Rad51-catalyzed DNA strand exchange is greatly enhanced by the single-stranded (ss) DNA binding factor RPA if the latter is introduced after Rad51 has already nucleated onto the initiating ssDNA substrate. Paradoxically, co-addition of RPA with Rad51 to the ssDNA to mimic the in vivo situation diminishes the level of strand exchange, revealing competition between RPA and Rad51 for binding sites on ssDNA. Rad52 promotes strand exchange but only when there is a need for Rad51 to compete with RPA for loading onto ssDNA. Rad52 is multimeric, binds ssDNA, and targets Rad51 to ssDNA. Maximal restoration of pairing and strand exchange requires amounts of Rad52 substoichiometric to Rad51 and involves a stable, equimolar complex between Rad51 and Rad52. The Rad51-Rad52 complex efficiently utilizes a ssDNA template saturated with RPA for homologous pairing but does not appear to be more active than Rad51 when an RPA-free ssDNA template is used. Rad52 does not substitute for RPA in the pairing and strand exchange reaction nor does it lower the dependence of the reaction on Rad51 or RPA.

In eukaryotic organisms, genetic recombination is mediated by genes of the RAD52 epistasis group. These genes were first identified in Saccharomyces cerevisiae and consist of RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RAD54/ TID1, MRE11, and XRS2. Mutations in these genes very often result in severe meiotic phenotypes including an arrest in meiotic prophase, low sporulation efficiency, and spore inviability. These ancillary protein factors, or mediators (15, 16), are functionally equivalent to the E. coli RecO-RecR complex (17) and T4 UvsY protein (18–21), which allow their cognate recombinases RecA and UvsX to gain access to ssDNA already coated with the ssDNA binding factor. However, the manner in which Rad52 and the Rad55-Rad57 heterodimer overcome the competition by RPA is not known at the present time. Here we describe biochemical studies that enable us to begin understanding the biochemical properties and the mediator function of Rad52 in greater detail.

MATERIALS AND METHODS

Purification of Recombination Proteins

Rad52—E. coli strain M15(pREP4) harboring the plasmid expressing His6-tagged Rad52 under the control of the T5 promoter (gift from Rodney Rothstein) (22) was used. Extract was made from 15 g of cell paste (from 8 liters of culture) in 120 ml of cell breakage buffer (50 mM Tris-Cl, pH 7.5, 10% sucrose, 150 mM KCl, 3 mM EDTA, 1 mM 2-mercaptoethanol) in the presence of protease inhibitors using a French press (23). The lysate was clarified by centrifugation (100,000 × g, 90 min), and the supernatant (fraction I, 120 ml) was applied onto a column of SP-Sepharose (2.5 × 61 cm; 30 ml total) equilibrated with 150–650 mM KCl in buffer K. Rad52 elutes from SP-Sepharose at ~360 mM KCl, the pool of which (fraction II, 30 ml) was diluted with 2 volumes of K buffer and applied to a Q-Sepharose column (1.5 × 5.5 cm; 8 ml total), which was developed with a 100-ml gradient of 120–450 mM KCl in K buffer, collecting 2-ml fractions. The pool of Rad52 (fraction

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III; 10 ml, eluting from Q-Sepharose at about 300 mM KCl, was mixed with 1 ml of nickel-NTA-agarose (Qiagen) for 2 h at 4 °C. The nickel matrix was poured into a glass column (1 cm diameter) and washed with 10 volumes each of 10, 20, and 30 mM imidazole in buffer K containing 500 mM KCl. Rad52 was eluted with 4 ml of 200 mM imidazole in buffer K containing 500 mM KCl and then concentrated to 1 ml using a Centricon microconcentrator (Amicon). The concentrated nickel pool (fraction IV) was subject to sizing in a column of Sepharose 6B (1.6 x 40 cm; 80 ml matrix) in buffer K containing 150 mM KCl. The Rad52 pool (fraction V, 8 ml) was loaded directly onto a Mono S column (HR5/5), which was developed with a 30-ml gradient of 150–500 mM KCl in buffer K. Fractions containing the peak of Rad52 protein, eluting at ~350 mM KCl, were pooled (fraction VI; 4 ml) and concentrated to 5 mg/ml and stored in small portions at −70 °C. The concentration of Rad52 protein was measured by densitometric comparison of multiple loadings of Rad52 protein against known amounts of bovine serum albumin and ovalbumin in a Coomassie Blue R-stained polyacrylamide gel.

Rad51 Protein—Rad51 was purified to near-homogeneity from yeast strain LP749-9B harboring the plasmid pRS1 (3 μm, PGK-RAD51), using a combination of ammonium sulfate precipitation and chromatographic fractionation steps in columns of Q-Sepharose, hydroxyapatite, Bio-Rex 70, and Mono Q (7). Rad51 was stored in K buffer containing 350 mM KCl. The concentration of Rad51 protein was measured using a molar extinction coefficient of 1.29 x 10^10 M^-1 cm^-1 at 280 nm (33).

RPA—RPA was purified from a yeast strain genetically tailored to co-overexpress the three subunits of RPA (a gift from Richard Kolodner). Extract was prepared and then subjected to fractionation in columns of Affi-Gel Blue, ssDNA cellulose, hydroxyapatite, and Mono Q as described (14). The RPA purified this way was nearly homogeneous and was stored in K buffer containing 200 mM KCl. The concentration of RPA was measured by densitometric comparison of multiple loadings of RPA against known amounts of bovine serum albumin and ovalbumin in a Coomassie Blue R-stained polyacrylamide gel.

Rad51-Rad52 Complex—Purified Rad51 (3 mg) and Rad52 (3.25 mg) were incubated in 5 ml of buffer K with 300 mM KCl for 12 h on ice and then mixed with 1 ml of nickel-NTA-agarose to immobilize the Rad51-Rad52 complex, which was eluted from the nickel matrix with 3 ml of 200 mM imidazole in buffer K containing 300 mM KCl. The eluate was concentrated to 0.3 ml in a Centricon-30 microconcentrator, diluted to 3 ml with buffer K containing 300 mM KCl, and reconstituted to 0.3 ml. This filter-dialysis step was repeated in the same concentrator, and the final concentrate, containing Rad51-Rad52 complex at 150 μg/ml, was stored in small portions at −70 °C.

Nucleic Acids

δX174 viral (+) strand was purchased from New England Biolabs, and the replicative form (about 90% supercoiled form and 10% nicked circular form) was from Life Technologies, Inc. The 83-mer oligonucleotide used in the standard reaction above.

Sizing by Gel Filtration

A Sepharose 6B column (1 x 45 cm; 35 ml total) was used to monitor the migration of Rad51, Rad52, and the Rad51-Rad52 complex in the experiment shown in Fig. 2A, Rad51-Rad52 complex (11.6 μm in panels II and III) and Rad52 protein (11.6 μm in panels IV and V) in 1 ml of buffer T (20 mM Tris-HCl, pH 7.5, 0.75 M nucleotides) at 150 mM KCl, were pooled (fraction VI; 4 ml) and concentrated to 5 ml of 3% SDS by boiling for 1 min, and then filtered through the sizing column at 0.2 ml/min, collecting 0.5-ml fractions. The indicated column fractions were subject to immunoblotting to examine the migration of Rad51, Rad52, and the Rad51-Rad52 complex in the standard reaction above.

Binding to Nickel-Agarose

In Fig. 2B, Rad51 and Rad52 proteins were incubated at various molar ratios (0.5 μm Rad51 and 2 μm Rad52 or 1:4; 1.5 μm Rad51 and 1.25 μm Rad52 or 1:2.1; 5 μm Rad51 and 1.5 μm Rad52 or 3:1; or 2 μm Rad51 alone) in 1 ml of K buffer containing 300 mM KCl and 0.01% Nonidet P-40 at 4 °C for 1 h. In Fig. 2C, reaction mixtures containing 1.5 μm Rad51 or a combination of 1.5 μm Rad51 and 1.5 μm Rad52 were incubated in the same buffer with or without 2.5 mM ATP and 3 mM MgCl2 for 1 h. All the mixtures were gently mixed with 200 μl of nickel-NTA-agarose beads at 4 °C for 3 h. The beads were washed with 1.0 ml of ssDNA/MgCl2 before eluting the bound proteins with 400 μl of 3% SDS by boiling for 1 min.

DNA Binding

DNA Mobility Shift—The substrates used were δX174 viral (+) strand and the replicative form linearized with PstI. In Fig. 3, A–C, the reactions contained both ssDNA (30 μm nucleotides) and dsDNA (20 μm nucleotides) with the indicated molar ratios (0.5 μm Rad51 and 2 μm Rad52 or 1:4; 1.5 μm Rad51 and 1.5 μm Rad52 complex in 10 μl of reaction buffer (35 mM K-MOPS, pH 7.2, 1 mM DTT, and 100 μg/ml BSA, with or without 2.5 mM ATP and 3 mM MgCl2, as indicated). The reaction mixtures were incubated at 25 °C for 10 min, mixed with 2 μl of loading buffer (0.1% Orange G in 30 mM Tris-HCl, pH 7.5, containing 50% glycerol), and then subjected to electrophoresis in 9.5% agarose gels at 100 mA in TAE buffer at 25 °C until the dye front had migrated 4 cm. The gels were stained with ethidium bromide to reveal the DNA species.

Binding to ssDNA Cellulose—In Fig. 3D, Rad51 (0.45 μm), Rad52 (0.45 μm), or a mixture of these two proteins in 150 μl of buffer T (20 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA, 1 mM DTT, and 0.01% Nonidet P-40) containing 150 mM KCl and 100 μg/ml BSA was incubated with or without 1.5 μl of dsDNA (30 μm nucleotides) in 1 ml of buffer T containing 300 mM KCl, and treated with 35 μl of 3% SDS by boiling for 1 min to elute bound proteins. Equivalent amounts of the input material, the supernatant that contained unbound proteins, the KCl wash, and ssDNA eluate were subject to immunoblotting to examine the Rad51 and Rad52 contents.

ATPase Assay

Rad51 protein (10 μm) with or without Rad52 protein (4 μm) was incubated with or without δX ssDNA (30 μm nucleotides) and 1 mM γ32P[ATP using the buffer conditions employed in the strand exchange assay. At the indicated times, 1-μl aliquots were removed and directly spotted on a polyethyleneimine-cellulose sheet, which was developed in 0.75 M potassium phosphate. The polyethyleneimine-cellulose sheets were analyzed in the PhosphorImager.

Homologous Pairing and Strand Exchange

δX ssDNA-based System—The indicated amounts of Rad51 protein in 1 μl of storage buffer were incubated with ssDNA (30 μm) added in 1 μl of TE buffer (10 mM Tris-HCl, pH 7.5, 0.2 mM EDTA) in 10 μl of buffer R (35 mM K-MOPS, pH 7.2, 1 mM DTT) containing 50 mM KCl, 2.5 mM ATP, and 0.5 mM MgCl2, for 5 min at 37 °C. After the addition of the indicated amounts of RPA in 0.5 μl of storage buffer, reaction mixtures were incubated at 37 °C for another 5 min before the incorporation of δX ssDNA (30 μm) in 1 μl of TE and 1 μl of 50 mM spermidine hydrochloride. The reaction mixtures were incubated at 37 °C and stopped by the addition of an equal volume of 1% SDS containing 1 mg/ml proteinase K. Deproteinization of the reaction mixtures was carried out at 37 °C for 20 min. After the addition of 0.2 volume of gel loading buffer, samples were run in 0.9% agarose gels in TAE buffer, stained with ethidium bromide for 60 min, and then destained for at least 4 h in a large volume of H2O. Images were recorded in a NucleoTech Gel documentation system and analyzed with the software provided. In the time course experiments, the reaction mixtures were scaled up accordingly, and control reactions of the mixtures were withdrawn for analysis at each time point.

Co-addition of Components—Reaction mixtures (12.5 μM final volume) containing the indicated amounts of Rad51, Rad52, and RPA were incubated on ice for 45 min, followed by the addition of δX ssDNA. The reaction mixtures were then incubated at 37 °C for 10 min, followed by the incorporation of the linear δX ssDNA and spermidine, as described in the standard reaction above.

Oligonucleotide-based System—In Fig. 6A, 1.0 μM Rad51 or Rad51-Rad52 complex was incubated with Oligo 2 (3 μm) in 10.5 μl of buffer R containing 20 mM KCl, 2.5 mM ATP, and 3 mM MgCl2, at 37 °C for 5 min, followed by the addition of 1 μl of 50 mM spermidine and the homologous duplex (6 μm) consisting of unlabeled Oligo 1 and 32P-labeled Oligo 2 in 1 μl. At the indicated times, 4 μl of the reaction mixture was...
deproteinized and resolved in a 10% polyacrylamide gel in TAE buffer, which was dried onto a sheet of DEAE paper, and the DNA species was quantified in a PhosphorImager. In the experiment in Fig. 7, A and B, unlabeled Oligo 2 (5 μM) was incubated with Rad51 or Rad51-Rad52 complex in 10 μl of buffer R at 37 °C for 45 min before an increasing concentration of RPA (0.07, 0.14, 0.28, and 0.56 μM) was added at 14°C. After a further 5 min, 1 μl of 50 mM spermidine hydrochloride and the homologous 32P-labeled duplex in 1 μl were incorporated to complete the reactions (12.5 μl final volume). Alternatively, Oligo 2 was co-incubated in 10.5 μl of buffer R with RPA and Rad51 or Rad51-Rad52 complex for 5 min or preincubated with RPA for 5 min before Rad51 or Rad51-Rad52 complex was added and then followed by an additional 5 min of incubation. Subsequent to the addition of labeled duplex DNA and spermidine, all the reaction mixtures (12.5 μl final volume) were incubated at 37°C for 15 min before being deproteinized and analyzed in 10% polyacrylamide gels in TAE buffer. The gels were dried and the DNA species quantified in the PhosphorImager.

**Rescue of Strand Exchange with Non-homologous Duplex**

All the reaction mixtures in the experiment in Fig. 5 had a final volume of 37.5 μl. In the experiments in panels I and II of Fig. 5A, Rad51 was preincubated with either dsDNA (30 μM nucleotides) and then with RPA (1.5 μM) as in the standard reaction. Following the preincubations, 1 μl of either TE or TE containing BsaI-linearized pBluescript DNA (75 μM nucleotides) was added to the reaction mixtures, which were incubated at 37 °C for another 2 min, before the dsDNA (30 μM nucleotides) and spermidine were incubated to complete the reaction. In the experiments in panel III of Fig. 5A, Rad51 (10 μM) and Rad52 proteins (2.5 μM) were preincubated with dsDNA (30 μM nucleotides) and then with RPA (1.5 μM). Following these preincubations, 1 μl of either TE or TE containing linear pBluescript duplex DNA (75 μM nucleotides) was added to the reaction mixtures, which were incubated at 37 °C for 2 additional min before dsDNA duplex DNA (30 μM nucleotides) and spermidine were incorporated to complete the reaction. As in the standard reaction, the portion of the reaction mixture was withdrawn at the indicated times and processed for gel electrophoresis.

**Self-aggregation Assay**

Self-aggregation reaction (25 μl final volume) was carried out by following the order of addition of reaction components and using the buffer described for the standard strand exchange reaction. After the addition of dsDNA, reaction samples were incubated at 37 °C for 2 min and immediately spun at 12,000 × g for 2 min at 25°C. After wash, 20 μl of the supernatant was mixed with 20 μl of 1% SDS, and 35 μl of 0.5% SDS was added to the pellet fraction to dissolve the precipitated protein-DNA complex. The supernatant and pellet fractions, 8 μl each, were analyzed for their protein contents by SDS-PAGE and Coomasie Blue staining. To examine the DNA contents, a 10-μl portion of the supernatant and pellet fractions was treated with 0.5 mg/ml proteinase K at 37 °C for 30 min and then subjected to agarose gel electrophoresis as described for the strand exchange experiments. Rad51, RPA, and DNA (ss and ds) were omitted from some of the experiments (Fig. 6, C and D), as indicated, and Oligo 2 replaced the 32P ssDNA in Fig. 6E.

**RESULTS**

**Effects of RPA and Rad52 on Rad51-mediated Strand Exchange**—We examined the level of strand exchange reaction products (Fig. 1A) by fixing the amount of Rad51 (10 μM), and we varied the concentration of RPA (0.4–2.8 μM), added either with Rad51 (Fig. 1B, panel I) or after Rad51 has already nucleated onto the ssDNA (Fig. 1B, panel II), as in the standard reaction. At levels of RPA of 2 μM and above, pronounced inhibition of the reaction was observed with the co-addition of components (Fig. 1, B and C). However, at amounts of RPA lower than 2 μM, the extent of suppression of reaction products was much less severe (Fig. 1, B and C). It seems likely that at lower concentrations of RPA, enough Rad51 can still nucleate onto the ssDNA to prime the assembly of the presynaptic nucleoprotein filament.

We have previously described expression of Rad52 in yeast and its purification to near-homogeneity (15). Since much larger amounts of functionally active and nearly homogeneous Rad52 can be obtained by expression in *E. coli* (12, 13, 22), we have since used the *E. coli* system for purifying Rad52 (22). As indicated from recent work (12, 13, 15) and reiterated here (Fig. 1D), the addition of Rad52 at an amount substoichiometric (1.2 μM) to that of Rad51 (10 μM) restored strand exchange to a level comparable to what was obtained in the standard reaction (Fig. 1D). Rad52 by itself, with or without RPA, is devoid of homologous DNA pairing activity (12, 13, 15).

**Rad52 Is Multimeric and Forms a Stable, Stoichiometric Complex with Rad51**—As shown in Fig. 2A, panel I, Rad51 (43 kDa) eluted from a Sepharose 6B column with an average Vₑ/Vᵣ (elution volume/total column volume) of ~0.7, which is slightly before the elution position of catalase (232 kDa), suggesting a multimeric structure under the conditions used. Rad52 (56 kDa) eluted from Sepharose 6B with an average Vₑ/Vᵣ of ~0.6, slightly before the elution volume of thyroglobulin (669 kDa), indicating that Rad52 also exists as a multimeric structure (Fig. 2A, panel II); the same results were obtained with Rad52 purified from yeast cells (data not shown).

When mixed with an equimolar amount of Rad52, the majority of Rad51 (>85%) emerged from the sizing column at a much earlier position (average Vₑ/Vᵣ of ~0.55) than free Rad51 (Vₑ/Vᵣ of ~0.7). Consistent with the formation of a stable complex of Rad51 and Rad52, the Rad52 peak was also shifted slightly (Fig. 2A, panel III). When the amount of Rad52 was lowered to one-fifth of the previous level, the portion of the
Rad51 shifted to the earlier elution position dropped accordingly to about 20% of the total (see Fig. 2A, panel IV). Similarly, when Rad52 was fixed at the previous level but with the amount of Rad51 being increased five times, the portion of Rad51 found in association with Rad52 was once again about 20% of the total (data not shown). Thus, Rad51 and Rad52 form a stable complex consisting of approximately equimolar amounts of the two proteins. Judging from the elution position of the Rad51-Rad52 complex, it appears that the complex contains multiple molecules of the two proteins.

Complex formation between Rad51 and Rad52 was also examined by mixing different molar amounts of the two proteins and then immobilizing the complex on nickel-NTA-agarose through the histidine tag on Rad52. The results shown in Fig. 2B are again consistent with an approximately equimolar complex between Rad51 and Rad52. The addition of ATP and magnesium did not alter the amount of the stoichiometry of Rad51-Rad52 complex formed, assessed both by immobilizing the complex via the histidine tag on Rad52 (Fig. 2C) or by sizing in Sepharose 6B (data not shown).

Rad52 Targets Rad51 to ssDNA—The same molar concentrations of Rad51, Rad52, and Rad51-Rad52 complex were incubated with a mixture of ssDNA and dsDNA in the presence of ATP and magnesium, and nucleoprotein complexes were separated from free DNA in an agarose gel and visualized by staining with ethidium bromide. Fig. 3A shows that both Rad52 and the Rad51-Rad52 complex bound specifically to the ssDNA. The experiment presented in Fig. 3A was done in the presence of ATP and magnesium, but we have detected no difference in terms of the amount of ssDNA shifted or the binding specificity of the Rad51-Rad52 complex when ATP was omitted from the reaction mixture (Fig. 3B). No significant shifting of either the ss or ds form of DNA was observed with the concentrations of Rad51 used (Fig. 3A). Much higher concentrations of Rad51 are needed to see ATP-dependent binding to the ssDNA and dsDNA (Fig. 3C).

In the DNA mobility shift experiments (Figs. 3, A and B), we could not ascertain that Rad51 was present in the nucleoprotein complex. To demonstrate directly that Rad51 is being targeted to ssDNA by Rad52, we co-incubated Rad51 and Rad52 with ssDNA cellulose and then analyzed the bound proteins by immunoblot analyses after their elution from the matrix by SDS treatment. Under the same conditions, Rad51 alone did not bind to ssDNA cellulose, but Rad52 alone did (Fig. 3D). Importantly, very similar amounts of Rad51 and Rad52 proteins were found in the ssDNA cellulose eluates, indicating that Rad51 bound along with Rad52 to the ssDNA on the matrix. In these experiments, ATP and magnesium were added to all the buffers used. However, the same results were obtained when either ATP or magnesium was omitted from the buffers (data not shown). Taken together, the results suggest that Rad51 is being targeted to ssDNA via complex formation with Rad52.

Excessive Rad52 Inhibits DNA Strand Exchange—Interestingly, quantities of Rad52 in excess of what was required to give maximal restoration of strand exchange in fact resulted in pronounced inhibition of the reaction. Specifically, at 3 μM Rad52, the level of reaction products was reduced more than 10-fold compared with the maximally restored level (Fig. 4, A and B). This inhibitory effect of Rad52 was also seen in experiments wherein RPA was incorporated after a preincubation of Rad51 and Rad52 with the ssDNA template (data not shown).

We considered the possibility that the suppression of strand exchange by excessive Rad52 might have been due to an exclusion of Rad51 from the ssDNA substrate. Since Rad52 by itself has no ATPase activity and the ssDNA-dependent ATPase activity of Rad51 provides a reliable means for estimating the level of Rad51-ssDNA nucleoprotein filament (8), we measured the Rad51-mediated ssDNA-dependent ATP hydrolysis in the presence of increasing amounts of Rad52 protein; the results of this experiment are shown in Fig. 4C. We found that an amount of Rad52 (4 μM) that would result in almost complete inhibition of pairing and strand exchange did not cause any noticeable inhibition of the Rad51 ATPase activity, strongly suggesting that Rad51 can still gain access to the ssDNA even when Rad52 is present in excess. Consistent with this deduction, we have found that a preassembled Rad51-Rad52 complex shows the same level of ssDNA-dependent ATPase activity as free Rad51 (data not shown).

Rad51 above the level of three nucleotides per protein monomer inhibits strand exchange by binding the duplex molecule (6), and this inhibition is effectively reversed by the addition of the unrelated pBluescript dsDNA to the reaction (Fig. 5, panel II in A and B). Since Rad52 also binds dsDNA, albeit with a much lower affinity than binding to ssDNA (Ref. 24, Fig. 3A), we thought it was possible that the inhibition of strand exchange by excessive Rad52 might have been due to coating of the dsDNA by Rad52. However, the addition of a relatively...
large amount of pBluescript dsDNA to reaction mixtures containing an inhibitory level of Rad52 did not restore strand exchange (Fig. 5, panel III in A and B; data not shown). We also considered the possibility that an excess of Rad52 may bind to and sequester RPA from the ssDNA, as an interaction between Rad52 and RPA has been described (24, 25). However, increasing the RPA concentration to exceed that of Rad52 was also completely ineffective in restoring strand exchange, regardless of whether the excess of RPA was added with Rad51 and Rad52 to the ssDNA or after a preincubation of Rad51 and Rad52 with the ssDNA (data not shown).

**Inhibition by Rad52 Is Likely Due to Intramolecular Aggregation**—One possible explanation for the inhibition seen (Figs. 4A and 5A) was that Rad51-Rad52 complex was inactive in DNA strand exchange and that an excess of it could impair the functionality of the nucleoprotein filament. If this was the cause of inhibition seen with the dsX substrates, then the complex of Rad51-Rad52 should be inactive in the strand exchange system that employs oligonucleotide-based substrates. To test directly this idea, we assembled and purified the Rad51-Rad52 complex (see “Experimental Procedures”) and then compared its ability to promote strand exchange between oligonucleotide-based substrates to that of Rad51. Surprisingly, the Rad51-Rad52 complex was just as active as free Rad51 in promoting strand exchange between the oligonucleotide-based substrates, regardless of whether relatively low concentrations of reactants (3 μM ssDNA, 6 μM dsDNA, 1 μM Rad51 or Rad51-Rad52 complex, as shown in Fig. 6A) or 10 times higher concentrations of reactants were used (data not shown). These results indicate that there is no functional deficit in the Rad51-Rad52 complex compared with free Rad51 in strand exchange activity.

As expected, purified Rad51-Rad52 complex was completely inactive in the strand exchange system that employs dsX DNAs, with or without RPA (data not shown). Thus, it appears that the inhibitory effect of Rad52 on strand exchange is specific for long DNA molecules, as we did not observe strand exchange inhibition with short oligonucleotide substrates (Fig. 6A). Because of this observation, we considered the possibility that perhaps multimers of Rad52 may have a tendency to interact with one another, thereby sequestering Rad51 and ssDNA through intramolecular aggregation. Indeed, when reaction mixtures containing increasing amounts of Rad52 were spun for a brief time in a bench top centrifuge, we found a Rad52 concentration-dependent aggregation of Rad51, Rad52, and ssDNA in a form recoverable in the bottom of the Eppendorf tubes (Fig. 6B). At 3 μM Rad52 (10 nucleotides per Rad52 monomer), where complete inhibition of strand exchange occurs, essentially all of the ssDNA and Rad51 co-aggregated with Rad52, whereas the expected amount of the duplex DNA remained in the supernatant (Fig. 6B, lanes 5 and 6). The amount of RPA that co-aggregated with the other reaction components varied between experiments but in general was much less than the amount of co-aggregating Rad51, Rad52, and ssDNA. The aggregation seen was induced by binding of Rad52 to the ssDNA because (i) in the absence of ssDNA the protein components remained in the supernatant after centrifugation (lanes 1 and 2 in Fig. 6, C and D), (ii) Rad52 by itself underwent DNA-dependent aggregation (lanes 3 and 4 in Fig. 6D), and (iii) Rad51, with or without RPA, did not undergo aggregation (lanes 1 and 2 in Fig. 6B, data not shown).

We have also examined whether relatively high amounts of Rad52 would inhibit strand exchange when the concentrations of the reactants were one-third those used in Fig. 6B. In these experiments, Rad52 again inhibited strand exchange when its concentration exceeded a ratio of 15 nucleotides per protein monomer, resulting in Rad51-Rad52-sDNA aggregates that were readily pelleted by a brief spin in a microcentrifuge (data not shown). As expected, incubation of concentrations of Rad52 protein and the oligonucleotide used in strand exchange experiments did not result in aggregate formation (Fig. 6E), confirming that Rad52-mediated co-aggregation of Rad51 and ssDNA occurs efficiently only with long DNA molecules and that this aggregation results in inactivation of strand exchange.

**Rad51-Rad52 Complex Can Utilize a Template Coated with**
Function of Rad52 in Recombination

Co-incubation of Rad51 and RPA also resulted in inhibition of the pairing reaction mediated by Rad51, although not to the same extent as when RPA was preincubated with the ssDNA substrate (Fig. 7A). As expected, RPA did not inhibit homologous pairing when added together with the Rad51-Rad52 complex to the ssDNA substrate (Fig. 7B) or added after Rad51 has already nucleated onto the oligonucleotide (Fig. 7A).

Rad52 Does Not Replace RPA nor Does It Lower the Dependence of Strand Exchange on RPA and Rad51—Human Rad52 enhances the efficiency of homologous DNA pairing when human RPA is absent and human Rad51 is limiting relative to the ssDNA (26). To examine whether yeast Rad52 would exert a similar stimulatory effect on yeast Rad51, we fixed the quantity of the ssDNA and used a wide range of Rad51 concentrations from ratios of 7.5 nucleotides/protein monomer to 1.9 nucleotides/protein monomer, with or without Rad52 at below the optimal level to above the optimal level for its mediator function. As shown previously (6), elevating the level of Rad51 up until three nucleotides per Rad51 monomer results in increasing extent of pairing and strand exchange, whereas ex-
Aggregation is DNA-dependent. Reactions containing Rad51 (10 μM) with Rad52 alone and is DNA-dependent. Reaction mixtures were centrifuged, and the supernatant (S) fractions were analyzed for their protein contents. C, reaction with Rad51; D, reaction with Rad51-Rad52 complex. B, aggregation of ϕX ssDNA, Rad51, and Rad52. Strand exchange reactions with final volumes of 25 μl containing ssDNA (30 μM nucleotides), dsDNA (30 μM nucleotides), RPA (2 μM), Rad51 (10 μM), and with or without Rad52 (lanes 1 and 2), 1.3 μM Rad52 (lanes 3 and 4), or 3 μM Rad52 (lanes 5 and 6) were incubated at 37 °C for 2 min and then centrifuged at 12,000 × g for 2 min at 25 °C. After centrifugation, 20 μl of the supernatant (S) was mixed with an equal volume of 1% SDS, whereas the material that remained in the tubes was mixed with 35 μl of 0.5% SDS and designated the pellet fraction (P). Portions of these fractions were analyzed by agarose gel electrophoresis for their DNA contents (I) and by SDS-PAGE for their protein contents (II), as shown. C, Rad52-mediated aggregation is DNA-dependent. Reactions containing Rad51 (10 μM), Rad52 (3 μM), RPA (2 μM), and with or without ssDNA (30 μM nucleotides) were assayed for aggregation. D, aggregation occurs with Rad52 alone and is DNA-dependent. Reaction mixtures containing 3 μM Rad52 protein and ssDNA (30 μM nucleotides) were assayed for aggregation. Symbols are the same as in C. E, aggregation does not occur with an oligonucleotide. Rad51 (10 μM), Rad52 (3 μM), and RPA (2 μM) were incubated with or without Oligo 2 (30 μM nucleotides), centrifuged, and the supernatant (S) and pellet (P) fractions were analyzed for their protein contents. B, C, and E, only the largest subunit of RPA is shown.

In progressive inhibition of the reaction, such that at 3.2 μM Rad52, little reaction product was seen (Fig. 9, A and B). As shown earlier (Fig. 6), the inhibition of strand exchange is due to intramolecular aggregation mediated by Rad52. We have also investigated whether Rad52 would lessen the dependence of the pairing and strand exchange reaction on RPA (Fig. 9, C and D), but we have found that Rad52 at a level optimal for its mediator function has no measurable effect on the RPA requirement (Fig. 9C, panel II), and higher Rad52 amounts progressively inhibit strand exchange at all concentrations of RPA tested (Fig. 9C, panel III; data not shown).

**DISCUSSION**

Rad52 as Mediator of Strand Exchange—Since the RAD52 gene is indispensable for recombination and the Rad52 protein interacts with Rad51 in a two-hybrid system (27) and in vitro (Refs. 4 and 15 and this work), it seems possible that Rad52 protein may help overcome the competition posed by RPA for binding to ssDNA. Consistent with this idea, the inclusion of Rad52 protein in a pairing and strand exchange reaction neutralizes the inhibitory effect of RPA (12, 13, 15). New et al. (12) have also demonstrated that a RecA-mediated strand exchange reaction is in fact inhibited by the inclusion of Rad52 protein. Taken together, the biochemical studies have indicated that Rad52 protein fulfills the role of a molecular mediator, functionally linking the Rad51 recombinase and the ssDNA binding factor RPA (15, 16).

The results from our immunoprecipitation studies have revealed that (i) Rad52 is of lower abundance than Rad51 pro-
creasing concentrations of Rad51 protein (4, 6, 8, 10, 12, and 16 μM in lanes 2–7, respectively) were preincubated with ssDNA (30 μM nucleotides) before the incorporation of RPA (1.5 μM), followed by dsDNA (30 μM nucleotides). The reactions in panels II–IV were set up exactly as described for panel I, except that 0.8, 1.2, and 1.8 μM Rad52 was added with Rad51 to the ssDNA in II–IV, respectively. The reaction time for these strand exchange experiments was 60 min. *jm*, joint molecules; *nc*, nicked circular duplex; *ds*, input linear duplex; *ss*, viral (+) strand and displaced linear (+) strand. B, the results shown in A are plotted: ○, results in I; [], results in II; ▲, results in III; △, results in IV.

**Fig. 8.** Effects of Rad52 on the dependence of strand exchange on Rad51. A, panel I shows strand exchange reactions in which increasing concentrations of Rad51 protein (4, 6, 8, 10, 12, and 16 μM in lanes 2–7, respectively) were preincubated with ssDNA (30 μM nucleotides) before the incorporation of RPA (1.5 μM), followed by dsDNA (30 μM nucleotides). The reactions in panels II–IV were set up exactly as described for panel I, except that 0.8, 1.2, and 1.8 μM Rad52 was added with Rad51 to the ssDNA in II–IV, respectively. The reaction time for these strand exchange experiments was 60 min. *jm*, joint molecules; *nc*, nicked circular duplex; *ds*, input linear duplex; *ss*, viral (+) strand and displaced linear (+) strand. B, the results shown in A are plotted: ○, results in I; [], results in II; ▲, results in III; △, results in IV.

protein, (ii) the majority or maybe all of the cellular Rad52 protein is associated with Rad51, and (iii) the majority of cellular Rad51 is in fact free from Rad52 (15). In apparent congruence with the immunoprecipitation data, we have demonstrated that in mediating the reversal of inhibition by RPA, amounts of Rad52 protein substoichiometric to that of Rad51 are already sufficient for the full restoration of pairing and strand exchange. We have presented results from sizing experiments that indicate a multimeric nature of Rad52 protein and that the Rad52 multimer forms a stable, approximately equimolar complex with Rad51 protein. Since maximal mediator activity is seen at Rad52 amounts about one-seventh to one-tenth that of Rad51, we deduce that perhaps only a small fraction of Rad51 is stably associated with Rad52 protein under conditions of maximal restoration of DNA strand exchange. Rad52 binds both ssDNA and dsDNA but shows higher affinity for ssDNA (Refs. 22 and 24, Fig. 3A). Whereas free Rad51 protein by itself requires ATP to bind to DNA, with apparently an equal affinity for ssDNA and dsDNA, the Rad51-Rad52 complex does not need ATP to bind DNA and appears to show a much higher affinity for ssDNA than dsDNA. It is apparent that in the presence of ATP, Rad51 is in contact with the DNA, as indicated by a normal level of ATPase activity (Fig. 4C). However, we do not yet know whether actual loading of Rad51 present in the Rad51-Rad52 complex to the ssDNA requires ATP binding and hydrolysis and whether upon binding of Rad51 to the ssDNA, Rad52 is displaced off the DNA substrate.

Although Rad52 protein enhances the level of pairing and strand exchange reaction products in the absence of RPA, even the maximally stimulated level is rather insignificant compared with what can be achieved with RPA (Fig. 9). The results from other related experiments have also indicated that the presence of Rad52 protein does not alter the amount of RPA required for maximal pairing and strand exchange (Fig. 9, C and D), thus eliminating the possibility Rad52 might have acted synergistically with RPA in the removal of secondary structure in the ssDNA substrate. We also considered the possibility that Rad52 protein may in fact lessen the reliance of the DNA strand exchange reaction on the Rad51 protein, especially that such an effect of human Rad52 on the equivalent reaction mediated by the human RAD51 protein has recently been reported by Benson et al. (26). In contrast to what has been observed with the equivalent human recombination factors, not only that yeast Rad52 does not lower the dependence of strand exchange on Rad51, it in fact suppresses this reaction significantly at suboptimal concentrations of Rad51 protein (Fig. 8, A and B). At suboptimal levels of Rad51 and without Rad52, we suspect that Rad51 could bind cooperatively to some of the ssDNA molecules and thus assemble into a limited number of functional presynaptic filaments. In the presence of Rad52 protein, however, the Rad51-Rad52 complex may nucleate randomly onto all of the ssDNA molecules and prime the assembly of partial Rad51 filaments that have only a limited ability to mediate pairing and strand exchange. Recently, an inverse DNA strand exchange reaction mediated by a RecA-dsDNA complex was reported (34). We have examined the possibility that Rad52 protein bound to a ss oligonucleotide may function with a Rad51-ds oligonucleotide complex in a similar inverse strand exchange reaction. The results indicate that the nucleoprotein complex of Rad51 with ds oligonucleotide has at most a low level of strand exchange activity. More importantly, Rad52 prebound to ssDNA in fact suppresses this already negligible level of strand exchange activity further. Although the results do not eliminate the possibility that Rad51 could promote limited inverse strand exchange, they indicate that Rad52 bound to ssDNA does not enhance a possible inverse strand exchange reaction. We have also tested whether Rad52 prebound to a ds oligonucleotide would enhance strand exchange with the Rad51-ss oligonucleotide complex. The results indicate that Rad52 suppresses strand exchange when bound to duplex oligonucleotide.

**How May Rad52 Work?—**Thus, Rad52 and RPA fulfill highly specialized functions in Rad51-mediated strand exchange, with RPA acting primarily to melt secondary structure in the ssDNA template and Rad52 functioning to facilitate Rad51-ssDNA nucleoprotein filament assembly when RPA is competing for binding sites on the template. Collectively, the results suggest that a preassembled, multimeric complex consisting of Rad51 and Rad52 binds to available sites on the ssDNA substrate, and we speculate that the DNA-bound Rad51-Rad52 complex could play two distinct roles to maximize the likelihood of assembling a functional presynaptic filament. First, the Rad51-Rad52 complex could displace RPA from the ssDNA within the vicinity of its initial loading site(s). Second, the stably bound Rad51-Rad52 complex could provide a priming effect for the recruitment of free Rad51 molecules. The growing chain of Rad51

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2 B. W. Song and P. Sung, unpublished observations.
protein filament then gradually displaces the bound RPA molecules in its path without additional assistance from Rad52 protein. It seems reasonable to suggest that the multimeric structure of Rad52 protein is relevant for its mediator function. Possibly, the oligomeric structure noted enables Rad52 protein and the Rad51-Rad52 complex to make multiple contacts with the DNA, resulting in a higher affinity of these protein species for DNA and enhanced stability of the nucleoprotein complexes that form. In addition, it is conceivable that the oligomeric structure helps ensure that one single nucleation event will lead to the loading of multiple Rad51 molecules onto the ssDNA, thus maximizing the chance for the assembly of a nascent Rad51 filament.

Interestingly, the mediator function of Rad52 described here is very familiar to those of UvsY in bacteriophage T4 and RecO-RecR complex in E. coli. These prokaryotic recombination factors promote the assembly of the recombinase-ssDNA nucleoprotein filament by overcoming the inhibition of an ssDNA-binding protein. The conservation of function of these mediators across divergent species is consistent with their importance in recombination (28).

Our studies have indicated that the complex of Rad55 and Rad57 proteins also functions as a mediator during the presynaptic phase of the pairing and strand exchange reaction. Whereas Rad55 is multimeric and associates stably with Rad51, Rad55-Rad57 is heterodimeric and interacts only weakly with Rad51 (14). Rad55-Rad57 heterodimer may act via a different mechanism or it may assist Rad51 at a stage in the assembly of the presynaptic filament temporally distinct from the reaction step that is dependent on Rad52.

RAD52-specific Recombination—In addition to functioning as a mediator in Rad51-mediated homologous DNA pairing and strand exchange, Rad52 protein also mediates the annealing of complementary single strands (22) in a reaction that is accelerated by RPA (24, 25). The DNA strand annealing activity of Rad52 is likely to be relevant for its involvement in the single-strand annealing pathway of deletion-associated recombination between direct DNA repeats (see Ref. 29 for a discussion). Rad52 is also involved in a long tract gene conversion pathway known as break-induced replication or BIR (30, 31). In BIR, a heteroduplex DNA joint is formed between an initiating single-strand and a duplex donor molecule at a region of homology, to prime DNA synthesis for copying the genetic information in the donor DNA molecule. Whether or not the ssDNA annealing activity of Rad52 is utilized for making the heteroduplex joint in BIR remains to be determined. Recently, Van Dyck et al. (32) reported that human RAD52 protein binds specifically to the ssDNA tail portion of a partially duplex DNA molecule, consistent with the postulated role of Rad52 in promoting the utilization of the ssDNA tail arising from the end-processing reaction for recombination events in vitro.

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