Functional Cross-talk among Rad51, Rad54, and Replication Protein A in Heteroduplex DNA Joint Formation*

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Saccharomyces cerevisiae Rad51, Rad54, and replication protein A (RPA) proteins work in concert to make heteroduplex DNA joints during homologous recombination. With plasmid length DNA substrates, maximal DNA joint formation is observed with amounts of Rad51 substantially below what is needed to saturate the initiating single-stranded DNA template, and, relative to Rad51, Rad54 is needed in only catalytic quantities. RPA is still indispensable for optimal reaction efficiency, but its role in this instance is to sequester free single-stranded DNA, which otherwise inhibits Rad51 and Rad54 functions. We also demonstrate that Rad54 helps overcome various reaction constraints in DNA joint formation. These results thus shed light on the function of Rad54 in the Rad51-mediated homologous DNA pairing reaction and also reveal a novel role of RPA in the presynaptic stage of this reaction.

Aside from contributing to the creation of genetic diversity, homologous recombination is indispensable for DNA double-stranded break repair, meiosis I, and for various aspects of telomere homeostasis. Genetic studies in Saccharomyces cerevisiae have been chiefly responsible for identifying the components of the recombination machinery. These recombination genes (RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54/TID1, MRE11, and XRS2) are collectively referred to as the RAD52 epistasis group. Gene cloning, genetic analyses, and biochemical studies have revealed a remarkable degree of conservation of the RAD52 group, from yeast to humans (1–4). In mammals, members of the RAD52 group interact with the tumor suppressors BRCA1 and BRCA2, which in turn influence the activities of their partner recombination factors and the efficiency of recombination (5–8). The latter observations underscore the importance for deciphering the functions of individual recombination factors and the mechanism of the protein machine comprising these factors.

Results from combined genetic and biochemical studies have suggested the following sequence of events in recombination. Following the introduction of a DNA double-stranded break, the ends of the break are processed nucleolytically to generate long single-stranded tails that have a 3’ extremity. Mre11, working in conjunction with Rad50 and Xrs2, provides the nuclease function for the formation of the 3’ ssDNA tails. Rad51, the eukaryotic equivalent of the Escherichia coli general recombinase RecA, nucleates onto the ssDNA tails to form a right-handed nucleoprotein filament. The Rad51-ssDNA nucleoprotein filament then conducts a search for a chromosomal homolog, either the sister chromatid or the homologous chromosome. Pairing between the initiating ssDNA tails and the complementary strand in the duplex partner yields heteroduplex DNA joints, followed by extension of the joints by branch migration. The biochemical reaction responsible for DNA homology search and the formation of heteroduplex DNA joints is commonly called “homologous DNA pairing and strand exchange” (1, 4, 9).

In the homologous DNA pairing and strand exchange reaction, the assembly of the Rad51-ssDNA nucleoprotein filament is referred to as the presynaptic phase. Rad52 and the Rad55-Rad57 complex are recombination mediators that promote the assembly of the Rad51-ssDNA presynaptic nucleoprotein filament (4, 10, 11). In the post-synaptic phase, the Rad51-ssDNA nucleoprotein filament cooperates with Rad54 and Rdh54 (also called Tid1) to form DNA joints (12, 13). Rad54 and Rdh54, both members of the Swi2/Snf2 protein family (4), utilize the free energy from ATP hydrolysis to produce compensatory negative and positive supercoils in duplex DNA, which probably result from a tracking motion of these proteins on DNA (13–15). The negative supercoils produced by Rad54 and Rdh54 lead to transient DNA strand opening, believed to be germane for the promotion of DNA joint formation (15). Interestingly, Rad51 enhances the DNA supercoiling and DNA strand opening activities of Rad54 (15, 16).

Whereas a good body of information concerning the biochemical properties of the RAD52 group proteins has accumulated in recent years, the manner in which these recombination factors functionally interact with one another and with the DNA substrates to achieve the maximal efficiency of DNA joint formation has remained mysterious. Here we present results that shed light on the synergistic interactions among Rad51, Rad54, RPA, and the ssDNA substrate in the initial stages of recombination. The results also reveal a new role of RPA in the homologous DNA pairing reaction.

EXPERIMENTAL PROCEDURES

Recombination Proteins—Rad51 and Rad54 proteins were overexpressed in yeast cells and purified to near homogeneity as described

* This work was supported by United States Public Health Service Grants ROI58507061 and ROI1GM57814. 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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previously (12, 17). RPA was overexpressed in yeast using three plasmids that code for the three subunits of RPA (18) and purified to near homogeneity as described (19). The concentrations of Rad51 and RPA were determined using extinction coefficients of 1.29 \times 10^4 and 8.8 \times 10^4 at 280 nm, respectively (20). The concentration of Rad54 was determined by densitometric scanning of SDS-PAGE gels of multiple loadings of purified Rad54 against known quantities of bovine serum albumin and ovalbumin.

DNA Substrates—The εX74 (++) strand and replicative form DNA were purchased from New England Biolabs and Invitrogen, respectively. The replicative form DNA was linearized by treatment with ApolI or StuI to yield linear duplex substrates that have either 3′-4-base overhangs or blunt ends, respectively. Linearization of the viral (++) strand formed a linear duplex in 3 μl of TE. The reactions were incubated for 4 min in 10 μl of buffer R (55 mM Tris-HCl, pH 7.2, 60 mM KCl, 2.5 mM ATP, 3 mM MgCl, 1 mM dithiothreitol, and an ATP-regenerating system consisting of 20 mM creatine phosphate and 300 ng of creatine kinase) with the indicated amounts of Rad51 added in 0.5 μl of storage buffer. Following the incorporation of RPA in 0.5 μl of storage buffer, the indicated amounts of ATP and RPA (9 μM) were added and an additional 4-min incubation, ApolI linearized dsDNA in 0.7 μl, and 1 μl of 50 mM spermidine hydrochloride (4 mM final concentration) was added to complete the reaction. For time course experiments, the reactions were scaled up accordingly, and unless stated otherwise, the same order of addition of reaction components was used. At the times indicated, 5-μl portions of the reaction mixtures were mixed with an equal volume of 1% SDS and then treated with proteinase K (0.5 mg/ml) for 10 min at 37 °C before being run in 0.9% agarose gels in TAE buffer (40 mM Tris acetate, pH 7.4, 0.5 mM EDTA) at 23 °C. The gels were stained with ethidium bromide and recorded in a Nucleotech gel documentation system. Quantitation of the data was done using the Gel Expert software.

L-loop Reactions with Plasmid Length ssDNA—The standard D-loop reaction was assembled by preincubating PolII-linearized εX ssDNA with Rad51 for 3 min, followed by the incorporation of RPA and an additional 3-min incubation. Rad54 was then added, and, following a 2-min incubation, εX replicative form DNA was incorporated to complete the reaction. All of the incubations were carried out at 23 °C, and the reaction mixtures were processed for electrophoresis as described above. Other details are given in the figure legends.

D-loop Reactions with Oligonucleotide as Single-stranded Substrate—Unless stated otherwise, Rad51 (2 μM) and the indicated amounts of Rad54 were incubated with the 5’-end-labeled 90-mer oligonucleotide (67 μM) with 67 nM RPA and 1.5 mM [γ-^32P]ATP (Amersham Biosciences) in 2 μl. Where indicated, dsDNA or ATP was also present during the preincubation of Rad54 at 37 °C. The reactions (10 μl) were incubated at 37 °C, and at the indicated times a 1.5-μl aliquot was removed and mixed with an equal volume of 500 mM EDTA to halt the reaction. The amount of ATP hydrolysis was determined by thin layer chromatography, as described (12). To examine the effect of Rad51 on the thermal stability of Rad54 (Fig. 1D), the combination of Rad51 (2 μM) and Rad54 (75 nM), in 8 μl of buffer A, that had or had not been preincubated at 23 °C or 37 °C as above was mixed with εX replicative form DNA (30 μM base pairs) and 1.5 mM [γ-^32P]ATP in 2 μl.

In Fig. 5D, Rad54 (150 μM) was incubated for 15 min at 23 °C with 1.5 mM [γ-^32P]ATP and the indicated amounts of ssDNA (0.9, 3.6, 7.2, 14.4, or 28.7 μM nucleotides) in 10 μl of buffer A. Rad54 (150 μM) was also similarly incubated with εX replicative form DNA (30 μM base pairs) and the indicated amounts of pBluescript ssDNA (0.9, 3.6, 7.2, 14.4, and 28.7 μM nucleotides). To examine the effect of preincubating the pBluescript ssDNA competitor with RPA, the ssDNA (150 μM nucleotides) was incubated with RPA (9 μM), as described above for Fig. 4B, and then added in 2 μl with the εX linear duplex in 1 μl of TE to the reaction.

ATPase Assay—In Fig. 1A, Rad54 (150 μM), in 5 μl of buffer A (30 mM Tris-HCl, pH 7.2, 1 mM dithiothreitol, 5 mM MgCl, 45 mM KCl, and 200 μg/ml bovine serum albumin), that had or had not been preincubated either at 37 or 23 °C was mixed with εX replicative form DNA (30 μM base pairs) and 1.5 mM [γ-^32P]ATP (Amersham Biosciences) in 2 μl. Where indicated, dsDNA or ATP was also present during the preincubation of Rad54 at 37 °C. The reactions (10 μl) were incubated at 37 °C, and at the indicated times a 1.5-μl aliquot was removed and mixed with an equal volume of 500 mM EDTA to halt the reaction. The amount of ATP hydrolysis was determined by thin layer chromatography, as described (12). To examine the effect of Rad51 on the thermal stability of Rad54 (Fig. 1D), the combination of Rad51 (2 μM) and Rad54 (75 nM), in 8 μl of buffer A, that had or had not been preincubated at 23 °C or 37 °C as above was mixed with εX replicative form DNA (30 μM base pairs) and 1.5 mM [γ-^32P]ATP in 2 μl.

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In Fig. 3A, panel III, the ATPase assay was performed as described for the model D-loop reaction with 660 nM Rad54, 1.4 μM Rad51, 1.3 μM RPA, and [γ-^32P]ATP, either with or without the ATP-regenerating system consisting of 20 mM creatine phosphate and 300 ng of creatine kinase.

RESULTS

Rad54 Is Prone to Thermal Inactivation—The rate of dsDNA-activated ATP hydrolysis by Rad54 was linear with time for at least 12 min at 23 °C (Fig. 1A). Preincubation of Rad54 at 37 °C in the absence of ATP and dsDNA for brief periods caused a dramatic decrease in ATP hydrolysis in reactions conducted at 23 °C, whereas preincubation of Rad54 at 23 °C had no effect on the ATPase activity (Fig. 1A). These results indicate that the Rad54 ATPase function is prone to denaturation at 37 °C. Whereas ATP had only a very slight protective effect against thermal denaturation of Rad54 (Fig. 1A), dsDNA exhibited a modest protective effect (Fig. 1A). Although Rad51 physically interacts with Rad54 (12, 22, 23) and stimulates the Rad54 ATPase and DNA supercoiling activities (15, 16), it did not prevent thermal denaturation of Rad54 (Fig. 1A).

In the homologous DNA pairing reaction, preincubation of Rad54 or the combination of Rad51 and Rad54 at 37 °C for brief periods also greatly diminished the extent of the reaction (see below). By contrast, Rad51 is stable for at least 30 min at 37 °C, as gauged by its ATPase and homologous DNA pairing and strand exchange activities (data not shown). Thus, Rad54 is quite unstable at 37 °C, and because of this, all of the reactions involving Rad54 were routinely carried out at 23 °C, where it is
Mechanism of DNA Joint Formation

Fig. 1. Thermal inactivation of Rad54 ATPase activity. A, Rad54 (150 nM) was incubated with dX dsDNA (30 μM base pairs) and 1.5 mM [γ-32P]ATP (dark diamonds) at 23 °C for the indicated times. Alternatively, the same amount of Rad54 was preincubated at 23 °C for 10 min (gray inverted triangles), at 37 °C for 5 min (open squares) or 10 min (gray squares), with the dsDNA at 37 °C for 5 min (open triangles) or 10 min (gray triangles), and with ATP at 37 °C for 5 min (open circles) or 10 min (gray circles), prior to mixing with the remaining reaction components and continuing the incubation at 23 °C for the indicated times. B, graphical representation of time courses of ATP hydrolysis by Rad54 (75 nM), Rad51 (2 μM), and dX dsDNA (30 μM base pairs) with no preincubation (dark diamonds) or preincubation at 23 °C for 10 min (gray inverted triangles) and at 37 °C for 5 min (open squares) or 10 min (gray squares), as done in A.

much more stable. Furthermore, since homologous pairing by Rad51 and Rad54 occurs efficiently, the use of a relatively low reaction temperature also allowed us to follow the reaction kinetics with greater ease.

With Rad54, a Contiguous Rad51-ssDNA Nucleoprotein Filament Is Not Needed for Homologous Pairing—In the model homologous DNA pairing and strand exchange reaction that employs circular ssDNA and linear duplex (see Fig. 2A, panel I, for schematic) but contains no Rad54, maximal reaction efficiency is observed at 3 nucleotides per Rad51 monomer (Fig. 2B, panels I and II) (20, 24). The optimal ratio of Rad51 to ssDNA in this model reaction corresponds to the ssDNA binding site size of Rad51 (25). Based on these observations, it has been generally assumed that a contiguous Rad51-ssDNA nucleoprotein filament is needed for achieving maximal homologous DNA pairing and strand exchange.

The stoichiometric relationship between Rad51 and the ssDNA substrate in the D-loop reaction (see Fig. 2B, panels I and II) (20, 24). The optimal ratio of Rad51 to ssDNA in this model reaction corresponds to the ssDNA binding site size of Rad51 (25). Based on these observations, it has been generally assumed that a contiguous Rad51-ssDNA nucleoprotein filament is needed for achieving maximal homologous DNA pairing and strand exchange.

The stoichiometric relationship between Rad51 and the ssDNA substrate in the D-loop reaction (see Fig. 2A, panel II, for schematic) was examined here. Amounts of Rad51 varying from 30 to 3.2 nucleotides of ssDNA per protein monomer (0.66–6.1 μM Rad51 and 19.6 μM nucleotides of ssDNA) were used with a concentration of Rad54 (150 nM) sufficient to afford robust homologous DNA pairing. Surprisingly, maximal homologous pairing (see Fig. 2B, panels II and III) occurred over the range of 15 to 9 nucleotides/Rad51 monomer, which is substantially below the ratio of 3 nucleotides/Rad51 monomer needed for the formation of a contiguous Rad51 filament. In fact, increasing the Rad51 amount to 3 nucleotides/protein monomer consistently led to a greater than 3-fold decrease in the amount of D-loop (Fig. 2B, panels II and III). We note that whereas at 30 nucleotides/Rad51 monomer a substantial level of D-loop was obtained (Fig. 2B, panel II, lane 2), only a trace of reaction product was formed by an amount of Rad51 corresponding to 7.4 nucleotides/Rad51 monomer in the model reaction that did not contain Rad54 (Fig. 2B, panel I, lane 2). We have also examined the dependence of D-loop formation on the Rad51 amount with concentrations of Rad54 higher and lower than that used in the experiment above. Under those conditions, we again found that Rad51 amounts from −15 to 9 nucleotides per protein monomer were optimal and that increasing the Rad51 level beyond this optimal range resulted in a similar degree of reduction of D-loop as in Fig. 2B (data not shown).

Turnover of RecA from the bound ssDNA occurs when the ssDNA is linear (9). If there had been sufficient Rad54 dissociating from the linear ssDNA substrate in the D-loop reaction, the free Rad51 pool could have sequestered the dsDNA from pairing with the ssDNA (24). To help eliminate this caveat, we examined the pairing between a circular ssDNA with linear duplex (Fig. 2A, panel I) in the presence of Rad54. In this case, the stoichiometric relationship between Rad51 and the ssDNA (Fig. 2C) closely resembled that seen in the D-loop experiment (Fig. 2B, panel II) (i.e. with the optimal concentration range of Rad51 at −15 to 9 nucleotides per protein monomer and the reaction efficiency gradually decreasing with elevating Rad51 amounts).

In aggregate, the new data demonstrate that homologous pairing catalyzed by the combination of Rad51 and Rad54 does not require a contiguous Rad51 filament. In fact, suppression of DNA joint formation occurs when a full Rad51 filament is allowed to assemble.

Rad54 Is Required in Only Catalytic Amounts—Although a quantity of Rad54 substoichiometric to Rad51 affords a robust homologous pairing reaction (12, 15, 26) (see Fig. 2), we examined whether higher amounts of Rad54 would further enhance the reaction. Although an ATP-regenerating system was included in the pairing assays, to ensure that the increased amounts of Rad54 did not cause a depletion of the ATP pool, we also monitored the level of free ATP by thin layer chromatography. As shown in Fig. 3A, panels I and II, the optimal level of Rad54 was between 82.5 and 300 nM, substantially below that of Rad51 (1.4 μM) used. Importantly, increasing the amount of Rad54 to 660 nM in fact suppressed joint formation greatly. For example, after 2.5 min of incubation, whereas −40% of the replicative form DNA had been converted to D loop at 82.5 nM of Rad54, a ratio of Rad51/Rad54 of 17 (Fig. 3A, panel I, lane 3; panel II), only −4% of D-loop formation was observed at 660 nM.
FIG. 2. Subsaturating Rad51 amounts give maximal DNA pairing. A, the pairing systems used. In I, pairing between the circular ssDNA and the linear duplex yields a joint molecule, which has the potential of generating a nicked circular duplex and a linear single strand as products, if branch migration of the DNA joint is successful over the 5.4 kb of dX ssDNA. css, circular single strand; lds, linear duplex; jm, joint molecules; nc, nicked circular duplex; lss, linear single-stranded DNA; sc, replicative form I DNA. B, stoichiometric relationship between Rad51 and ssDNA in homologous DNA pairing and strand exchange. In panel I, circular dX ssDNA (19.6 μM nucleotides) was first incubated with Rad51 (2.6, 3.5, 4.5, 5.3, 6.1, 7, 7.8, and 8.9 μM in lanes 2–9, respectively) and then with RPA (1.3 μM) at 37 °C, before the linear duplex (10 μM base pairs) was incorporated to complete the reaction, which was stopped after 50 min of incubation at 37 °C. In panel II, linear dX ssDNA (19.6 μM nucleotides) was incubated with Rad51 (0.66, 0.88, 1.3, 1.7, 2.2, 2.6, 3.5, 4.5, 5.3, and 6.1 μM in lanes 2–11, respectively) at 23 °C and then with RPA (1.3 μM) and Rad54 (150 nM) at 23 °C before the dX replicative form I DNA (12.3 μM base pairs) was added to complete the reaction. The completed reaction mixtures were incubated at 23 °C for 5 min before electrophoresis. The efficiency of joint formation is plotted against the nucleotides (nt) to Rad51 monomer ratio in panel III. Closed circles, results from panel I of B; open squares, results from panel II. C, circular dX ssDNA (19.6 μM nucleotides) was incubated with Rad51 (0.66, 0.88, 1.3, 1.7, 2.2, 2.6, 3.5, 4.5, 5.3, and 6.1 μM in lanes 2–11, respectively) at 23 °C and then with RPA (1.3 μM) and Rad54 (150 nM) at 23 °C before the linear dX duplex (10 μM base pairs) was added. The completed reaction mixtures were incubated at 23 °C for 10 min before electrophoresis.

FIG. 3. Catalytic amounts of Rad54 are sufficient for maximal DNA joint formation. A, all of the steps were carried out at 23 °C. In panel I, linear dX ssDNA (19.6 μM nucleotides) was incubated with Rad51 (1.4 μM) and then with RPA (1.3 μM) and the indicated concentrations of Rad54 before the dX replicative form I DNA (12.3 μM base pairs) was incorporated. After 2.5 min and 5 min of incubation, a 5-μl aliquot was withdrawn and processed for electrophoresis. The agarose gel containing the samples from the 2.5-min time point is shown. lss, linear single-strand DNA; sc, replicative form DNA. The results are graphed in panel II. Panel III shows the level of ATP in D-loop reactions containing 660 nM Rad54 either with (open squares) or without (closed circles) the ATP-regenerating system. The reaction mixtures in panel III contained [γ-32P]ATP and were assembled in the same manner as those in panel I. B, the results from another series of D-loop reactions that contained 4 μM Rad51, 1.3 μM RPA, and varying amounts of Rad54 are graphed. C, the results from the 5-min time point in panel I of A (closed circles) and in B (open triangles) are plotted as percentage of D-loop against the Rad51/Rad54 ratio.
Rad54, a ratio of Rad51/Rad54 of 2.1 (Fig. 3A, panel I, lane 10; panel II). Even at the highest concentration of Rad54, the ATP level never dropped below 98% of the nucleotide pool (Fig. 3A, panel III).

We also investigated whether maximal D-loop formation would require more Rad54 at a Rad51 concentration (4 μM) significantly higher than that used in Fig. 3A (1.4 μM). However, even with the increased Rad51 amount, the optimal concentration range of Rad54 (Fig. 3B) remained essentially the same as that observed before (Fig. 3A). Once again, elevating the Rad54 amount above the optimal range resulted in a precipitous decrease in DNA joint formation (Fig. 3B). It is important to note that in this particular instance (Fig. 3B), optimal D-loop formation was at ratios of Rad51/Rad54 of 48 to 16 (Fig. 3, B and C).

Taken together, we were able to conclude that only a catalytic quantity of Rad54 is needed for robust DNA joint formation and that there does not appear to be a formal stoichiometric relationship between Rad51 and Rad54 in order to achieve maximal reaction efficiency. We will discuss under “Discussion” why D-loop formation is suppressed by relatively high Rad54 concentrations.

RPA Shields the Presynaptic Complex from Free ssDNA—In the model reaction, RPA is indispensable for maximal reaction efficiency. By helping minimize secondary structure in the ssDNA template, RPA allows for the assembly of a contiguous Rad51-ssDNA nucleoprotein filament (20, 24). The results in Fig. 2 have shown that in the D-loop reaction, Rad51 amounts...
substantially below that required to saturate the ssDNA template in fact yield significantly more D-loop than when a saturating amount of Rad51 is used. As shown in Fig. 4A, at these low Rad51 concentrations, RPA was still needed for maximal DNA joint formation. Specifically, while greater than 60% of the input substrate had been converted into D-loop after 5 min, less than 5% of D-loop was seen with the omission of RPA.

In the optimized D-loop reaction there is insufficient Rad51 to make a contiguous nucleoprotein filament, yet RPA is still needed for optimal efficiency. Therefore, we concluded that RPA probably plays another role in this reaction. We considered the possibility that perhaps by sequestering free ssDNA left uncovered by Rad51, RPA might prevent the naked ssDNA from interfering with the homologous pairing reaction. To test this hypothesis directly, we carried out a reaction in which the φX ssDNA was incubated with Rad51, Rad54, and RPA as before (e.g. Fig. 2B, panel II), but an increasing amount of the unrelated pBluescript ssDNA was added with the φX replicative form. Severe inhibition of DNA loop formation by the pBluescript ssDNA was seen (Fig. 4, B (panel I) and C). For
instance, the level of D-loop was reduced from ~60% (Fig. 4B, panel I, lane 2) after 5 min of reaction to 11 and 4% by 20 and 34 μM of the pBluescript ssDNA, respectively (Fig. 4, B (panel I, lanes 4 and 6, respectively) and C). By contrast, the addition of equivalent amounts of pBluescript dsDNA did not cause inhibition of the D-loop reaction (Fig. 4, B (panel II) and C). Importantly, preincubation of the pBluescript ssDNA competitor with an amount of RPA sufficient to completely coat the ssDNA competitor proved to be highly effective in abating the inhibitory effect of the DNA (Fig. 4, B (panel III) and C). Taken together, the results support the notion that free ssDNA left-uncovered by Rad51/Rad54 constitutes a strong inhibitor of homologous pairing. The data also lent credence to the suggestion that the main role of RPA in the D-loop reaction is to sequester protein-free ssDNA and prevent inhibition of the pairing reaction by the DNA.

Single-stranded DNA Compromises Rad51 and Rad54 Functions—The results above indicated that naked ssDNA inhibits the D-loop reaction markedly but did not address whether the ssDNA inhibitor compromises the functional integrity of the Rad51 presynaptic filament and/or Rad54 function. To identify the target(s) of inhibition by ssDNA, we first tested the effect of ssDNA on the Rad51-mediated strand exchange reaction that used φX174 DNA substrates. As shown in Fig. 5, A (lanes 3–6) and B, the addition of free pBluescript ssDNA strongly inhibited strand exchange between the φX DNA substrates. By contrast, equivalent amounts of free pBluescript dsDNA had little or no effect on the reaction efficiency (Fig. 5C). Once again, preincubation of the ssDNA competitor with RPA (Fig. 5, A (lanes 7–10) and B) was sufficient to ablate its inhibitory effect.

Rad54 has a robust ATPase activity that is dependent on DNA for activation, and dsDNA is more effective than ssDNA in supporting ATP hydrolysis (12, 27). To assess whether ssDNA can also interfere with the binding of dsDNA by Rad54, we examined the effect of adding ssDNA on the Rad54 dsDNA-dependent ATPase activity. The results, as summarized in Fig. 5D, indicated that ATP hydrolysis by Rad54 was suppressed by concentrations of ssDNA (0.9–7.2 μM nucleotides) substantially below that of the duplex (30 μM base pairs). Here too, incubating the ssDNA with RPA can effectively reverse the suppression by ssDNA (Fig. 5D). In control experiments, the addition of extra dsDNA (45 μM base pairs) had no effect on the level of ATP hydrolysis (data not shown). Taken together, the results indicated that the Rad54 dsDNA-activated ATPase activity is strongly inhibited by ssDNA. Other experiments have found that Rad54 in fact has a higher affinity for ssDNA, which, when present, prevents Rad54 from binding to dsDNA (data not shown).

Rad54 Helps Overcome Various Reaction Constraints—The Rad51-mediated homologous DNA pairing and strand exchange reaction is normally conducted at 37 °C (Fig. 6A, panel I), since lowering the reaction temperature to 23 °C greatly diminishes product formation (Fig. 6A, panels II and III). As demonstrated before (15) and reiterated here (Figs. 6 and 7), homologous DNA pairing reactions that contain Rad54 proceed efficiently at 23 °C. In the reaction that does not contain Rad54, a low level of magnesium is present during the preincubation of Rad51 with ssDNA, but the addition of either spermidine or extra magnesium with the duplex substrate is critical for robust pairing and strand exchange (17, 19) (Fig. 6B, panels I, II, and III), with spermidine being more effective than magnesium in this regard (Fig. 6B, panel III). With the inclusion of Rad54, even in the absence of spermidine or additional magnesium, a highly significant amount of DNA joints is obtained (Fig. 6B, panels V and VI). Interestingly, with Rad54, higher rates of homologous pairing are seen with the addition of magnesium (Fig. 6B, panels IV and VI) than spermidine (Fig. 6A, panel IV). The duplex substrate used in the standard DNA strand exchange reaction has either 3' or 5' overhangs, since Rad51 has very limited capacity to utilize duplex DNA with blunt ends (28) (Fig. 7, panel I). By contrast, with Rad54 in the reaction, a blunt-ended DNA substrate is efficiently used for homologous pairing (Fig. 7, panels III, IV, and V).
D-loop Reaction with an Oligonucleotide as Initiating Substrate—Recently, Mazin et al. (16) reported that maximal D-loop formation required an amount of Rad54 equivalent to that of Rad51. In this work, the D-loop reaction was carried out with an oligonucleotide as the initiating substrate (see Fig. 8A for schematic). The combination of Rad51 (2 μM) and Rad54 (0.04–1.6 μM) was mixed with the 90-mer oligonucleotide (6 μM nucleotides) in the presence of ATP. The reactions were preincubated for 10 min at either 23 or 37 °C prior to the addition of target duplex. The completed reaction mixtures were incubated at 23 °C for 5 min and processed for electrophoresis in 0.9% agarose gels. Panel II displays the reactions in which Rad51 and Rad54 were preincubated with the 90-mer oligonucleotide at 23 °C, whereas panel III shows the reaction with the preincubation step done at 37 °C. C, the results in panels II (lanes 5–14) and III (lanes 5–14) of B are graphed as a function of Rad54 concentration and against the Rad51/Rad54 ratio, as shown. The level of reaction product is expressed as the percentage of the input single-stranded oligonucleotide incorporated into the D-loop structure. D, in one reaction (closed square), Rad51 was incubated with the 90-mer oligonucleotide for 8 min at 23 °C, followed by the addition of Rad54 and a 2-min incubation at 23 °C, before the pBluescript DNA was incorporated to complete the reaction mixture. In another reaction (closed circle), Rad51 and Rad54 were coincubated with the oligonucleotide for 10 min at 23 °C, and then the pBluescript DNA was incorporated to complete the reaction. In the third reaction (open triangle), Rad51 and Rad54 were coincubated with the oligonucleotide for 10 min at 37 °C, and then the pBluescript DNA was incorporated to complete the reaction. In all three cases, the completed reaction mixture was incubated at 23 °C, and aliquots were withdrawn at the indicated times and analyzed as described for B. The concentrations of reaction components were as follows: Rad51, 2 μM; Rad54, 150 nM; 90-mer oligonucleotide, 6 μM nucleotides or 67 nM oligonucleotides; pBluescript DNA, 65 μM base pairs or 22 nM plasmid molecules.

Mazin et al. (16) also suggested that GST-Rad54 was targeted to the site of homologous pairing by the Rad51-ssDNA nucleoprotein filament. This conclusion was drawn from experiments in which the order of addition of GST-Rad54, Rad51, and ssDNA was varied. Specifically, preincubation of GST-Rad54 with the ssDNA substrate at 37 °C for 10 min, regardless of whether Rad51 was present, resulted in a substantial drop in reaction efficiency, as compared with the addition of GST-Rad54 after the formation of the Rad51 presynaptic complex (16). We wished to reexamine this issue, since we know that Rad54 is quite unstable at 37 °C (Fig. 1), which was the reaction temperature used in the work of Mazin et al. (16). Importantly, when all the reaction steps were carried out at 23 °C, preincubation of Rad51, Rad54, and the oligonucleotide resulted in nearly identical levels of D-loop as when Rad54 was added to a preassembled Rad51-ssDNA nucleoprotein complex (Fig. 8D). In sharp contrast, preincubation of Rad51, Rad54, and the oligonucleotide at 37 °C resulted in almost complete suppression of the D-loop reaction (Fig. 8, B (panel II) and C (panel I)).
ablation of D-loop formation (Fig. 8, B (panel III, lane 6) and D). The lack of D-loop formation in this instance was due to thermal inactivation of Rad54, since incubating Rad51 with the ssDNA at 37 °C for 20 min before adding Rad54 and then continuing the incubation at 37 °C did not result in inhibition of the D-loop reaction (data not shown).

We have also investigated whether increasing amounts of Rad54 would at least partially compensate for the thermal denaturation of Rad54. Although some D-loop was seen with higher concentrations of Rad54 preincubated at 37 °C (Fig. 8, B (panel III) and C (panel I)), its final level was substantially lower than what was attained when the preincubation step was done at 23 °C (Fig. 8, B (panel II) and C (panel I)). Importantly, with plasmid length ssDNA, preincubation of Rad51 and Rad54 with the ssDNA at 23 °C also did not diminish the efficiency of the D-loop reaction, whereas when 37 °C was used as the preincubation temperature, a dramatic decrease in the level of D-loop was again seen (data not shown).

To further delineate the stoichiometric relationship between Rad51 and Rad54 as a function of the reaction temperature, we carried out another series of experiments in which the Rad51 presynaptic filament was preassembled at 37 °C and then mixed with Rad54 that had not previously been exposed to 37 °C, with the actual D-loop reactions being carried out at 23, 30, and 37 °C, respectively (Fig. 9). At all three reaction temperatures, maximal D-loop formation occurred at amounts of Rad54 substoichiometric to that of Rad51. Interestingly, significantly more Rad54 was needed to achieve maximal D-loop formation at 37 °C than at 23 °C; this could be due to rapid thermal denaturation of Rad54 that was offset by increasing amounts of this protein.

Taken together, the results clearly indicate that Rad54 is equally effective in homologous pairing whether it is added with Rad51 to the ssDNA or to a preformed Rad51-ssDNA nucleoprotein complex. Our results also provide evidence that the decrease in D-loop formation seen with preincubation of Rad54 with Rad51 and the ssDNA substrate as reported by Mazin et al. (16) was probably due to thermal inactivation of Rad54.

DISCUSSION

Stoichiometric Relationship among Rad51, Rad54, and the ssDNA Substrate—We have demonstrated that in the Rad51/Rad54/RPA-mediated homologous DNA pairing reaction that utilizes plasmid length DNA substrates, a contiguous Rad51-ssDNA filament is not needed for maximal DNA joint formation. In fact, the reaction efficiency decreases significantly when an amount of Rad51 sufficient to yield a contiguous filament is used. Equally important, our results indicate that amounts of Rad54 substantially below that of Rad51 can achieve highly robust DNA joint formation and that increasing the Rad54 concentration beyond the optimal level results in a lower reaction efficiency.

Our observation that catalytic amounts of Rad54 are sufficient for attaining the maximal rate of homologous pairing is seemingly at odds with the work of Mazin et al. (16), who suggested that the assembly of a 1:1 complex of Rad51 and Rad54 was required for maximal efficiency of DNA joint formation. We do not yet have a definitive answer to this discrepancy between the two studies, but it is possible that the GST-Rad54 used in the work of Mazin et al. behaves differently than the six histidine-tagged Rad54 employed in our work. In addition, the different purification protocols used in the two studies could have resulted in Rad54 preparations with different specific activities. Last, it remains possible that the GST-Rad54 protein is even more prone to thermal denaturation than our histidine-tagged Rad54, such that higher amounts of the GST-Rad54 fusion protein could be needed for achieving optimal homologous pairing at the reaction temperature of 37 °C. Regardless of the reason(s) for the discrepancy between the two studies, we note that in the work of Mazin et al. (16), significant D-loop formation was seen at levels of Rad54 sevenfold below that of Rad51 (16). Together with our results reported here and elsewhere (12, 15), it seems clear that efficient homologous pairing is not contingent upon the assembly of an equimolar complex of Rad51 and Rad54.

Mazin et al. (16) also reported that incubation of Rad54 with the ssDNA led to a greatly diminished reaction efficiency. By contrast, we find that with both plasmid length ssDNA and an
Modulation of Homologous DNA Pairing Efficiency by ssDNA and RPA—RPA is known to promote Rad51 presynaptic filament assembly by effecting the removal of secondary structure in the DNA (20, 24). We have found that free ssDNA greatly diminishes the ability of a preassembled Rad51-ssDNA nucleoprotein filament to conduct the homologous DNA pairing and strand exchange reaction. Our results thus reveal a novel role of RPA, not in the removal of secondary DNA structure in the ssDNA template, but in sequestering ssDNA and preventing it from occupying the secondary DNA binding site in the Rad51-ssDNA nucleoprotein filament. In addition, RPA could effect the sequestering of Rad51 molecules at the end of the linear single strand, which would enhance the probability for the formation of a stable DNA joint with the homologous duplex.

We have also asked whether ssDNA affects Rad54 functions. At the expense of ATP hydrolysis, Rad54 tracks on duplex DNA and generates negatively and positively supercoiled domains in the DNA (14, 15). Furthermore, as a result of negative superhelical stress, the DNA strands in the duplex molecule undergo transient separation, resulting in a marked sensitivity to the single-stranded specific nuclease P1 (15). Regrettfully, we have been unable to ascertain whether free ssDNA inhibits Rad54-mediated DNA supercoiling and DNA strand opening, because the E. coli topoisomerase I used in monitoring DNA supercoiling is completely inhibited by ssDNA, and the P1 nuclease employed in the detection of DNA strand opening digests the ssDNA competitor rapidly. However, since both DNA supercoiling and DNA strand opening by Rad54 are strictly coupled to the hydrolysis of ATP, it seems reasonable to suggest that free ssDNA would also adversely affect the ability of Rad54 to supercoil and transiently unwind duplex DNA.

A Model for DNA Strand Invasion—The available results indicate that Rad54 tracks on the incoming duplex, producing compensatory negative and positive supercoils (14, 15). The tracking motion probably enhances the rate at which the incoming duplex molecule can be sampled for homology by the presynaptic complex. The negative supercoils produced lead to transient opening of the DNA strands that is thought to facilitate the formation of the nascent DNA joint upon locating DNA homology (14, 15).

Although it can be expected that long heteroduplex joints may only occur with a contiguous Rad51 filament, our results strongly suggest that a nascent DNA joint can be made before a contiguous filament of Rad51 is assembled on the initiating ssDNA substrate. In fact, as indicated from our biochemical experiments, the assembly of a contiguous Rad51 nucleopro-tein filament at the very initial stage of the recombination reaction may compromise the formation of the nascent DNA joint. We speculate that extensive interactions between the incoming duplex and the “secondary” DNA binding site within the presynaptic Rad51 filament may actually impede scanning of the duplex molecule for DNA homology and DNA supercoiling by Rad54. We envision that at a later stage of the recombination reaction, the branch migration of the nascent DNA joint to extend the region of heteroduplex DNA will probably require the assembly of a contiguous Rad51 nucleoprotein filament. The assembly of a contiguous Rad51 nucleoprotein filament is expected to depend on the mediator function of Rad52 and the Rad55-Rad57 heterodimer (4).

Our results have shown that an excess of Rad54 is inhibitory to DNA joint formation, suggesting that uncoordinated movement of the incoming duplex molecule relative to the presynaptic nucleoprotein complex may diminish the ability of the nucleoprotein complex to conduct DNA homology search and joint formation. Alternatively, or in addition, the ssDNA that results from extensive unwinding of the DNA duplex by Rad54 may inhibit DNA joint formation by compromising the functional integrity of the presynaptic protein complex through inhibition of Rad51 and Rad54 functions.

Acknowledgments—We are grateful to Lumir Krejci and Kelly Trujillo for reading the manuscript.

REFERENCES

1. Bianco, P. R., Tracy, R. B., and Kowalczykowski, S. C. (1998) Front. Biosci. 3, 579–603.
2. Cromie, G. A., Connelly, J. C., and Leach, D. R. (2001) Mol. Cell 8, 1163–1174.
3. Paques, F., and Haber, J. E. (1999) Microbiol. Mol. Biol. Rev. 63, 349–404.
4. Sung, P., Trujillo, R. M., and Van Komen, S. (2000) Mutat. Res. 451, 257–275.
5. Basika, G. K., Lin, S. C., Zhao, S., Sung, P., Tomkinson, A., and Lee, E. Y. (1999) Oncogene 18, 7883–7899.
6. Moynahan, M. E., Chiu, J. W., Kohler, B. H., and Jasin, M. (1999) Mol. Cell 4, 511–518.
7. Moynahan, M. E., Pierce, A. J., and Jasin, M. (2001) Mol. Cell 7, 263–272.
8. Pierce, A. J., Stark, M. J., Araujo, F. D., Moynahan, M. E., Berwick, M. B., and Jasin, M. (2001) Trends Cell Biol. 11, 52–59.
9. Rocca, A. I., and Cox, M. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8454–8460.
10. Van Komen, S., Petukhova, G., Sigurdsson, S., Stratton, S., and Sung, P. (2000) Mol. Cell 6, 583–592.
11. Mazin, A. V., Bornarth, C. J., Solinger, J. A., Heyer, W. D., and Kowalczykowski, S. C. (2000) Mol. Cell 6, 583–592.
12. Sung, P. (1994) Science 265, 1241–1243.
13. Nakagawa, T., Flores-Rozas, H., and Kolodner, R. D. (2001) J. Biol. Chem. 276, 31487–31493.
14. Namsaraev, E. A., and Berg, P. (1997) Mol. Cell 11, 1111–1121.
15. Petukhova, G., Van Komen, S., Vergano, S., Klein, H., and Sung, P. (1999) J. Biol. Chem. 274, 33839–33842.
Functional Cross-talk among Rad51, Rad54, and Replication Protein A in Heteroduplex DNA Joint Formation
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J. Biol. Chem. 2002, 277:43578-43587.
doi: 10.1074/jbc.M205864200 originally published online September 10, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M205864200

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