Rad52 promotes second-end DNA capture in double-stranded break repair to form complement-stabilized joint molecules

Amitabh V. Nimonkar[1], R. Alejandro Sica[1], and Stephen C. Kowalczykowski[2]

Departments of Microbiology and of Molecular and Cellular Biology, University of California, Davis, CA 95616-8665

Contributed by Stephen C. Kowalczykowski, December 25, 2008 (sent for review December 18, 2008)

Saccharomyces cerevisiae **Rad52 performs multiple functions during the recombinational repair of double-stranded DNA (dsDNA) breaks (DSBs). It mediates assembly of Rad51 onto single-stranded DNA (ssDNA) that is complexed with replication protein A (RPA); the resulting nucleoprotein filament pairs with homologous dsDNA to form joint molecules. Rad52 also catalyzes the annealing of complementary strands of ssDNA, even when they are complexed with RPA. Both Rad51 and Rad52 can be envisioned to promote "second-end capture," a step that pairs the ssDNA generated by processing of the second end of a DSB to the joint molecule formed by invasion of the target dsDNA by the first processed end. Here, we show that Rad52 promotes annealing of complementary ssDNA that is complexed with RPA to the displaced strand of a joint molecule, to form a complement-stabilized joint molecule. RecO, a prokaryotic homolog of Rad52, cannot form complement-stabilized joint molecules with RPA–ssDNA complexes, nor can Rad52 promote second-end capture when the ssDNA is bound with either human RPA or the prokaryotic ssDNA-binding protein, SSB, indicating a species-specific process. We conclude that Rad52 participates in second-end capture by annealing a resected DNA break, complexed with RPA, to the joint molecule product of single-end invasion event. These studies support a role for Rad52-promoted annealing in the formation of Holliday junctions in DSB repair.**

DNA annealing | DNA renaturation | Rad51 | Rad54 | replication protein A

Double-stranded DNA breaks (DSBs) can arrest cell growth, lead to loss of genetic integrity, and if unrepaired, cause cell death (1). Repairing such DSBs is a function of homologous recombination. In *Saccharomyces cerevisiae*, homologous recombination is mediated by members of the *RAD52* epistasis group (2, 3). In the classical DSB repair (DSBR) model (4, 5), repair first involves processing of the broken dsDNA ends to generate 3′-ended single-stranded DNA (ssDNA) overhangs (6), which are initially bound by replication protein A (RPA) (7, 8). RPA not only protects ssDNA from nucleases, but also prevents formation of DNA secondary structures. The binding of RPA to ssDNA is, however, nonproductive with regard to recombinational DNA repair because it blocks the binding of Rad51 protein onto the ssDNA (8). Rad51 needs to assemble on the resected ssDNA to form a nucleoprotein filament that has the capability both to find homology within an intact double-stranded (dsDNA) template and mediate the exchange of DNA strands to form a joint molecule (also referred to as a D-loop) (2, 3, 7–9). At this stage, Rad52 protein plays an important role by mediating the displacement of RPA from ssDNA by Rad51 to generate the nucleoprotein filament (10–12). Subsequently, Rad54 associates with the Rad51 nucleoprotein filament, stabilizing it and enhancing its ability to form joint molecules (13–15). This interaction with the Rad51 nucleoprotein delivers Rad54 to the dsDNA target, where the translocation capability of Rad54 extends the DNA heteroduplex (16–19). Joint molecule formation results in displacement of a DNA strand from the dsDNA target, which is then bound by RPA (8, 20).

At this point, the second end of the broken DNA molecule needs to be engaged in the repair process. The simplest possibility is that the ssDNA, formed by processing at the other end of the DSB, invades the dsDNA target to form two separate joint molecules. Rad51 would next be removed from the DNA heteroduplex by the action of Rad54 to expose the 3′ ends of both invading strands (21). The invading strands can then prime DNA synthesis to extend the length of the DNA heteroduplex (22, 23), ultimately forming two Holliday junctions (4, 24).

Alternatively, it is equally plausible that instead of a second independent invasion event, DNA synthesis extends the first joint molecule before engagement of the second end of the DSB. This would have the effect of enlarging the length of the displaced DNA strand or migrating the position of the displaced strand ["bubble-migration" (25)] toward the sequences that are homologous to the second processed DNA end. In this case, the next step in the repair process would require annealing of this displaced strand to the ssDNA tail created by resection at the other DNA end of the break (4, 26). Rad52 is a protein that is both essential to recombinational DNA repair (27) and has ssDNA-annealing activity (28). The annealing activity of Rad52 is unique among the many proteins that promote DNA annealing and renaturation because it can anneal ssDNA that is complexed with RPA (29–31). Functional homologs of Rad52 can be found in bacteria and phage, and, interestingly, annealing of protein–ssDNA complexes by these proteins displays the unique requirement for the cognate ssDNA-binding protein (SSB) (32). These facts suggested to us that ssDNA annealing was a common universal step in recombination and that this capability could be used for "second-end capture" (32, 33). Subsequent DNA synthesis from this captured second end would generate two four-way structures composed of DNA strands base paired with old and new partners simultaneously. This intermediate structure comprises a double Holliday junction (dHJ) structure (4, 24), which can then be resolved to produce either cross-over or non-cross-over products, thereby completing the repair of the DNA break.

An alternative to the DSBR pathway proposes that, after DNA synthesis, the joint molecule is disrupted to liberate the polymerase-extended invading strand that then anneals with the ssDNA formed by processing of the other end of the DSB; this pathway is termed synthesis-dependent strand-annealing (SDSA) (34). Genetic studies established that *RAD52* function was essential for SDSA (2, 3). Because these ssDNA intermediates would be bound by RPA protein, the annealing of ssDNA in the SDSA pathway also could be mediated by Rad52 (31, 32),

Author contributions: A.V.N., R.A.S., and S.C.K. designed research; A.V.N. and R.A.S. performed research; A.V.N. and R.A.S. contributed new reagents/analytic tools; A.V.N., R.A.S., and S.C.K. analyzed data; and A.V.N., R.A.S., and S.C.K. wrote the paper.

The authors declare no conflict of interest.

[1]A.V.N. and R.A.S. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: sckowalczykowski@ucdavis.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0813247106/DCSupplemental.

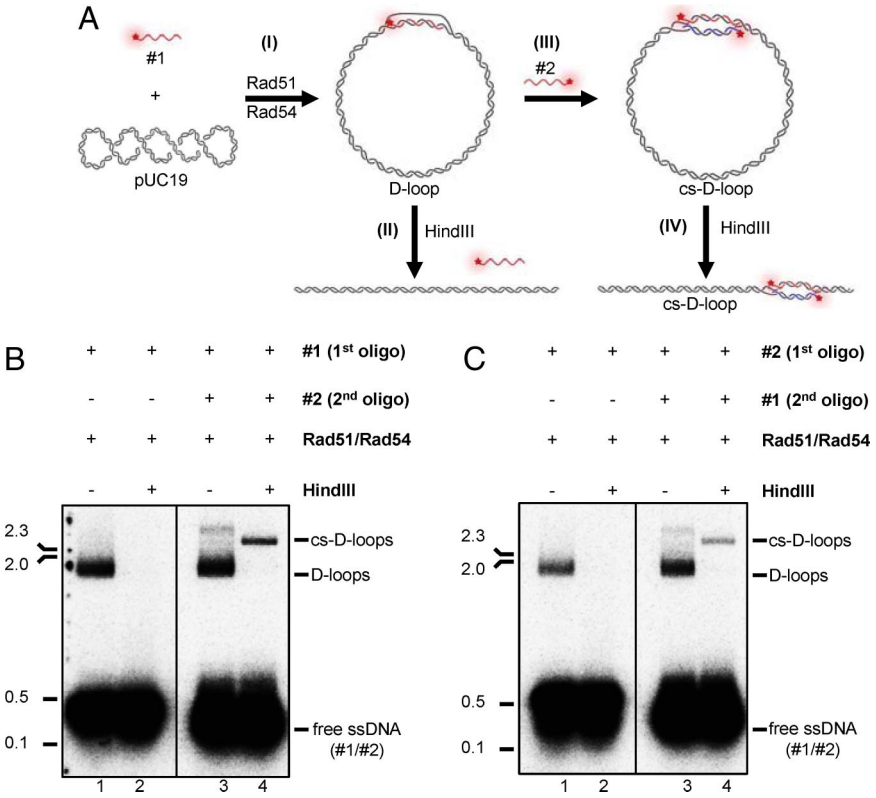

Fig. 1. Rad51 and Rad54 generate complement-stabilized D-loops. (*A*) Schematic representation of cs-D-loop formation. (*I*) Rad51 and Rad54 promote D-loop formation. (*II*) Single D-loops are unstable upon restriction. (*III*) The single D-loop can be stabilized by pairing the displaced strand in the D-loop with a complementary oligonucleotide. (*IV*) This complement-stabilized D-loop is stable upon restriction. Red star indicates position of ^{32}P label on the 5′ ends of ssDNA. (*B*) D-loops and cs-D-loops were formed as described in *Materials and Methods* and analyzed by electrophoresis. Lane 1, single D-loops; lane 2, single D-loops treated with HindIII; lane 3, cs-D-loops; lane 4, cs-D-loops treated with HindIII. The positions of D-loops, cs-D-loops, free oligonucleotides, and molecular size markers (kbp) are indicated. (*C*) The order of oligonucleotide addition was interchanged; lanes are as in *B*. The amounts of cs-D-loops formed in lane 4 of *B* and *C* were 3 ± 0.5% and 1.2 ± 0.3%, respectively, expressed relative to the scDNA concentration that is limiting; error is the SEM.

and an in vitro reaction that established key biochemical elements of this process was reported (33). DSBR via SDSA produces only non-cross-over products.

In the DSBR pathway, the notion that assimilation of the processed second DNA end is sequential rather than simultaneous with invasion of the first end (4, 5, 35) and that annealing proteins could be involved in second-end capture (32, 33, 36) prompted us to evaluate the role of Rad52 in more detail. Specifically, models of second-end capture by Rad52 require that it has the ability to anneal an RPA–ssDNA complex to the displaced stand of a joint molecule made by Rad51 and Rad54. In this work, we establish an in vitro reaction by using Rad51, Rad52, Rad54, and RPA that establishes the capacity of Rad52 to mediate annealing of an RPA–ssDNA complex to the displaced strand of the D-loop formed by Rad51 and Rad54. The product of this reaction is a distinct homologously paired joint molecule known as a complement stabilized D-loop (cs-D-loop) (37, 38). This activity provides further evidence for a pivotal role of Rad52 in homologous recombination.

Results

Rad51 and Rad54 Generate Complement-Stabilized D-Loops. To mimic second-end capture, we devised an experimental system (Fig. 1*A*) based on the described properties of cs-D-loops (37, 38). A cs-D-loop is formed when two complementary ssDNA molecules pair with the two strands of a dsDNA molecule; being complementary strands, the pairing is at the same site. Unlike single D-loops, the resulting cs-D-loops are stable upon linearization of supercoiled DNA (scDNA), which allows convenient discrimination between single and double D-loops. In our experiments, Rad51 and Rad54 catalyze single D-loop formation between an ssDNA 90-mer (oligonucleotide 1) and pUC19 scDNA (Fig. 1*B*, lane 1). As expected, these D-loops dissociate completely upon linearization with HindIII (Fig. 1*B*, lane 2), a restriction endonuclease that cleaves 24 bp away from the region of pairing. The dissociation of D-loops upon deproteinization is driven by the release of superhelical energy within the negatively super coiled DNA (39).

When a second ssDNA 90-mer (oligonucleotide 2) that is complementary to the displaced strand of the single D-loop is added, a joint molecule is generated whose mobility is the same as the single D-loop (Fig. 1*B*, lane 3). However, in contrast to the single D-loops, a fraction of the resultant joint molecules are now stable upon cleavage with HindIII (Fig. 1*B*, lane 4). Based on this characteristic, we conclude that the pairing of oligonucleotide 2 to a joint molecule formed with oligonucleotide 1 and scDNA results in the formation of a cs-D-loop. Furthermore, in agreement with previous observations (37, 38), the linearized cs-D-loops possess the same mobility as linear pUC19 (Fig. 1*B*, lanes 3 and 4) and do not dissociate during electrophoresis. When both oligonucleotides are present, approximately one-fifth of the joint molecules are stable upon linearization (compare Fig. 1*B*, lanes 3 and 4). This is because the joint molecules (lane 3) comprise both single D-loops and cs-D-loops, and the single D-loops dissociate after linearization (lane 2). Similar results were obtained upon interchanging the order of addition of the two oligonucleotides (Fig. 1*C*), although the efficiency of both D-loop and cs-D-loop formation was consistently ≈2-fold lower

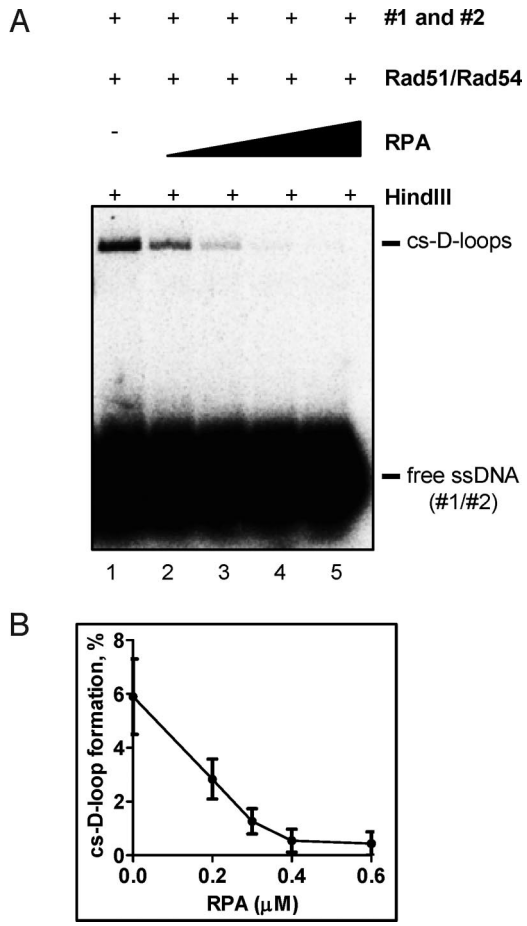

Fig. 2. RPA inhibits complement-stabilized D-loop formation in the absence of Rad52. (*A*) Gel showing inhibition of cs-D-loop formation by RPA. Standard reactions were performed with the following amounts of RPA: 0, 0.2, 0.3, 0.4, and 0.6 μM in lanes 1–5, respectively. The positions of cs-D-loops and free oligonucleotides are indicated. (*B*) Quantification of cs-D-loop formation. The percentage of cs-D-loop formation from experiments as shown in *A* was expressed with respect to the limiting scDNA concentration.

because of sequence differences affecting the initial D-loop formation (40–42). Finally, cs-D-loop formation required both Rad51 and Rad54 because both are known to be required for single D-loop formation (13, 15). Because the reaction yield was higher when D-loops were formed with oligonucleotide 1, it was used as the ssDNA for assembly of presynaptic complexes in all subsequent pairing reactions.

RPA Inhibits Complement-Stabilized D-Loop Formation. Initially, the DNA-pairing experiments were conducted in the absence of RPA because it is not essential for D-loop formation (15, 43). However, we found that when the second oligonucleotide was added to single D-loop that was formed by using Rad51 and Rad54, and then deproteinized, cs-D-loops were still formed [supporting information (SI) Fig. S1]. This finding showed that pairing of the second oligonucleotide to the single D-loops is independent of Rad51 or Rad54 and suggested that it might be occurring by spontaneous annealing of the complementary ssDNA to the D-loop. RPA is known to inhibit spontaneous annealing between two complementary oligonucleotides (30). To determine whether RPA also blocked cs-D-loop formation, oligonucleotide 2 was preincubated with varying amounts of yeast RPA before addition to D-loops formed by using oligonucleotide 1 and pUC19. Fig. 2 shows that with increasing concentrations of RPA, the efficiency of cs-D-loop formation decreased (Fig. 2*A*, lanes 1–5). Similar results were obtained when RPA was added to both the D-loop and the incoming strand, before addition of oligonucleotide 2 (data not shown). Blocking of spontaneous cs-D-loop formation was not species-specific because both *Escherichia coli* SSB and human RPA (hRPA) inhibited the reaction (Fig. S3, lanes 3 and 5). We therefore conclude that in the absence of RPA, cs-D-loop formation occurs by Rad51/Rad54-mediated D-loop formation followed by spontaneous annealing of the complementary ssDNA to the displaced strand of the D-loop. However, RPA blocks this annealing, regardless of the ssDNA strand to which it binds.

Rad52 Promotes Complement-Stabilized D-Loop Formation in the Presence of RPA. Previous work established that the displaced DNA strand in a D-loop is stabilized by an SSB or RPA proteins, which will prevent reannealing and dissociation of the nascent joint molecule (20, 44). Because Rad52 interacts with RPA to form a complex that can anneal complementary ssDNA–RPA complexes (10, 29, 30, 45), we wished to determine whether Rad52 could promote cs-D-loop formation in the presence of RPA. Consequently, cs-D-loop reactions, inhibited by RPA, were performed by using increasing concentrations of Rad52. Oligonucleotide 2 was preincubated with both RPA (0.4 μM) and Rad52, and then the complex was added to D-loops formed by Rad51 and Rad54 between oligonucleotide 1 and pUC19. This concentration of RPA completely blocked spontaneous annealing of oligonucleotide 2 to the D-loop (Fig. 3*A*, lanes 1 and 6). As anticipated, despite the presence of RPA, Rad52 enabled cs-D-loop formation (Fig. 3*A*, lanes 1–5). The formation of cs-D-loop was a function of Rad52 concentration and time, with product formation saturating at 0.8 μM and at 15 min, respectively (Fig. 3*B* and Fig. S2).

Complement-Stabilized D-Loop Formation by Rad51, Rad52, Rad54, and RPA Requires Species-Specific Interactions. We next wanted to determine whether an annealing protein that is unable to interact with RPA could mediate second-end capture as measured by cs-D-loop formation. We therefore tested the ability of RecO protein, the *E. coli* homolog of Rad52 (32), to form cs-D-loops. At all concentrations tested, RecO failed to promote cs-D-loop formation in the presence of RPA (Fig. 3*A*, lanes 6–10). This observation is consistent with the previous findings that both Rad52- and RecO-mediated annealing of ssDNA in the presence of an SSB requires the cognate binding protein (30, 32). This finding shows that the mechanism by which Rad52 mediates the second-end capture of RPA–ssDNA complexes also entails a species-specific interaction with RPA (29, 30).

To provide further evidence for the requirement of species specificity in cs-D-loop formation, we tested the ability of Rad52 to form cs-D-loops in the presence of *E. coli* SSB and hRPA. Fig. 4*A* shows that SSB is as effective as RPA in blocking spontaneous cs-D-loop formation (lanes 1 and 5). Although Rad52 could promote cs-D-loop formation in the presence of RPA (Fig. 4*A*, lanes 1–4), it was much less efficient in presence of SSB (Fig. 4*A*, lanes 5–8; Fig. 4*B*). Similar results were obtained when RPA was replaced with hRPA (Fig. S3). These finding demonstrate that the mechanism by which Rad52 accelerates second-end capture is the annealing of ssDNA with the D-loop, a reaction that requires a specific protein interaction with RPA (29, 30).

Discussion

The integrated cascade of events during DSBR that initiates with the resection of ends and culminates with recombinant DNA molecules requires the concerted action of many proteins involved in homologous recombination (2, 3). After DNA strand invasion and synthesis primed by the invading strand, the D-loop can be progress via two distinct pathways. In the SDSA pathway, the extended invading arm is displaced from the D-loop and then

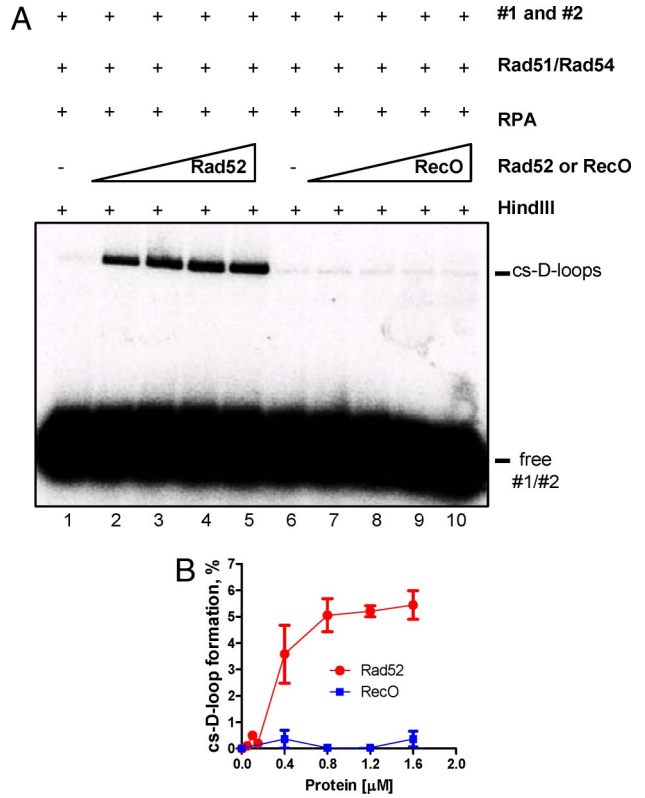

Fig. 3. Rad52 permits formation of complement-stabilized D-loops in the presence of RPA, but *E. coli* RecO does not. (*A*) Gel comparing the ability of Rad52 and RecO to mediate cs-D-loop formation in the presence of RPA (0.4 μM). Standard reactions were performed by using Rad52 or RecO: lanes 1–5: 0, 0.4, 0.8, 1.2, and 1.6 μM, Rad52, respectively; lanes 6–10: 0, 0.4, 0.8, 1.2, and 1.6 μM RecO, respectively. The NaCl added by Rad52 and RecO storage buffers was 80 mM. The positions of cs D-loops and free oligonucleotides are indicated. (*B*) Quantification of cs-D-loop formation. The percentage of cs-D-loops from experiments as shown in *A* was expressed with respect to the limiting scDNA concentration.

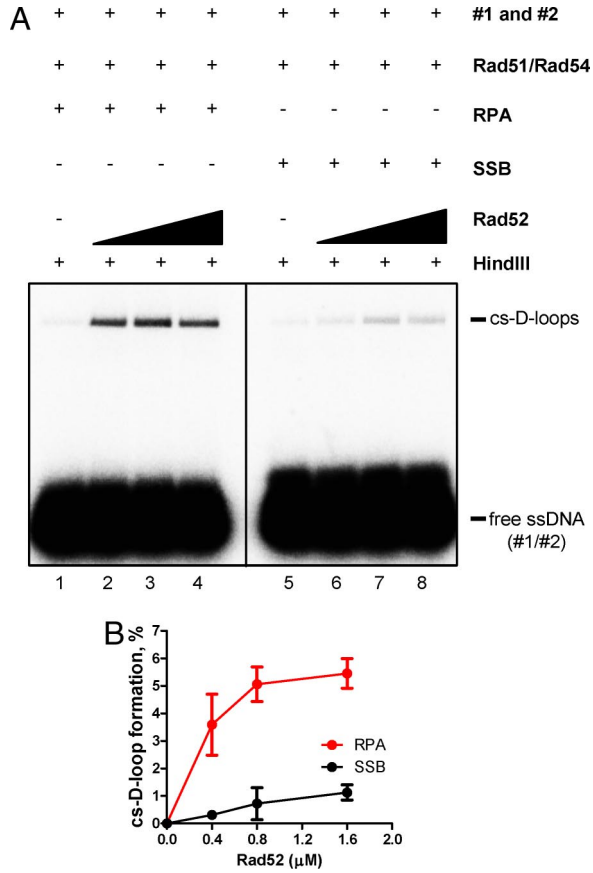

Fig. 4. Rad52 cannot efficiently form complement-stabilized D-loop in the presence of *E. coli* SSB. (*A*) Gel comparing the ability of Rad52 to mediate cs-D-loop formation in the presence of RPA or SSB. Standard reactions were performed by using RPA or SSB: lanes 1–4, RPA (0.4 μM) and 0, 0.4, 0.8, and 1.2 μM Rad52, respectively; lanes 5–8, SSB (0.4 μM) and 0, 0.4, 0.8, and 1.2 μM Rad52, respectively. The NaCl added by the Rad52 storage buffer was 80 mM. The positions of cs-D-loops and free oligonucleotides are indicated. (*B*) Quantification of cs-D-loop formation. The percentage of cs-D-loops from experiments as shown in *A* was expressed with respect to the limiting scDNA concentration.

annealed to the second resected DNA end, generating non-cross-over repair products. Alternately, in the canonical DSBR pathway, the second resected end can either invade the dsDNA target or it can anneal with the displaced strand of the D-loop (4). Further extension and branch migration of this doubly paired intermediate results in the formation of dHJs. Although dHJs can potentially be resolved to generate cross-over or non-cross-over recombinants (reviewed in ref. 46), the temporal appearance of the recombinant products during meiosis suggests that dHJs are mostly resolved to form cross-overs (5). Thus, the two ends of resected DNA can participate in two biochemically distinct reactions that dictate the nature of resolution products, i.e., cross-over or non-cross-overs. It is now evident that the primary decision point as to whether a particular recombination event will generate a cross-over or a non-cross-over is not necessarily the resolution step, but an earlier step that determines whether the second-end is captured (5, 35, 36, 47). The pairing of the second processed end to the target duplex DNA leads to dHJs and to cross-overs, whereas the renaturation of the second end to the evicted invading strand after DNA synthesis (SDSA) will result in non-cross-overs.

Our data show that productive engagement of an RPA-coated strand that is complementary to the D-loop requires Rad52. Rad52 functions in presynapsis by mediating Rad51-dependent displacement of RPA (10, 11, 48). The observation that Rad52 remains at a DNA break longer than Rad51 suggested that this protein also plays a role in a step subsequent to DNA strand invasion and DNA synthesis (7, 49). Also, our work is in agreement with recent findings that demonstrated the role of Rad52 during the postinvasion steps of recombination (36, 50) and fits with the DSBR model depicted in Fig. 5. After processing of a DNA break to create a 3′-ssDNA overhang, RPA is the first protein to bind (Step I). A species-specific interaction then recruits Rad52 (Step II). Rad52 facilitates the formation of a Rad51–ssDNA nucleoprotein filament by mediating the displacement of RPA at either or both ends of the DSB (Step III) (49, 51). The newly formed Rad51–ssDNA filament is stabilized by Rad54 binding (Step III) (52), and Rad54 stimulates joint molecule formation (13–15) via its capacity to translocate on dsDNA (19). The Rad51–Rad54–ssDNA complex then catalyzes invasion of the homologous dsDNA target, generating a displaced strand (Step IV). The displaced ssDNA in the joint molecule is a substrate for RPA and Rad52 (Step IV) (20, 30). Subsequent translocation by Rad54 clears Rad51 from the heteroduplex DNA (21), which makes the 3′ end of the invading strand accessible to DNA polymerases (53). Elongation from the 3′ end of the invading ssDNA by DNA synthesis results in further DNA strand displacement and additional binding of RPA and Rad52 (49, 51). Once a region of ssDNA that is complementary to the other side of the DNA break is exposed, annealing between the displaced strand and the resected DNA end can

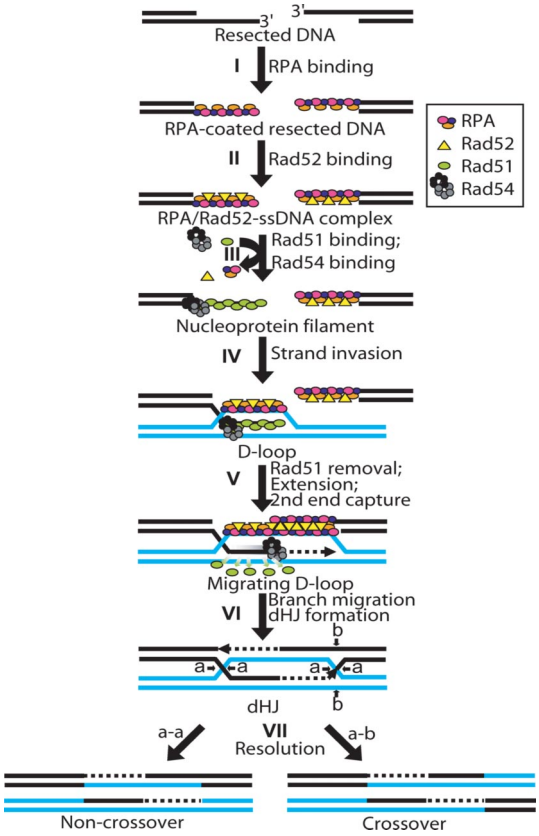

Fig. 5. Rad52-mediated second-end capture as part of DSBR. (Step I) RPA binds to the 3′-terminated ssDNA tails of the resected DSB. (Step II) Rad52 binds to the RPA–ssDNA complex. (Step III) Rad52 mediated displacement of RPA by Rad51 to form a Rad51 nucleoprotein filament (*Left end*); Rad54 binds and stabilizes the Rad51 nucleoprotein filament. (Step IV) The Rad54–Rad51–DNA nucleoprotein filament catalyzes DNA strand invasion to form a D-loop, displacing the resident strand in the target dsDNA; the displaced strand is bound by RPA and Rad52. (Step V) Rad54 clears Rad51; the invading 3′ end primes DNA synthesis; extension causes the displaced strand to advance until an ssDNA region complementary to second resected DNA end is exposed; Rad52 mediates annealing of the RPA-complexed displaced strand to second end (*Right end*). (Step VI) Additional DNA synthesis, branch migration, and ligation generate dHJs. (Step VII) Resolution to produce cross-overs or non-cross-overs.

ensue because both ssDNA molecules are covered with RPA and Rad52 [Step V; the oligomeric form of Rad52 required for DNA annealing is not illustrated (54)] (32, 33). After DNA synthesis from the second end, branch migration, and subsequent ligation, a dHJ is generated (Step VI). The dHJs can then be resolved as cross-overs or non-cross-overs (Step VII).

Using an in vitro assay, we have provided direct biochemical evidence for the ability of Rad52 to capture a processed DSB via annealing of the RPA–ssDNA complex to the displaced strand of a joint molecule. This ability of Rad52 to mediate second-end capture in the presence of RPA is specific because neither *E. coli* SSB nor hRPA can substitute in the pairing reaction. In addition, our observation that *E. coli* RecO mediates second-end capture only in the presence of RecA and SSB (E. Valencia-Morales and S.C.K., unpublished results), but not in the presence of Rad51, Rad54, and RPA further illustrates the specificity and general conservation of this process. This work and recent related work (33, 36, 50) demonstrate the importance of Rad52 in second-end capture and establish an alternative to direct DNA strand invasion by the second processed end in DNA break repair.

Materials and Methods

Enzymes and Reagents. T4 polynucleotide kinase and HindIII were purchased from New England Biolabs. ATP (disodium salt), phosphoenolpyruvate, and pyruvate kinase were purchased from Sigma. [γ-^{32}P]ATP (6,000 Ci/mmol) was purchased from NEN. Proteinase K was purchased from Roche.

DNA. Covalently closed supercoiled plasmid DNA (pUC19) was purified from *E. coli* DH5α cells by detergent lysis followed by equilibrium centrifugation in a CsCl-ethidium bromide gradient (55). Its concentration was determined by using an extinction coefficient of 6.6×10^3 M^{-1} cm^{-1} at 260 nm. Two complementary 90-mer oligonucleotides, 1 (5′-CGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGT-3′) and 2 (5′-ACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCG-3′) were synthesized and gel-purified by Sigma–Genosys. The two oligonucleotides have the same sequence as residues 471–560 of pUC19. The concentrations of the oligonucleotides were determined spectrophotometrically by using molar (nucleotide) extinction coefficients of 9.7×10^3 and 9.3×10^3 M^{-1} cm^{-1} at 260 nm, respectively. Oligonucleotides were 5′-^{32}P-labeled with T4 polynucleotide kinase and purified by using MicroSpin G-25 columns (GE Healthcare). DNA concentrations are expressed in moles of nucleotide as well as molecules.

Proteins. *S. cerevisiae* Rad51 (56), Rad52 (11), RPA (57), Rad54 (17), *E. coli* RecO (58), *E. coli* SSB (59), and hRPA (A. Carreira and S.C.K., unpublished data) were purified as described. Rad54 was purified as described in ref. 17 except for two modifications: the ammonium sulfate precipitation step was omitted, and a Sephacryl S300HR column was used instead of Sephacryl S400. Protein concentrations were determined by using the extinction coefficients provided in each reference; the concentration of SSB is given as a monomer.

Joint Molecule Formation. Unless stated otherwise, Rad51 (0.12 μM) and Rad54 (0.12 μM) were preincubated with 5′-^{32}P-labeled oligonucleotide 1 (0.9 μM nt, 10 nM molecules) in a buffer containing 25 mM Tris acetate (pH 7.5), 10 mM magnesium acetate, 1 mM DTT, 100 μg/mL BSA, 2 mM ATP, 3 mM phosphoenolpyruvate, and 20 units/mL pyruvate kinase for 15 min at 30 °C. The concentration of NaCl contributed by the Rad51 and Rad54 storage buffers were 2 mM and 66 mM, respectively. Unless indicated otherwise, when present, the RPA and Rad52 storage buffers added 3 mM and 40 mM, respectively, to the reaction buffer. D-loop formation was initiated by the addition of pUC19 (26 μM nt, 5 nM molecules), and incubation was continued at 30 °C for 15 min. For cs-D-loop formation, 5′-^{32}P-labeled oligonucleotide 2 (0.9 μM nt, 10 nM molecules) was added, and incubation was continued at 30 °C for 15 min. When stated, oligonucleotide 2 was preincubated with the indicated amounts of RPA and Rad52 at 30 °C for 15 min before addition. After cs-D-loop formation, the reaction products were treated with 10 units of HindIII at 37 °C for 45 min. Finally, reactions were stopped by the addition of termination buffer (final concentration, 7 μg/μL proteinase K, 50 mM EDTA, and 2% SDS), with further incubation at 37 °C for 30 min. The reaction products were analyzed by 1% agarose gel electrophoresis (9 V/cm for 1 h 15 min). After electrophoresis, the gels were dried on DE81 paper (Whatman) and quantified by storage phosphor analysis using a Molecular Dynamics Storm 860 PhosphorImager (GE Healthcare). Errors are reported as the standard error of the mean (SEM) and were determined by using GraphPad Prism v.5.

ACKNOWLEDGMENTS. We thank Dr. Wolf Heyer (University of California, Davis) and laboratory members (Ichiro Amitani, Aura Carreira, Petr Cejka, Anthony Forget, Edgar Valencia-Morales, and Jody Plank) for comments on the manuscript. We are especially indebted to Petr Cejka (discussions), Jody Plank (discussions and art work), and Aura Carreira (hRPA). This work was supported by National Institutes of Health Grant GM-62653 (to S.C.K.) and Tobacco-Related Disease Research Program postdoctoral fellowship 16FT-0069 (to A.V.N.).

1. Rudin N, Haber JE (1988) Efficient repair of HO-induced chromosomal breaks in *Saccharomyces cerevisiae* by recombination between flanking homologous sequences. *Mol Cell Biol* 8:3918–3928.
2. Pâques F, Haber JE (1999) Multiple pathways of recombination induced by double-strand breaks in *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* 63:349–404.
3. Symington LS (2002) Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. *Microbiol Mol Biol Rev* 66:630–670.
4. Szostak JW, Orr-Weaver TL, Rothstein RJ, Stahl FW (1983) The double-strand break repair model for recombination. *Cell* 33:25–35.
5. Allers T, Lichten M (2001) Differential timing and control of non-cross-over and cross-over recombination during meiosis. *Cell* 106:47–57.
6. Sun H, Treco D, Szostak JW (1991) Extensive 3′-overhanging, single-stranded DNA associated with the meiosis-specific double-strand breaks at the *ARG4* recombination initiation site. *Cell* 64:1155–1161.

7. Lisby M, Barlow JH, Burgess RC, Rothstein R (2004) Choreography of the DNA damage response: Spatiotemporal relationships among checkpoint and repair proteins. *Cell* 118:699–713.
8. Wang X, Haber JE (2004) Role of *Saccharomyces* single-stranded DNA-binding protein RPA in the strand invasion step of double-strand break repair. *PLoS Biol* 2:E21.
9. Bianco PR, Tracy RB, Kowalczykowski SC (1998) DNA strand exchange proteins: A biochemical and physical comparison. *Front Biosci* 3:D570–D603.
10. Sung P (1997) Function of yeast Rad52 protein as a mediator between replication protein A and the Rad51 recombinase. *J Biol Chem* 272:28194–28197.
11. New JH, Sugiyama T, Zaitseva E, Kowalczykowski SC (1998) Rad52 protein stimulates DNA strand exchange by Rad51 and replication protein A. *Nature* 391:407–410.
12. Shinohara A, Ogawa T (1998) Stimulation by Rad52 of yeast Rad51-mediated recombination. *Nature* 391:404–407.
13. Petukhova G, Stratton S, Sung P (1998) Catalysis of homologous DNA pairing by yeast Rad51 and Rad54 proteins. *Nature* 393:91–94.
14. Petukhova G, Van Komen S, Vergano S, Klein H, Sung P (1999) Yeast Rad54 promotes Rad51-dependent homologous DNA pairing via ATP hydrolysis-driven change in DNA double helix conformation. *J Biol Chem* 274:29453–29462.
15. Mazin AV, Bornarth CJ, Solinger JA, Heyer WD, Kowalczykowski SC (2000) Rad54 protein is targeted to pairing loci by the Rad51 nucleoprotein filament. *Mol Cell* 6:583–592.
16. Solinger JA, Heyer WD (2001) Rad54 protein stimulates the postsynaptic phase of Rad51 protein-mediated DNA strand exchange. *Proc Natl Acad Sci USA* 98:8447–8453.
17. Solinger JA, Lutz G, Sugiyama T, Kowalczykowski SC, Heyer WD (2001) Rad54 protein stimulates heteroduplex DNA formation in the synaptic phase of DNA strand exchange via specific interactions with the presynaptic Rad51 nucleoprotein filament. *J Mol Biol* 307:1207–1221.
18. Bugreev DV, Mazina OM, Mazin AV (2006) Rad54 protein promotes branch migration of Holliday junctions. *Nature* 442:590–593.
19. Amitani I, Baskin RJ, Kowalczykowski SC (2006) Visualization of Rad54, a chromatin remodeling protein, translocating on single DNA molecules. *Mol Cell* 23:143–148.
20. Eggler AL, Inman RB, Cox MM (2002) The Rad51-dependent pairing of long DNA substrates is stabilized by replication protein A. *J Biol Chem* 277:39280–39288.
21. Solinger JA, Kiianitsa K, Heyer WD (2002) Rad54, a Swi2/Snf2-like recombinational repair protein, disassembles Rad51:dsDNA filaments. *Mol Cell* 10:1175–1188.
22. Bugreev DV, Hanaoka F, Mazin AV (2007) Rad54 dissociates homologous recombination intermediates by branch migration. *Nat Struct Mol Biol* 14:746–753.
23. McIlwraith MJ, *et al.* (2005) Human DNA polymerase η promotes DNA synthesis from strand invasion intermediates of homologous recombination. *Mol Cell* 20:783–792.
24. Holliday R (1964) A mechanism for gene conversion in fungi. *Genet Res* 5:282–304.
25. Formosa T, Alberts BM (1986) DNA synthesis dependent on genetic recombination: Characterization of a reaction catalyzed by purified bacteriophage T4 proteins. *Cell* 47:793–806.
26. Hastings PJ, McGill C, Shafer B, Strathern JN (1993) Ends-in vs. ends-out recombination in yeast. *Genetics* 135:973–980.
27. Malone RE, Esposito RE (1980) The *RAD52* gene is required for homothallic interconversion of mating types and spontaneous mitotic recombination in yeast. *Proc Natl Acad Sci USA* 77:503–507.
28. Mortensen UH, Bendixen C, Sunjevaric I, Rothstein R (1996) DNA strand annealing is promoted by the yeast Rad52 protein. *Proc Natl Acad Sci USA* 93:10729–10734.
29. Shinohara A, Shinohara M, Ohta T, Matsuda S, Ogawa T (1998) Rad52 forms ring structures and cooperates with RPA in single-strand DNA annealing. *Genes Cells* 3:145–156.
30. Sugiyama T, New JH, Kowalczykowski SC (1998) DNA annealing by RAD52 protein is stimulated by specific interaction with the complex of replication protein A and single-stranded DNA. *Proc Natl Acad Sci USA* 95:6049–6054.
31. Wu Y, Sugiyama T, Kowalczykowski SC (2006) DNA annealing mediated by Rad52 and Rad59 proteins. *J Biol Chem* 281:15441–15449.
32. Kantake N, Madiraju MV, Sugiyama T, Kowalczykowski SC (2002) *Escherichia coli* RecO protein anneals ssDNA complexed with its cognate ssDNA-binding protein: A common step in genetic recombination. *Proc Natl Acad Sci USA* 99:15327–15332.
33. Sugiyama T, Kantake N, Wu Y, Kowalczykowski SC (2006) Rad52-mediated DNA annealing after Rad51-mediated DNA strand exchange promotes second ssDNA capture. *EMBO J* 25:5539–5548.
34. Nassif N, Penney J, Pal S, Engels WR, Gloor GB (1994) Efficient copying of nonhomologous sequences from ectopic sites via P-element-induced gap repair. *Mol Cell Biol* 14:1613–1625.
35. Hunter N, Kleckner N (2001) The single-end invasion: An asymmetric intermediate at the double-strand break to double-Holliday junction transition of meiotic recombination. *Cell* 106:59–70.
36. Lao JP, Oh SD, Shinohara M, Shinohara A, Hunter N (2008) Rad52 promotes postinvasion steps of meiotic double-strand break repair. *Mol Cell* 29:517–524.
37. Jayasena VK, Johnston BH (1993) Complement-stabilized D-loop: RecA-catalyzed stable pairing of linear DNA molecules at internal sites. *J Mol Biol* 230:1015–1024.
38. Sena EP, Zarling DA (1993) Targeting in linear DNA duplexes with two complementary probe strands for hybrid stability. *Nat Genet* 3:365–372.
39. Holloman WK, Radding CM (1976) Recombination promoted by superhelical DNA and the *recA* gene of *Escherichia coli*. *Proc Natl Acad Sci USA* 73:3910–3914.
40. Tracy RB, Kowalczykowski SC (1996) In vitro selection of preferred DNA pairing sequences by the *Escherichia coli* RecA protein. *Genes Dev* 10:1890–1903.
41. Seitz EM, Kowalczykowski SC (2000) The DNA binding and pairing preferences of the archaeal RadA protein demonstrate a universal characteristic of DNA strand exchange proteins. *Mol Microbiol* 37:555–560.
42. Seitz EM, Kowalczykowski SC (2006) Human Rad51 protein displays enhanced homologous pairing of DNA sequences resembling those at genetically unstable loci. *Nucleic Acids Res* 34:2847–2852.
43. Raschle M, Van Komen S, Chi P, Ellenberger T, Sung P (2004) Multiple interactions with the Rad51 recombinase govern the homologous recombination function of Rad54. *J Biol Chem* 279:51973–51980.
44. Lavery PE, Kowalczykowski SC (1992) A postsynaptic role for single-stranded DNA-binding protein in RecA protein-promoted DNA strand exchange. *J Biol Chem* 267:9315–9320.
45. Sugiyama T, Kowalczykowski SC (2002) Rad52 protein associates with replication protein A (RPA)-single-stranded DNA to accelerate Rad51-mediated displacement of RPA and presynaptic complex formation. *J Biol Chem* 277:31663–31672.
46. Petes TD, Malone RE, Symington LS (1991) Recombination in yeast. *The Molecular Cellular Biology of the Yeast Saccharomyces*: *Genome Dynamics*, *Protein Synthesis*, *and Energetics*, eds Broach JR, Jones E, Pringle J (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol I, pp 407–521.
47. Allers T, Lichten M (2001) Intermediates of yeast meiotic recombination contain heteroduplex DNA. *Mol Cell* 8:225–231.
48. Song B, Sung P (2000) Functional interactions among yeast Rad51 recombinase, Rad52 mediator, and replication protein A in DNA strand exchange. *J Biol Chem* 275:15895–15904.
49. Miyazaki T, Bressan DA, Shinohara M, Haber JE, Shinohara A (2004) In vivo assembly and disassembly of Rad51 and Rad52 complexes during double-strand break repair. *EMBO J* 23:939–949.
50. McIlwraith MJ, West SC (2008) DNA repair synthesis facilitates RAD52-mediated second-end capture during DSB repair. *Mol Cell* 29:510–516.
51. Sugawara N, Wang X, Haber JE (2003) In vivo roles of Rad52, Rad54, and Rad55 proteins in Rad51-mediated recombination. *Mol Cell* 12:209–219.
52. Mazin AV, Alexeev AA, Kowalczykowski SC (2003) A novel function of Rad54 protein: Stabilization of the Rad51 nucleoprotein filament. *J Biol Chem* 278:14029–14036.
53. Li X, Heyer WD (2009) RAD54 controls access to the invading 3′-OH end after RAD51-mediated DNA strand invasion in homologous recombination in *Saccharomyces cerevisiae*. *Nucleic Acids Res* 37:638–646.
54. Lloyd JA, Forget AL., Knight KL (2002) Correlation of biochemical properties with the oligomeric state of human Rad52 protein. *J Biol Chem* 277:46172–46178.
55. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning*: *A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY), 2nd Ed.
56. Zaitseva EM, Zaitsev EN, Kowalczykowski SC (1999) The DNA binding properties of *Saccharomyces cerevisiae* Rad51 protein. *J Biol Chem* 274:2907–2915.
57. Kantake N, Sugiyama T, Kolodner RD, Kowalczykowski SC (2003) The recombination-deficient mutant RPA (rfa1–t11) is displaced slowly from single-stranded DNA by Rad51 protein. *J Biol Chem* 278:23410–23417.
58. Luisi-DeLuca C, Kolodner RD (1994) Purification and characterization of the *Escherichia coli* RecO protein: Renaturation of complementary single-stranded DNA molecules catalyzed by the RecO protein. *J Mol Biol* 236:124–138.
59. LeBowitz J (1985) Biochemical mechanism of strand initiation in bacteriophage λ DNA replication. PhD dissertation (Johns Hopkins University, Baltimore, MD).