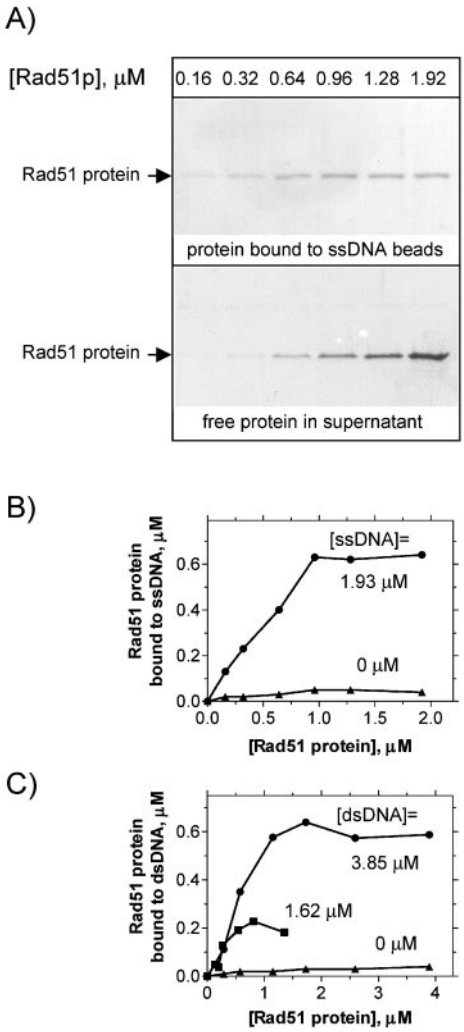

FIG. 2. **Rad51 protein binds stoichiometrically to DNA immobilized on polystyrene beads.** Bead-immobilized ssDNA or dsDNA was incubated with Rad51 protein at the indicated concentrations. The bound and free Rad51 protein fractions were separated by centrifugation and analyzed by SDS electrophoresis in 8% polyacrylamide gels. Coomassie-stained gels showing the binding of Rad51 protein to ssDNA (48-mer, oligonucleotide 48) (1.93 μM) are shown in *A*; quantification of these gels is shown in *B*. Note that only 55% of the fractions of bound protein were loaded on the gel; for the fractions of free protein, the entire fractions were loaded. The *graph* in *C* depicts binding of Rad51 protein to bead-immobilized dsDNA (100 base pairs, positions 77 and 78) at a nucleotide concentration of either 1.62 μM (*squares*) or 3.85 μM (*circles*). The nonspecific binding of Rad51 protein to the beads lacking DNA is shown by the *triangles*. Typically, experiments were repeated at least three times, with S.E. not exceeding 10%.

on the ssDNA. These preassembled Rad51-nucleoprotein filaments were incubated with increasing amounts of Rad54 protein. We found that Rad54 protein forms a stable complex with the Rad51-ssDNA complexes (Fig. 3*A*). The amount of Rad51 protein in these co-complexes does not decrease with increasing Rad54 protein binding, demonstrating that the Rad51 protein is not being displaced; if anything, there was a slight increase in the amount of bound Rad51 protein, suggesting stabilization (see below). At saturating Rad54 protein concentrations, these complexes contain equimolar amounts of Rad51 and Rad54 proteins (Fig. 3*B*). The level of nonspecific Rad54 protein binding to the polystyrene beads lacking DNA did not exceed 15% of Rad54 protein binding to the Rad51-ssDNA complexes (see Fig. 4*A*).

Similar experiments were conducted using Rad51-dsDNA filaments (gel data not shown). In this case, we also observed increased binding of Rad54 protein until saturation of the Rad51-dsDNA complexes was achieved (Fig. 3*C*). As was the case for ssDNA, saturation occurred at approximately one Rad54 monomer per Rad51 monomer. Also, it appeared that increasing amounts of Rad54 protein promoted a slight increase of Rad51 protein bound, particularly at the lower concentrations of Rad54 protein (see below). Thus, Rad54 protein forms a stable co-complex with the Rad51 nucleoprotein filament, assembled on either ssDNA or dsDNA, without the displacement of Rad51 protein.

Rad54 Protein Stabilizes the Rad51 Nucleoprotein Filament Assembled on Either ssDNA or dsDNA—The slight increase in bound Rad51 protein that was detected in Fig. 3, when Rad54 protein was present, suggested that Rad54 protein might affect the stability of the Rad51-ssDNA and Rad51-dsDNA nucleoprotein filaments. As a criterion for stability, we used the resistance of the filament to disruption by NaCl (36, 38, 39). Consequently, the nucleoprotein complexes were incubated in the presence of increasing concentrations of NaCl, and the amount of Rad51 protein bound to ssDNA was determined (Fig. 4*A*). We observed a decreased amount of Rad51 protein bound to dsDNA upon the addition of NaCl (36). The STMP, which is defined as the NaCl concentration at which one-half of the protein (relative to the fully saturated complex) is dissociated from the nucleoprotein complexes, is ~120 mM NaCl for Rad51 protein, when Rad54 protein was absent (Fig. 4*A*, *squares*). Interestingly, in the presence of 0.42 μM Rad54 protein (*triangles*), the STMP for the Rad51-ssDNA complexes increased to 300 mM.

Similar NaCl titration experiments were conducted using dsDNA instead of ssDNA (titrations not shown). We found that Rad54 protein also stabilized the Rad51-dsDNA filament in a similar manner (Fig. 4*B*). The STMP for the Rad51-dsDNA complexes is less than 50 mM NaCl for Rad51 protein, when

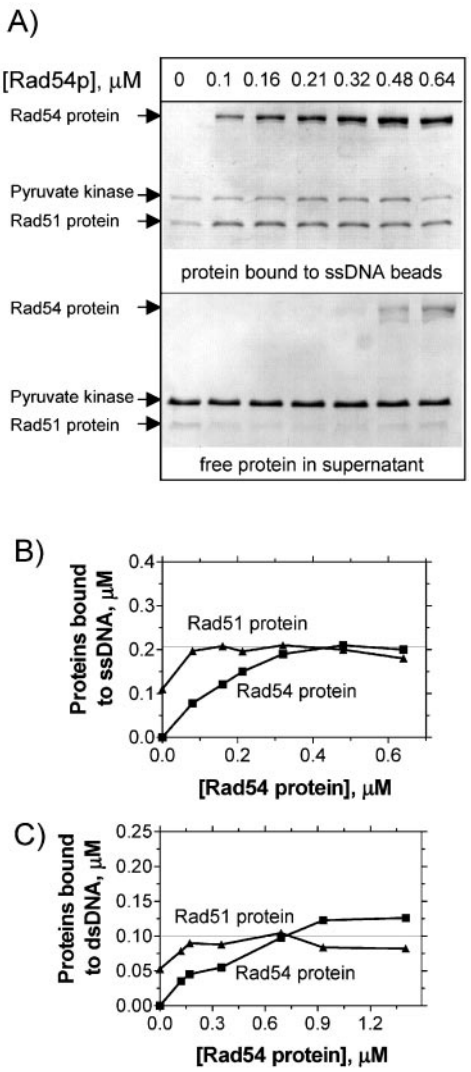

FIG. 3. **Rad54 protein binds stoichiometrically to the Rad51-DNA filament.** Bead-immobilized ssDNA (48-mer, oligonucleotide 48) (0.64 μM) or dsDNA (100 base pairs, oligonucleotides 77 and 78) (0.62 μM, nucleotide) was incubated first with Rad51 protein (0.32 μM) and then with Rad54 protein at the indicated concentrations as described under "Experimental Procedures." The mixture was supplemented with an ATP regeneration system containing 3 mM phosphoenolpyruvate and 20 units/ml pyruvate kinase. Proteins bound to ssDNA-beads or dsDNA-beads were separated from free protein by centrifugation and analyzed by SDS gel electrophoresis in 8% polyacrylamide gels. Coomassie-stained gels showing the binding of Rad54 and Rad51 proteins to ssDNA are shown in *A*. Note that relative to the fractions of free protein, only 80% of the volume of the fractions of bound protein was loaded on the gel. The pyruvate kinase in the fractions of bound protein results from the residual solution that remains associated with the beads. Graphs show the binding of Rad51 and Rad54 proteins to ssDNA-beads (*B*) and to dsDNA-beads (*C*). The *thin horizontal lines* in *B* and *C* indicate the theoretical stoichiometry for binding of 1 Rad51 protein monomer per 3 nucleotides or base pairs. The binding experiments were repeated three times; S.E. values for the measurements were smaller than 12%.

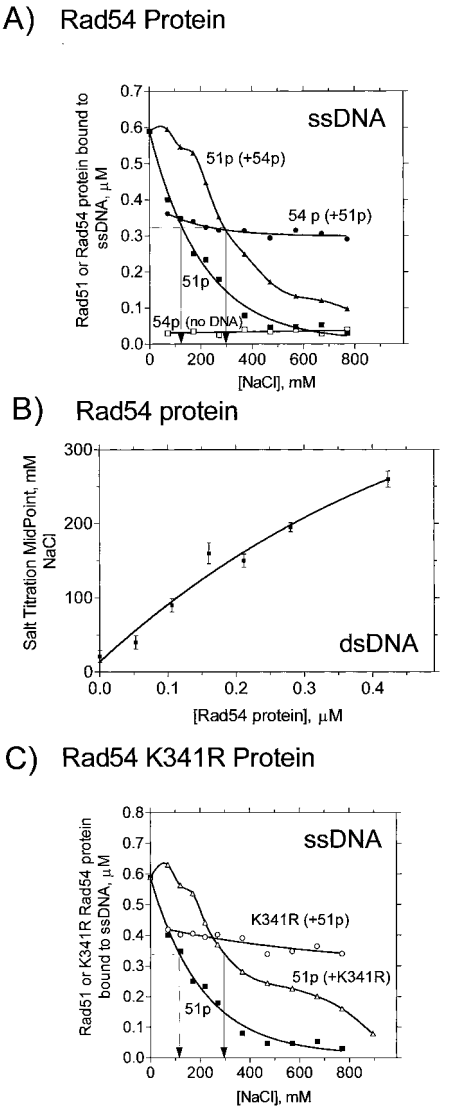

FIG. 4. **Rad54 protein stabilizes the Rad51 nucleoprotein filament on either ssDNA or dsDNA.** *A* and *C*, the Rad51-ssDNA filaments were assembled by incubating Rad51 protein (0.85 μM) with bead-immobilized ssDNA (48-mer, oligonucleotide 48) (1.73 μM). Rad54 protein (0.42 μM) (*A*) or mutant K341R Rad54 protein (0.42 μM) (*C*) was added to the Rad51-ssDNA nucleoprotein filaments to form Rad54-Rad51-ssDNA co-complexes. The stability of the nucleoprotein complexes was challenged by NaCl for 5 min at 37 °C. The amounts of Rad51 protein, Rad54 protein, and K341R Rad54 protein bound to ssDNA and dsDNA were determined using SDS-polyacrylamide gel electrophoresis. The levels of Rad51 protein binding either alone (*51p*) or in the presence of Rad54 protein (*51p* (*+54p*)) or of Rad54 K341R mutant protein (*51p* (*+K341R*)) are indicated by *squares* and *triangles*, respectively. The levels of Rad54 protein (*54p* (*+51*)) or Rad54 K341R mutant protein binding in the presence of Rad51 protein (*K341R* (*+51p*)) are indicated by *circles* and *open circles*, respectively. The nonspecific binding of Rad54 protein to beads devoid of DNA is indicated in *A* by *open squares*. The *arrows* indicate the STMP of the Rad51-ssDNA complexes. Typically, binding experiments were repeated three times; S.E. values for each point were smaller than 10%. *B*, Rad51-dsDNA filaments were assembled by incubating Rad51 protein (0.85 μM) with bead-immobilized dsDNA (48 base pairs, positions 48–26) (3.45 μM, nucleotides). Rad54 protein was added at the indicated concentrations. The stability of the nucleoprotein complex was challenged by NaCl for 5 min at 37 °C. For each Rad54 protein concentration, the STMP was determined from experiments done at 5–11 different NaCl concentrations. The STMP for each Rad54 protein-Rad51 protein-dsDNA complex is plotted.

Rad54 protein was absent. In the presence of Rad54 protein, we found that the addition of increasing amounts caused a gradual increase in the salt resistance of the Rad51-dsDNA complex. At the highest Rad54 protein concentration tested (0.42 μM), representing a stoichiometry of one Rad54 protein monomer per two Rad51 protein monomers), the STMP for Rad51 protein increased to 260 mM NaCl. Thus, Rad54 protein substantially stabilizes both the Rad51-ssDNA and the Rad51-dsDNA nucleoprotein complexes.

In the salt stability experiments described above, we also measured the binding of Rad54 protein to the Rad51-ssDNA and Rad51-dsDNA complexes at each of the NaCl concentrations. The Rad54 nucleoprotein complexes show a very high

resistance to NaCl: at 770 mM NaCl, about 80 and 50% of Rad54 protein remains complexed with ssDNA (Fig. 4*A*, *filled circles*) and dsDNA (data not shown), respectively. The effect of nonspecific binding of Rad54 protein to beads was also tested; it was negligible over the entire range of tested salt concentrations (Fig. 4*A*, *open squares*). Thus, Rad54 protein remains bound to DNA over the range of these experiments, and the stabilizing effect of Rad54 protein on the Rad51-DNA nucleoprotein complexes is probably associated with its high affinity for DNA.

Stabilization of the Rad51-DNA Complex Is Independent of the ATPase Activity of Rad54 Protein—Previously, it was shown that the Rad54 K341R mutant protein, which lacks ATP hydrolysis and dsDNA unwinding activities, is still proficient in binding to both DNA and Rad51 protein (29).[2] Therefore, we tested this mutant protein with regard to its capacity to stabilize Rad51 nucleoprotein filaments. We found that the K341R mutant protein also stabilized the Rad51-ssDNA nucleoprotein to the same degree as the wild type Rad54 protein and that it also showed a high affinity for DNA (Fig. 4*C*). These results show that neither ATP hydrolysis nor DNA unwinding activity is needed for stabilization of the Rad51 nucleoprotein filament. Rather, we conclude that the stabilizing effect of Rad54 protein on the Rad51 filament results from both its high affinity for DNA and its ability to bind DNA and Rad51 protein simultaneously within the filament.

Rad54 Protein Increases Protection of dsDNA within the Rad51 Filament from Restriction Endonucleases—As an independent measure of filament stability, we examined the ability of Rad54 protein to protect dsDNA, within a Rad51 filament, from restriction endonuclease cleavage. The nucleoprotein complexes were formed by incubating Rad51 and Rad54 proteins with dsDNA (63 base pairs), which was then incubated with *Rsa*I restriction endonuclease. The binding of Rad51 protein alone, at a stoichiometry of 1:3 base pairs, provided only partial protection, whereas the binding of Rad54 protein alone provided no protection to dsDNA against cleavage by *Rsa*I endonuclease (Fig. 5*A*). However, the addition of Rad54 protein to the Rad51-dsDNA filament led to an almost complete protection of the dsDNA against *Rsa*I cleavage. The Rad54 protein concentration dependence of this protection (Fig. 5*B*) also resembled the profile observed in the salt titration experiments; these results are consistent with Rad54 protein stabilizing the Rad51-dsDNA nucleoprotein, thereby reducing its dissociation from dsDNA and, consequently, protecting the dsDNA from endonuclease cleavage. Thus, using two independent approaches, we find that the binding of Rad54 protein increases the stability of the Rad51-dsDNA nucleoprotein.

Rad54 Protein Stabilizes the Rad51 Nucleoprotein Filament against Dissociation—The experiments described thus far were performed with relatively short DNA substrates. The Rad51 nucleoprotein filaments formed with such short fragments are intrinsically unstable. Therefore, we asked whether Rad54 protein also stabilizes longer Rad51-DNA nucleoprotein filaments. To answer this question, we studied the effect of Rad54 protein on the transfer of Rad51 protein from complexes formed with relaxed circular pUC19 dsDNA to bead-immobilized dsDNA (100 base pairs) (Fig. 6*A*). We found that even low Rad54 protein concentrations (0.034 μM; representing a stoichiometry of 1 Rad54 protein monomer per 50 Rad51 protein monomers) significantly inhibited transfer of Rad51 protein from the nucleoprotein complex with pUC19 DNA to the naked bead-immobilized dsDNA (Fig. 6*B*). Thus, Rad54 protein has significant stabilizing effect on both short and long Rad51 nucleoprotein filaments.

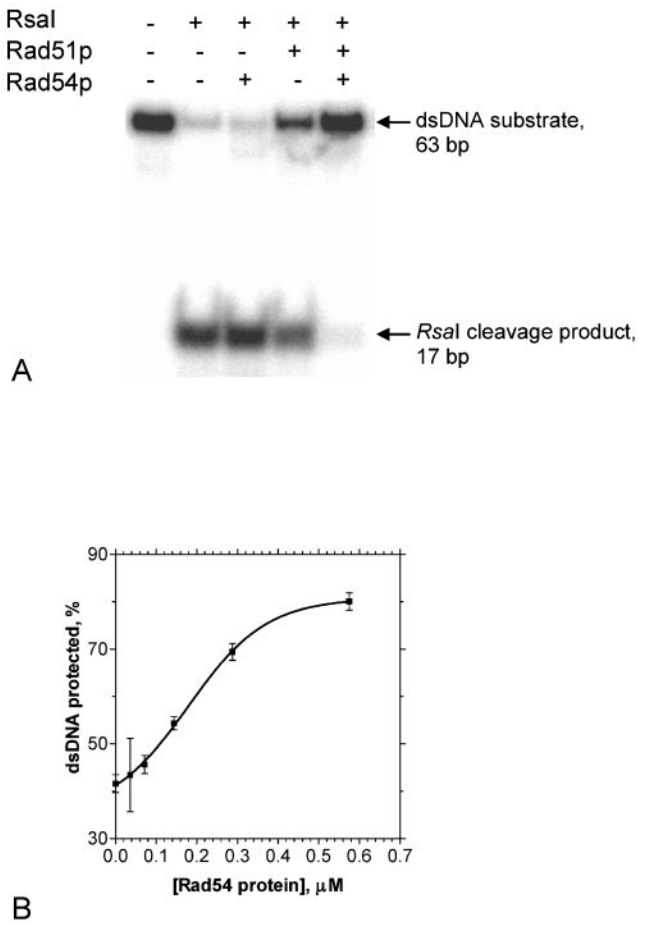

FIG. 5. **The binding of Rad54 protein to the Rad51-dsDNA nucleoprotein protects it against endonucleolytic cleavage.** *A*, the Rad51 and Rad54 nucleoprotein complexes were formed by incubating Rad51 protein (0.575 μM) or Rad54 protein (0.575 μM), respectively, with dsDNA (63 base pairs, oligonucleotides 109–111) (3.45 μM, nucleotides). Rad54-Rad51-dsDNA nucleoprotein co-complexes were formed by incubating the Rad51-dsDNA complexes with Rad54 protein (0.575 μM) for 5 min. *Rsa*I endonuclease (10 units) was added to each complex. DNA products were deproteinized and analyzed by electrophoresis in 10% polyacrylamide gels, with untreated dsDNA as a control (*left lane*). *B*, a titration with Rad54 protein was performed as in *A*, except the Rad51 concentration was 0.85 μM.

Rad54 Protein Increases the Salt Resistance of DNA Strand Exchange Promoted by Rad51 Protein—We next asked whether the stabilizing effect of Rad54 protein on the Rad51-DNA nucleoprotein filament contributes to the latter's capacity to promote DNA strand exchange. To answer this question, we measured the efficiency of DNA strand exchange in the presence of Rad54 protein at increasing NaCl concentrations. DNA strand exchange promoted by Rad51 protein alone showed a sensitivity to NaCl; the product yield decreased by one-half at 115 mM NaCl (Fig. 7). However, in the presence of Rad54 protein, both the yield of DNA strand exchange products was higher at all conditions, and the reaction itself was less sensitive to inhibition by NaCl; 220 mM NaCl was required for a 50% inhibition. Thus, we conclude that Rad54 protein not only stimulates DNA strand exchange under what might be considered "standard" *in vitro* reaction conditions, but it also specifically increases the resistance of the reaction to increasing salt concentrations. This result indicates that the stabilizing effect of Rad54 protein on the Rad51-ssDNA filament, indeed, contributes to the stimulation of DNA strand exchange.

[2] A. V. Mazin, A. A. Alexeev, and S. C. Kowalczykowski, unpublished observations.

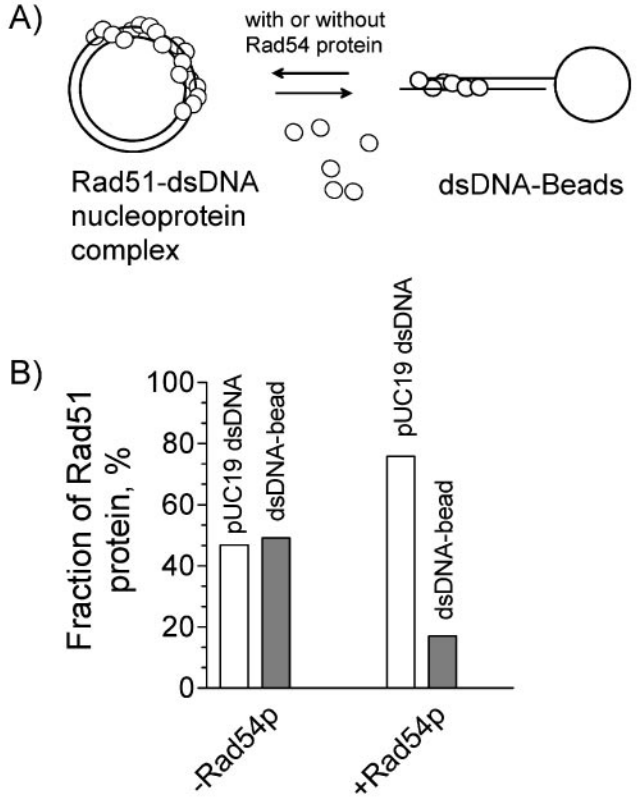

FIG. 6. **Rad54 protein inhibits transfer of Rad51 protein between DNA molecules.** The experimental outline is shown in *A*. The Rad51 nucleoprotein filament was formed by incubating Rad51 protein (1.7 μM) with relaxed circular pUC19 dsDNA (10 μM, nucleotides). The reaction mixture was divided in two, and Rad54 protein (0.034 μM) was added to one set. These two mixtures were incubated with dsDNA-beads (100 base pairs, oligonucleotides 77 and 78) (10 μM, nucleotides) for 30 min at 37 °C. *B*, the amounts of Rad51 protein remaining associated with the pUC19 dsDNA in solution (*white bars*) or transferred to the DNA-bead fraction (*gray bars*) were determined as described under "Experimental Procedures."

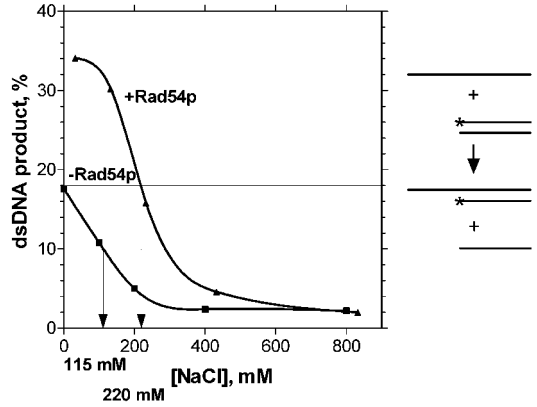

FIG. 7. **Rad54 protein increases the salt resistance of DNA strand exchange promoted by Rad51 protein.** The DNA strand exchange reaction employed is shown in the *right panel*. Nucleoprotein complexes were formed by incubating Rad51 protein (1 μM) with ssDNA (63-mer, oligonucleotide 2) (3 μM). The reaction mixture was divided in two, and Rad54 protein (0.125 μM) was added to one set. DNA strand exchange with dsDNA (32 base pairs, oligonucleotides 5 and 6) (3 μM, nucleotides) was carried out for 15 min at the indicated NaCl concentrations. The products of DNA strand exchange were deproteinized and analyzed by electrophoresis in a 10% polyacrylamide gel in TBE buffer. The *arrows* indicate the salt concentration at which the reaction is half-inhibited. Experiments were repeated three times, with S.E. values not exceeding 10–12%.

DISCUSSION

Recent studies have revealed extensive species-specific interactions between the proteins involved in homologous recombination. For instance, the Rad51 protein interacts with a number of other homologous recombination proteins, including Rad52 protein (8), Rad55 protein (40), and Rad54 protein (41, 42). In addition, Rad55 protein forms a stable heterodimer with Rad57 protein (21); Rad50 protein, Mre11 protein, and Xrs2 protein form a heterotrimer (43); and Rad52 protein interacts with RPA (44, 45). These multiple interactions permit formation of a variety of complexes, whose dynamic properties permit great flexibility in responding to various endogenous or exogenous DNA damages. It is important, therefore, to understand the consequences of these interactions on the DNA pairing activity of a protein central to the recombinational repair process, the Rad51 protein.

Our results demonstrate that Rad54 protein binds directly to the Rad51-DNA nucleoprotein filament to form a Rad54-Rad51-DNA co-complex. At saturation, the binding stoichiometry of Rad54 protein within this joint complex is ~1:1 relative to Rad51 protein. Previously, based on enzymatic assays, Rad54 was inferred to interact with the assembled Rad51-ssDNA filament (24, 31). This interaction leads to synergistic enhancement of the DNA pairing activity of Rad51 protein and, reciprocally, the dsDNA-dependent ATPase and the dsDNA topological unwinding activities of Rad54 protein. These findings were important primarily because they argued that Rad54 protein was not acting independently at the duplex DNA to promote DNA recombination but, rather, that it was acting as part of, and in concert with, the Rad51 presynaptic filament. Our findings here provide physical evidence for this novel stoichiometric interaction between a recombination protein and the Rad51 nucleoprotein filament, and they also provide a mechanistic explanation for enhancement of the enzymatic activities of both Rad51 and Rad54 proteins.

We should emphasize that, despite demonstrating the existence of saturated Rad54-Rad51-DNA complexes, the stimulatory effects are linear in response to increasing Rad54 protein concentration, and stimulation occurs over a broad range of concentrations. For example, our current results demonstrate that even when present at 1 Rad54 protein monomer per 50 Rad51 protein monomers, Rad54 protein exerts a stabilizing effect on the Rad51-DNA filament, and increasing the Rad54 protein concentration results in progressively increased stabilization. Similarly, increasing Rad54 protein concentrations progressively stimulate DNA strand exchange at protein ratios ranging from "catalytic" (1:50 Rad54/Rad51) to "stoichiometric" (1:1) (24, 28, 31). Thus, the maximal stimulation seen at equimolar amounts of Rad54 and Rad51 proteins in certain assays represents the limiting level rather than an obligatory requirement to form such saturated complexes. In this regard, it remains intriguing that the intracellular ratio of Rad54 protein to Rad51 protein for both yeast and mouse cells is approximately equimolar (46, 47), a physiological finding that is consistent with our biochemical observations. The observation that stimulation saturates at lower than equimolar in some *in vitro* experiments can result from any number of factors. For instance, the maximal level of the stimulation observed in experiments with longer (kb-sized) ssDNA substrates was at ~1 Rad54 protein per 20–50 Rad51 protein monomers (31). Because Rad51 protein forms more stable filaments with longer DNA substrates, saturating amounts of Rad54 protein would not necessarily be needed; furthermore, we found that with such DNA substrates (*e.g.* ϕX174 ssDNA), the higher Rad54 protein concentrations caused artifactual aggregation of the DNA and the consequent inhibition of DNA strand ex-

change.[3] Also, lowering either the magnesium ion concentration or the temperature, both factors that destabilize RecA- and Rad51-ssDNA filament, may result in less assembly of Rad51 protein into the presynaptic filament than expected, particularly with short DNA substrates, resulting in a concomitantly reduced concentration of Rad54 protein at saturation (28). The thermal instability of Rad54 protein (31, 48) cannot explain the observed stoichiometries in the oligonucleotide-based D-loop assays (28), because the assay time is short. Thus, both real and artifactual reasons can explain lower than maximal stoichiometries. Regardless, these collective experiments demonstrate that Rad54 protein interacts directly with the Rad51 nucleoprotein filament and that this co-complex is the active species in recombination processes.

We showed here that the binding of Rad54 protein to the Rad51-DNA nucleoprotein filament has a strong effect on its stability and that this is one element explaining the enhanced functionality of the co-complex. Our results provide three lines of evidence that the binding of Rad54 protein increases the structural stability of both the Rad51-ssDNA and Rad51-dsDNA nucleoprotein filaments. First, salt-titration experiments yield direct evidence that Rad54 protein has a stabilizing effect on the Rad51 nucleoprotein complex formed with either ssDNA or dsDNA. Second, the Rad54-Rad51-dsDNA co-complex provides much better protection to dsDNA against restriction endonucleases than does either protein alone. Finally, the binding of Rad54 protein significantly inhibits the transfer of Rad51 protein from one dsDNA molecule onto another. Stabilization does not depend on either the ATPase or on DNA topology-remodeling activities of Rad54 protein, because the Rad54 mutant protein, K341R, which lacks both of these activities, has a similar stabilizing effect on the Rad51 nucleoprotein filament. Hence, we believe that Rad54 protein stabilizes the Rad51-DNA nucleoprotein filament by virtue of its ability to interact simultaneously with Rad51 protein and the DNA molecule within the filament.

Experiments using the yeast two-hybrid system and immunoprecipitation proved that Rad51 protein can interact with Rad54 protein (41, 42). We demonstrated directly that Rad51 protein retains its ability to interact with Rad54 protein when it is assembled into a filament on DNA, and we also showed that Rad54 protein also interacts with the DNA component of the complex (Fig. 4). In addition, we observed that the Rad51-dsDNA nucleoprotein filament, formed with saturating concentrations of Rad51 protein, stimulates the ATPase activity of Rad54 protein better than naked dsDNA, especially at low Rad54 protein concentrations.[3] This stimulation is species-specific, because when yeast Rad51 protein was replaced in the nucleoprotein complex with its human homologue, an inhibition of the ATPase activity of Rad54 protein resulted.[3] Taken together, these results indicate that Rad51 protein within the filament interacts specifically with Rad54 protein and facilitates its incorporation onto the filament, and we conclude that Rad54 protein can interact with dsDNA within the Rad51-dsDNA nucleoprotein filament. In contrast, the binding of Rad54 protein to the Rad51-ssDNA does not activate ATP hydrolysis by Rad54 protein (24), indicating that the interaction of Rad54 protein with dsDNA within the filament is essential for its ATPase activity. The DNA-binding experiments show that, even in the context of the Rad51-DNA nucleoprotein filament, Rad54 protein displays very high affinity for DNA; incubation of the Rad54-Rad51-DNA complexes at 0.8 M NaCl, which removes virtually all Rad51 protein, could not remove Rad54 protein completely. This is true for both ssDNA (Fig. 4) and dsDNA (not shown). We believe that this high affinity for DNA is the factor responsible for the stabilizing effect of Rad54 protein on the Rad51 nucleoprotein filament.

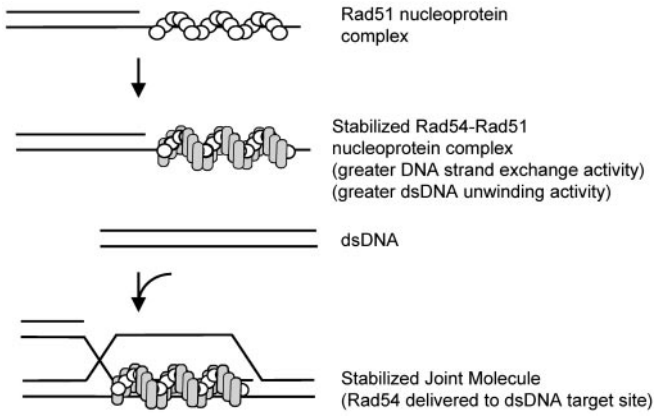

FIG. 8. **Proposed role of Rad54 protein: stabilization of the Rad51 nucleoprotein filament.** Shown is Rad51-ssDNA filament formed on ssDNA resulting from nucleolytic processing of a double-stranded break. Binding of Rad54 protein stabilizes the initially unstable Rad51-ssDNA filament and prevents Rad51 protein dissociation and transfer to undamaged dsDNA. This stabilization stimulates DNA strand exchange promoted by Rad51 protein, and Rad54 protein is delivered to the homologous target dsDNA by the Rad51 nucleoprotein filament. The enhanced stability of the Rad51-dsDNA product stabilizes the nascent DNA heteroduplex.

Genetic studies suggest that Rad54 protein functions after formation of the Rad51 presynaptic filament (49, 50). Our current results add to this now canonical view by establishing that the binding of Rad54 protein to the preformed Rad51-DNA filament stabilizes it against dissociation. Thus, the stabilization of existing Rad51-ssDNA filaments constitutes an additional role of Rad54 protein in a "late" step of the presynaptic stage of DNA strand exchange. There are *in vivo* observations regarding this proposed presynaptic function for Rad54 protein that are consistent with our results. First, in mouse embryonic stem cells or in meiotic *S. cerevisiae* cells, Rad51 protein foci formation is either Rad54 protein-dependent (23) or accelerated by Rad54 protein (51). Given that visible foci represent extensive protein-DNA complexes, our results suggest that the stabilization afforded by Rad54 protein binding allows detection of either presynaptic complexes (a stabilized Rad51-ssDNA filament) or synaptic complexes (a stabilized Rad51-heteroduplex DNA filament). Second, expression of human Rad54 K189R, which is completely deficient in the ATPase and DNA topology remodeling activities that are essential for stimulation of DNA strand exchange *in vitro*, partially rescues the γ-ray and mitomycin-C sensitivity of *mRAD54* knockout mouse ES cells (48). These observations point to an additional Rad54 protein function that is independent of its enzymatic activities; perhaps, *in vivo*, the stabilization of Rad51-DNA nucleoprotein complex by the Rad54 K341R protein that we detect might protect the processed ssDNA against nucleolytic degradation and thereby allow it to participate in repair through alternative mechanisms. Third, recently it was demonstrated that overexpression of Rad54 protein in *S. cerevisiae* suppresses the γ-ray sensitivity of *rad57* or *rad51-K191R* strains and that functional *RAD57* was needed for this suppression of the mutant *rad51-K191R* strain (52). These results indicate that the Rad51-K191R protein, which probably has a decreased affinity for DNA (53), requires Rad55/57 protein to form a presynaptic complex (21) and that this mutant Rad51 protein can function only when excess Rad54 is present. The authors concluded that these *in vivo* findings are consistent with a stabilizing effect of Rad54 protein on the Rad51-ssDNA filament (52).

[3] A. V. Mazin and S. C. Kowalczykowski, unpublished observations.

In summary, our results demonstrate that Rad54 protein forms a co-complex with the Rad51-DNA filament and stabilizes it. We imagine that co-complex formation and stabilization are important for several steps in genetic recombination (Fig. 8). By binding to the Rad51-ssDNA complex, Rad54 protein stabilizes the presynaptic complex. *In vitro*, this stabilization is especially important for stimulation of DNA pairing by Rad51 protein using short ssDNA substrates. *In vivo*, this mode of stimulation may act in a similar way by stabilizing short, and therefore intrinsically unstable, Rad51-ssDNA filaments and making them longer lived in both the homology search process and dsDNA invasion. Because Rad54 protein is part of the presynaptic filament, it can now participate directly in the homology search and DNA pairing steps, probably facilitating DNA strand exchange through its DNA topological unwinding ability. In addition, recently it was shown that Rad54 protein is needed for pairing when the target is chromatin (54); both that work and our own (55) have shown that that Rad54 protein can remodel chromatin. Thus, the removal of nucleosomal structure further increases the accessibility of dsDNA for homologous recognition and pairing *in vivo*. Furthermore, since Rad51 protein is bound to the heteroduplex DNA product, the continued binding of Rad54 protein to this heteroduplex product will help to stabilize this intrinsically labile nascent joint molecule; one suggested interpretation of recent mutational analysis is in accord with heteroduplex DNA stabilization (56). Finally, by virtue of having been delivered to the heteroduplex DNA, Rad54 protein can use its inferred translocation ability (26, 27) to migrate the heteroduplex joint as is observed (25). This movement of Rad54 protein on the duplex DNA can also help strip the stable Rad51-dsDNA complex that remains (57), especially if Rad54 protein were to start translocating through the region of DNA heteroduplex that is bound with Rad51 protein. Therefore, Rad54 protein is a multifunctional protein that can have a role in the presynaptic, synaptic, and postsynaptic steps of DNA strand exchange, acting in each step by a different mechanism: binding, unwinding, and translocation, respectively.

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