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Rad52-mediated DNA annealing after Rad51-mediated DNA strand exchange promotes second ssDNA capture

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Rad51, Rad52, and RPA play central roles in homologous DNA recombination. Rad51 mediates DNA strand exchange, a key reaction in DNA recombination. Rad52 has two distinct activities: to recruit Rad51 onto single-strand (ss)DNA that is complexed with the ssDNA-binding protein, RPA, and to anneal complementary ssDNA complexed with RPA. Here, we report that Rad52 promotes annealing of the ssDNA strand that is displaced by DNA strand exchange by Rad51 and RPA, to a second ssDNA strand. An RPA that is recombination-deficient (RPA(rfa1-t11)) failed to support annealing, explaining its *in vivo* phenotype. *Escherichia coli* RecO and SSB proteins, which are functional homologues of Rad52 and RPA, also facilitated the same reaction, demonstrating its conserved nature. We also demonstrate that the two activities of Rad52, recruiting Rad51 and annealing DNA, are coordinated in DNA strand exchange and second ssDNA capture.
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Introduction

Homologous recombination is a major mechanism employed in repair of DNA double-strand breaks (DSBs) (Pâques and Haber, 1999; Symington, 2002; West, 2003; Haber *et al*, 2004; Krogh and Symington, 2004). In humans, homologous recombination is required to maintain genome integrity, to prevent cancer development, and to survive DNA damage caused by ionizing radiation, stalled DNA replication, and inhibited DNA topoisomerase action (Liang *et al*, 1998; Johnson and Jasin, 2000; Pierce *et al*, 2001). Recombination is crucial for meiosis in most sexually reproducing organisms to ensure proper segregation of homologous chromosomes into germ cells (Carpenter, 1994; Kleckner, 1996; Roeder, 1997; Zickler and Kleckner, 1998; Keeney, 2001).

Recombination-dependent DSB repair and meiotic recombination have common genetic requirements (Petes *et al*, 1991) and biochemical steps (Krogh and Symington, 2004). The most accepted model, which is obtained mainly from studies of meiotic recombination of *Saccharomyces cerevisiae*, is summarized as follows (Figure 1) (Resnick, 1976; Szostak *et al*, 1983; Allers and Lichten, 2001, for a review of recombination pathways, see Haber *et al*, 2004). First, a DSB is resected to produce ssDNA tails with 3′-ends (processing), and then one ssDNA tail invades a homologous dsDNA molecule (first-strand invasion). The second ssDNA tail, derived from the other end of the DSB, may also invade the dsDNA (second-strand invasion) or anneals to the displaced DNA strand that is produced by the DNA synthesis from the 3′-end of the first ssDNA (second-strand annealing with the displaced strand). These pathways produce double Holliday junctions, which can be resolved to produce crossover products. Alternatively, the invading strand is unwound from its template after DNA synthesis and re-anneals with the other resected end of the DSB, producing only non-crossover products (synthesis-dependent strand annealing).

As a member of the RecA-family recombinases, Rad51 mediates *in vitro* DNA strand exchange between two homologous DNA molecules in the presence of ATP and the ssDNA-binding protein RPA (Sung, 1994; Sung and Robberson, 1995). Like *Escherichia coli* RecA, Rad51 binds ssDNA to form a helical filament (Ogawa *et al*, 1993; Sung and Robberson, 1995) to mediate DNA strand exchange. RPA greatly stimulates DNA strand exchange by removing DNA secondary structure that is inhibitory to contiguous filament formation (Sung and Robberson, 1995; Sugiyama *et al*, 1997). However, RPA inhibits DNA strand exchange when it saturates ssDNA before the addition of Rad51. Addition of Rad52 overcomes this inhibition and stimulates DNA strand exchange by recruiting Rad51 onto the RPA–ssDNA complex (Sung, 1997; New *et al*, 1998; Shinohara and Ogawa, 1998; Song and Sung, 2000). Rad52 binds to Rad51 (Shinohara *et al*, 1992; Sung, 1997) and to RPA (Shinohara *et al*, 1998), via direct protein–protein interaction. Rad52 and RPA form a co-complex on ssDNA (Sugiyama and Kowalczykowski, 2002) that recruits Rad51. The activity of Rad52 to facilitate loading of Rad51 onto the RPA–ssDNA complex is referred to as recombination mediator activity (for a review, see Sung *et al*, 2003). In agreement, *in vivo*, Rad51 cannot associate with DSB sites in the absence of functional Rad52 (Sugawara *et al*, 2003).

In addition to its recombination mediator activity, Rad52 also has ssDNA binding and annealing activities (Mortensen *et al*, 1996). Importantly, Rad52 mediates the annealing of ssDNA that is saturated with RPA (Shinohara *et al*, 1998;

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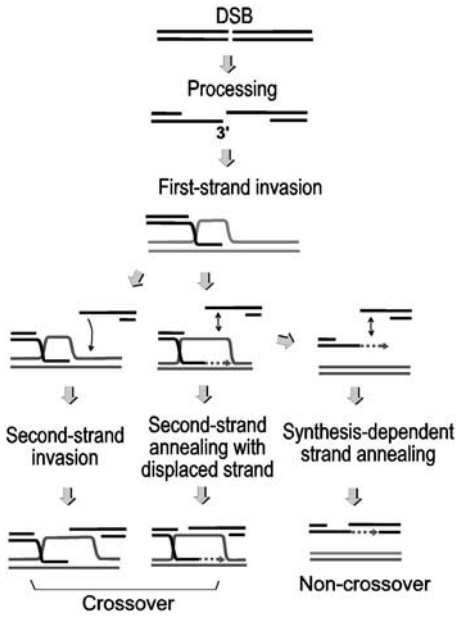

Figure 1 Illustration of error-free repair pathways of a DSB. See text for details.

Sugiyama *et al*, 1998). According to current models, the second ssDNA tail derived from a DSB anneals to either the displaced ssDNA strand or the unwound invading strand (Figure 1). Rad52 and RPA possibly mediate these annealing reactions (second ssDNA capture). Some *in vivo* studies are consistent with this idea. Immunostaining showed that Rad52 remains longer at the DSB site than Rad51 after a DSB is introduced by HO-nuclease (Miyazaki *et al*, 2004). Chromatin immunoprecipitation showed that RPA binds to the displaced ssDNA after strand invasion (Wang and Haber, 2004). In mouse spermatocytes, RPA foci stay longer than Rad51 foci (Moens *et al*, 2002). These data suggest that Rad52 and RPA are also involved in a later stage of recombination than Rad51-ssDNA filament formation. Physical analysis of meiotic chromosomes showed that DNA strand invasion and second ssDNA capture are temporally separate *in vivo* (Hunter and Kleckner, 2001), suggesting that the two events are distinct biochemical processes. However, the role of Rad52-mediated annealing in the second-ssDNA capture has not been directly demonstrated.

RPA consists of three subunits, RPA1 (70 kDa), RPA2 (32 kDa), and RPA3 (14 kDa). Cells with a mutation in RPA1 (*rfa1-t11*: K45E) have defects in both meiotic and mitotic recombination and the single-strand annealing pathway of DSB-repair, with no significant defect in DNA replication (Umezu *et al*, 1998; Soustelle *et al*, 2002). This mutant also has defects in damage checkpoint (Kim and Brill, 2001; Clerici *et al*, 2004) and adaptation (Lee *et al*, 2001, 2003; Pellicioli *et al*, 2001). Biochemical analyses showed that RPA(rfa1-t11) has a defect in Rad51-ssDNA filament formation under a certain conditions (Kantake *et al*, 2003). However, chromatin immunoprecipitation suggested that *rfa1-t11* strain has a defect in later stage of recombination (Wang and Haber, 2004).

Here, we provide biochemical evidence indicating that Rad52 and RPA can mediate the annealing of the displaced DNA strand that is produced by Rad51-mediated DNA strand exchange with a second ssDNA molecule. RPA(rfa1-t11) failed to support the annealing reaction under conditions that allowed Rad51-mediated DNA strand exchange, defining its inability to support the postsynaptic stage of DNA recombination. We also integrated the recombination mediator activity and ssDNA annealing activity of Rad52 into a single reaction to show that these two processes can be coordinated.

Results

Experimental design

We used pBluescript phagemid circular ssDNA and linear dsDNA as model substrates for DNA strand exchange and annealing (Figure 2A). To obtain a DNA strand exchange intermediate with a displaced strand, pBluescript SK− ssDNA (ssDNA(SK−)) and pBluescript SK+ linear dsDNA (dsDNA(SK+)) were used in Rad51-mediated DNA strand exchange reactions (Figure 2A, Step 1). These two model substrates are homologous except for a 0.5 kb region (Figure 2A, hatched and open boxes). This region prevents complete DNA strand exchange, producing instead joint molecules that contain the displaced ssDNA strand. Then, Bluescript SK+ ssDNA (ssDNA(SK+)) preincubated with saturating amount of RPA was added to the joint molecules together with Rad52 (Figure 2A, Step 2). As ssDNA(SK+) is complementary to the displaced DNA strand over its entire length, annealing between the displaced strand and ssDNA(SK+) should produce a new DNA product (referred to as 'double joint molecule' in the figure), which can be identified by agarose gel electrophoresis. If branch migration separates the double joint molecule, then two nonidentical circular DNA products (a nicked circle and a gapped circle) will form that can be distinguished by agarose gel electrophoresis. This reaction scheme is termed two-step DNA strand exchange and annealing.

Rad52 and RPA mediate annealing of the displaced ssDNA strand

First, to identify the joint molecules and nicked circle dsDNA products, we performed Rad51-mediated DNA strand exchange using partially homologous (Figure 2A, Step 1) or completely homologous substrates (Figure 2B), and analyzed the products by agarose gel electrophoresis (Figure 2C). With homologous substrates, Rad51 mediated formation of both nicked circular product (NC) and joint molecules (JM) as expected (Figure 2C, lane 2). With partially homologous DNA substrates in an otherwise identical reaction (Figure 2A, Step 1), DNA strand exchange produced only joint molecules that could not proceed beyond the heterologous region (Figure 2C, lane 4). To examine whether the joint molecules containing the displaced strand could be substrates for DNA annealing by Rad52, ssDNA(SK+) complexed with RPA was added to the joint molecules together with Rad52 (Figure 2A, Step 2). The displaced strand in the joint molecules is complementary to ssDNA(SK+) along its entire length. The amount of joint molecules decreased and two new products were formed, which migrated between linear dsDNA (L) and joint molecules on the gel (Figure 2C, lane 5). One of these products had the same mobility as the nicked circular dsDNA, and the other migrated slightly slower than the nicked circle (gapped circle; GC).

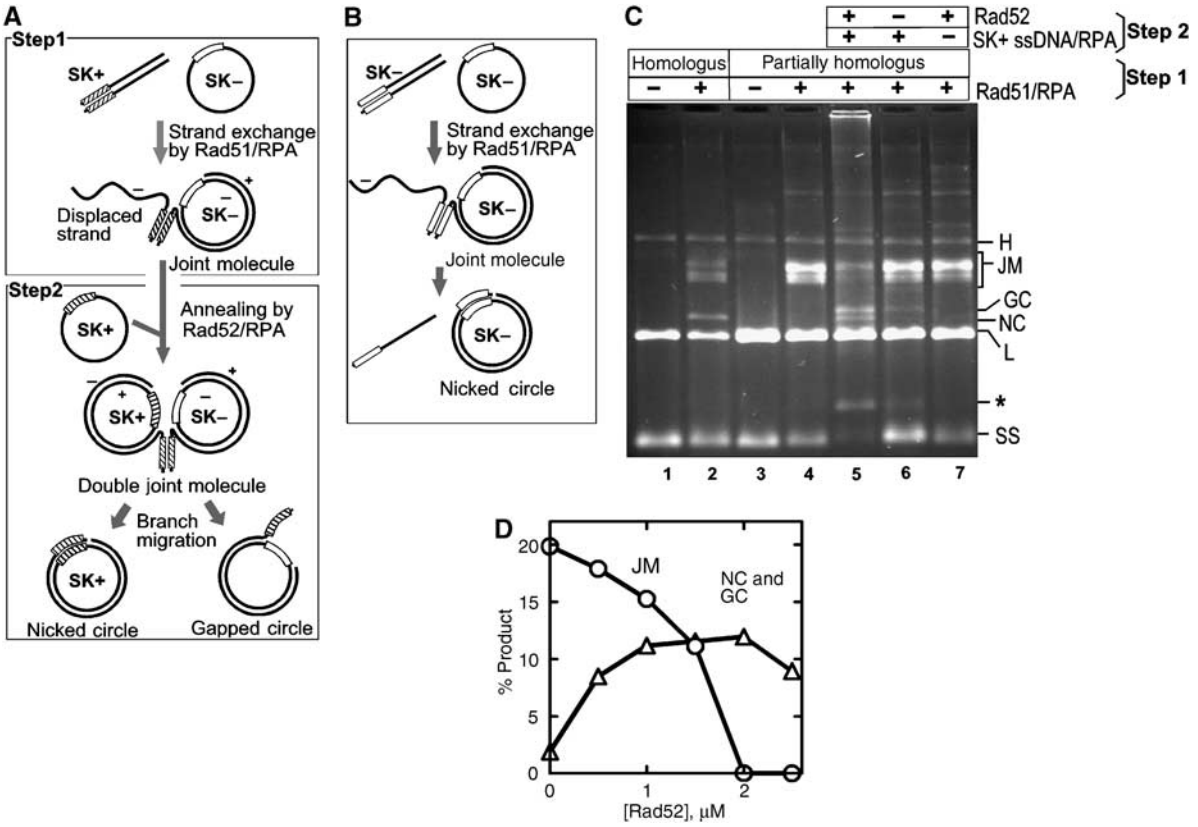

Figure 2 Rad52 mediates annealing of the displaced DNA strand that is produced by DNA strand exchange by Rad51. (**A**) Illustration of two-step DNA strand exchange and annealing. See text for details. (**B**) Illustration of DNA strand exchange between homologous ssDNA and dsDNA. Reaction produces joint molecules, nicked circular dsDNA, and linear ssDNA. (**C**) Lanes 1 and 2: DNA strand exchange between ssDNA and homologous dsDNA (illustrated in (B)) was performed in the absence (lane 1) or the presence (lane 2) of Rad51 and RPA. Lanes 3–7: Two-step DNA strand exchange and annealing (illustrated in (A)). DNA strand exchange between partially homologous substrates was performed by Rad51 and RPA (lane 4), and the reaction mixture was subsequently incubated for 60 min (Step 2 in panel A) with RPA–ssDNA(SK+) complex and Rad52 (lane 5), with only RPA–ssDNA(SK+) (lane 6), or with only Rad52 (lane 7). Lane 3 is no-protein control. Joint molecule (JM), gapped circle (GC), nicked circle (NC), linear double-strand (L), and single-stranded (ss) DNA are indicated. Besides the molecules mentioned in the text, the ssDNA of helper phage (VCSM13, indicated as 'H') was present in the SK− ssDNA preparation. Another band, which was indicated by an asterisk, was produced most evidently in lane 5. This is a product of partial annealing between SK− and SK+ circular ssDNA molecules, as this band also appeared when these ssDNA molecules were incubated with Rad52 without dsDNA (data not shown). This band is also visible in other gels (Figures 4, 5, 6, and 7), and indicated by asterisks. Linear ssDNA that was produced in the reaction comigrated with the circular ssDNA. (**D**) Same experiment as lane 5 of (C) was performed in the presence of various amount of Rad52, and joint molecule (open circle) and the sum of nicked and gapped circular DNA products (open triangle) were expressed as the percentage to the total amount of DNA.

To determine the identity of these products, we repeated the complete two-step reaction using dsDNA substrates that were labeled with ^{32}P in a strand-specific manner (Figure 3), and visualized the same gel by ethidium bromide staining (lanes 1) and by phosphor imaging (lanes 2). Radioactive labeling revealed the disposition of exchanged strands in the nicked and gapped circular DNA products (Figure 3A). The '+' strand, which is partially complementary to ssDNA(SK−), was incorporated into the gapped circular product (Figure 3B), and the '−' strand, which is fully complementary to ssDNA(SK+), was incorporated into the nicked circular product (Figure 3C). Based on these results, we concluded that these products were correctly identified as nicked and gapped circular dsDNA molecules. Therefore, these results demonstrated the annealing of the displaced DNA strand with ssDNA(SK+).

When Rad52 was omitted from the two-step reaction (Figure 2C lane 6, and D), very little circular product was produced, indicating that Rad52 is required for the annealing during the second step. Rad52 alone without the ssDNA(SK+) in the second step produced no detectable amount of circular product (lane 7). These results indicate that Rad52-mediated annealing can incorporate a second ssDNA molecule into the DNA strand exchange intermediate produced by Rad51-mediated DNA strand exchange. The optimum Rad52 concentration for the circular products formation was approximately 1.5–2 μM (Figure 2D). The putative intermediate molecule that contains two circular structures connected by short region of dsDNA ('double joint molecule' in Figure 2A) was not clearly identified on the gel. We suggest that it may be quickly converted to the final products by branch migration or was incorporated into larger DNA network that could not enter the agarose gel. The latter possibility is consistent with the observation that the complete reaction also produced a significant amount of DNA that was retained in the loading well (Figure 2C, lane 5).

Extension of Rad52-mediated DNA annealing promotes branch migration

In the two-step DNA strand exchange and annealing reaction (Figure 2A, Step 2), formation of the nicked and gapped

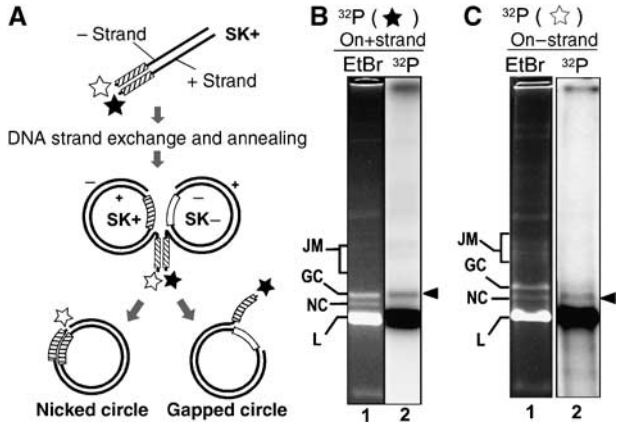

Figure 3 Identification of nicked and gapped circular DNA products by strand-specific labeling of dsDNA substrate. (**A**) The '+' strand of the linear dsDNA substrate (marked by filled star) is partially complementary to the ssDNA(SK–), and '–' strand (marked by open star) is fully complementary to ssDNA(SK+). DNA strand exchange followed by annealing of the displaced DNA strand produces nicked and gapped circular products that receive '–' and '+' strands from the dsDNA, respectively. (B, C) Two-step DNA strand exchange and annealing (same reactions as shown in Figure 2C, lane 5) were performed except that the dsDNA substrates were labeled with ^{32}P at '+' strand (**B**) or '–' strand (**C**). Gels were visualized by ethidium bromide staining (lanes 1) and then by phosphorimaging (lanes 2).

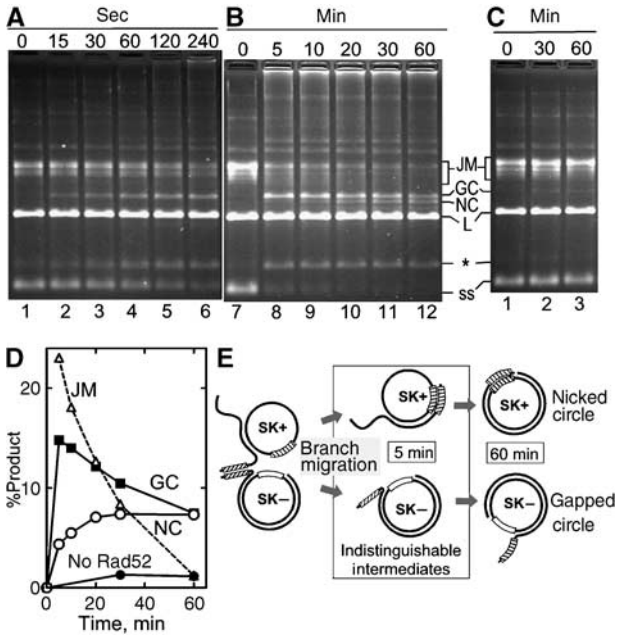

Figure 4 Rad52-mediated DNA annealing promotes quick separation of joint molecule into circular products. (**A–C**) Same reaction as shown in Figure 2C, lane 5 was repeated in a larger volume, and aliquots were withdrawn to stop the reaction at the indicated times after addition of Rad52. As a negative control, same reaction as (B) was performed without Rad52 (C). Products were analyzed by agarose gel electrophoresis and ethidium bromide staining. The reactions for lanes 1 and 7 were stopped before addition of Rad52. (**D**) Amount of nicked (open circle) and gapped (filled square) circular DNA products and joint molecules (open triangle) were quantified from (B), and amount of gapped circle was quantified from (C) (filled circle), and expressed as the percentage of the total amount of DNA. (**E**) Possible intermediates of the reaction.

circular DNA products should be accompanied by unwinding of the 0.5 kb region (hatched dsDNA region) in 'double joint molecule'. Our results suggest that Rad52-mediated DNA annealing could extend into the pre-existing dsDNA region, so that it could promote branch migration. However, we cannot eliminate the possibility that the nicked and gapped circular DNA were produced by Rad52-dependent DNA annealing followed by Rad52-independent thermodynamic branch migration, as the annealing reaction was carried out for 60 min in Figures 2 and 3. Therefore, we performed a kinetic analysis of the two-step DNA strand exchange and annealing (Figure 4). The joint molecule was produced in the Step 1 under the standard conditions, and the reaction was stopped at different times in the second step after addition of Rad52 (Figure 4A and B). In the first 4 min, unexpectedly, the gapped circle DNA product was formed without producing a detectable amount of nicked circle DNA product (Figure 4A). The amount of the gapped circle reached its maximum at 10 min, and then decreased slowly during next 50 min to approximately 50% of the maximum (Figure 4B and D). The amount of the nicked circle DNA increased gradually as the amount of the gapped circle DNA decreased, and after 60 min, approximately equal amounts of the gapped and nicked circle DNA products were produced. This result appears inconsistent with the reaction scheme shown in Figure 2A, which shows that the equimolar amount of both circular DNA molecules should be produced simultaneously. One possible explanation for this inconsistency is that the nicked circle DNA is formed via an intermediate molecule that is indistinguishable from the gapped circle DNA by the gel electrophoresis (Figure 4E). This intermediate may be produced by quick separation of putative double-joint molecule before annealing over entire length, which results in two circular DNA with similar shapes (Figure 4E, '5 min'). The intermediate containing ssDNA(SK+) forms a nicked circular product as the reaction progresses. However, if the reaction is stopped, the deproteinized ssDNA region forms a stable secondary structure that prevents formation of the nicked circular product. Time-course analyses with labeled substrates (Supplementary Figure S1) confirmed the existence of a 'gapped circle-like' intermediate in the process of the nicked circle formation. This also provides an explanation for detection of a faint band of the gapped circle DNA in Figure 3C, lane 2. As spontaneous branch migration in the presence of magnesium ion is very slow (Panyutin and Hsieh, 1994), these results suggest that Rad52 (or Rad51–Rad52 complex) can mediate DNA annealing that promotes branch migration for at least 0.5 kb in 5 min.

A recombination-deficient mutant RPA, RPA(rfa1-t11) fails to support Rad52-mediated annealing

The *rfa1-t11* mutant yeast is recombination-deficient (Umezu *et al*, 1998; Soustelle *et al*, 2002). However, purified RPA(rfa1-t11) stimulates DNA strand exchange by Rad51 as effectively as wild-type RPA under conditions that allowed saturation of the Rad51–ssDNA complex formation before the addition of RPA (Kantake *et al*, 2003). Therefore, we examined the effect of RPA(rfa1-t11) on the annealing of the displaced DNA strand (Figure 5).

In Step 1 of two-step DNA strand exchange and annealing, the mutant RPA efficiently supported joint molecule formation by Rad51 (Figure 5A, lane 2). This result is consistent

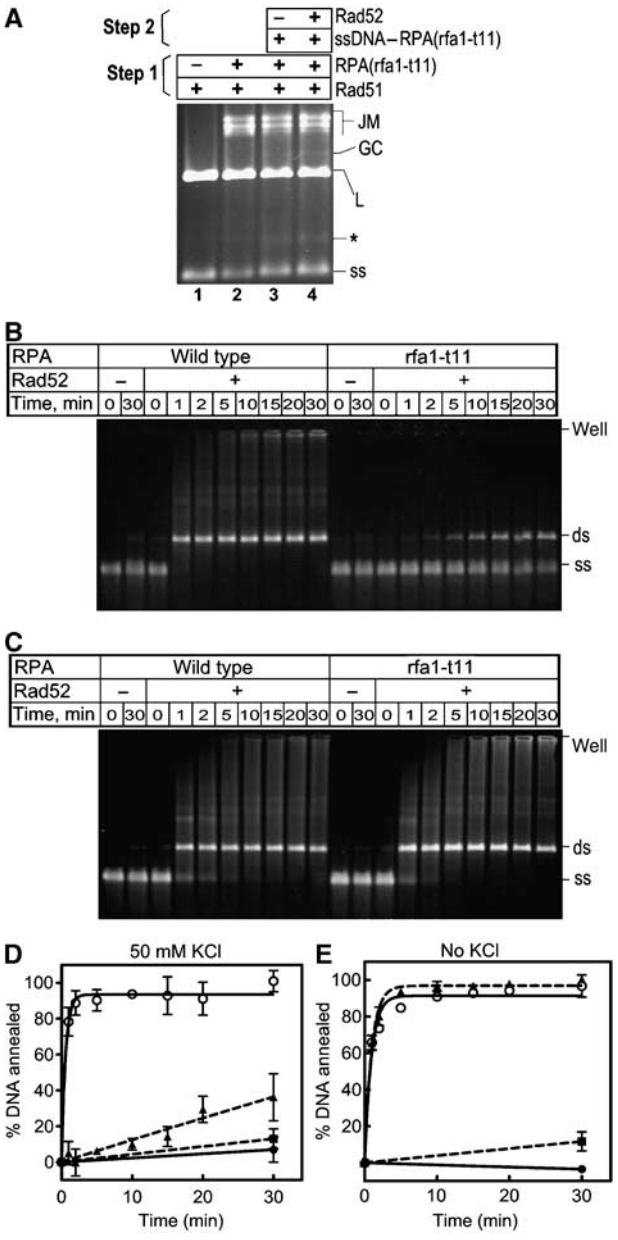

Figure 5 RPA(rfa1-t11) does not support DNA annealing by Rad52. (**A**) Lanes 1 and 2 show DNA strand exchange reaction between the partially homologous substrates with Rad51 only (lane 1) or with Rad51 and RPA(rfa1-t11) (lane 2). Lanes 3 and 4 show two-step DNA strand exchange and annealing with RPA(rfa1-t11). DNA strand exchange between the partially homologous substrates was performed with Rad51 and RPA(rfa1-t11), and then followed by an incubation (60 min) with RPA(rfa1-t11)–ssDNA(SK+) complex (lane 3) or with both RPA(rfa1-t11)–ssDNA(SK+) complex and Rad52 (lanes 4). (**B**) DNA annealing was analyzed using heat-denatured pBluescript SK− dsDNA in the identical buffer conditions to the two-step DNA strand exchange and annealing. DNA (10 μM) was preincubated with 0.5 μM of either wild-type RPA or RPA(rfa1-t11), then with (+) or without (–) 1.59 μM of Rad52 for the indicated periods, and the products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. Linear ssDNA substrate (ss), dsDNA product (ds), and loading well (well) are indicated. (**C**) Same experiments as (B) were performed, except that (C) contained no KCl in the reaction buffer. (D–E) Percentages of DNA annealing are calculated from duplicated experiments of panel B (**D**), and panel C (**E**). For both (D) and (E), curves represent the products formations with Rad52 and either wild-type RPA (open circle with solid line) or RPA(rfa1-t11) (filled triangle with dashed line), only with wild-type RPA (filled circle with solid line), or only with RPA(rfa1-t11) (filled square with dashed line). Error bars represent standard deviations.

with our previous observations (Kantake *et al*, 2003). In Step 2, however, very little nicked circle or gapped circle DNA products were produced (lane 4), indicating that Rad52 did not mediate annealing of the displaced DNA strand which was covered with RPA(rfa1-t11). To confirm that RPA(rfa1-t11) inhibited Rad52-mediated DNA annealing, we analyzed annealing of heat-denatured linear pBluescript SK− plasmid DNA, (Figure 5B–E). The heat-denatured dsDNA was first incubated with either wild-type RPA or RPA(rfa1-t11) and then Rad52 was added to start DNA annealing. When the reaction was performed in the buffer used in the two-step DNA strand exchange and annealing reaction, DNA annealing was much slower with RPA(rfa1-t11) than with wild-type RPA (Figure 5B and D), confirming that RPA(rfa1-t11) was defective in Rad52-mediated DNA annealing under these conditions.

We previously reported that RPA(rfa1-t11) supported Rad52-mediated DNA annealing as efficiently as wild-type RPA (Kantake *et al*, 2003). The experiments shown in Figure 5A and B were carried out in the presence 50 mM KCl, whereas our previous annealing assays did not contain KCl. To reconcile these results, we performed the similar DNA annealing experiments as in Figure 5B except that the reactions contained no KCl (Figure 5C and E). Under these conditions, both wild-type and the mutant RPA efficiently supported Rad52-mediated DNA annealing. These results indicate that the defect of the *rfa1-t11* mutation in Rad52-mediated DNA annealing is undetectable at low-salt concentrations, but is obvious in the presence of 50 mM KCl, more closely mimicking physiological conditions.

The second ssDNA capture requires species-specific protein–protein interactions between recombination mediator and ssDNA-binding protein

Rad52-mediated annealing of RPA–ssDNA complex involves protein–protein interaction between RPA and Rad52 (Shinohara *et al*, 1998; Sugiyama *et al*, 1998). To test whether this is also true for the second ssDNA capture, we used *E. coli* SSB protein, instead of RPA, in the two-step DNA strand exchange and annealing (Figure 6). In step 1, SSB protein stimulated Rad51-mediated DNA strand exchange with a slightly lower efficiency (Figure 6, lane 2) than RPA, as previously reported (Sugiyama *et al*, 1997). In Step 2, addition of SSB protein–ssDNA complex together with Rad52 produced very little nicked or gapped circular product (lane 3), showing that SSB protein could not substitute for RPA for the annealing of the displaced DNA strand.

We previously proposed that *E. coli* RecO is a functional homolog of yeast Rad52 (Kantake *et al*, 2002). Both RecO (or RecOR) and Rad52 have recombination mediator activity with their cognate DNA strand exchange proteins (Umezu *et al*, 1993; Umezu and Kolodner, 1994; Shan *et al*, 1997; Morimatsu and Kowalczykowski, 2003), and they also can mediate DNA annealing that is precoated with their cognate ssDNA-binding proteins (Luisi-DeLuca and Kolodner, 1994; Sugiyama *et al*, 1998; Kantake *et al*, 2002). To examine the ability of RecO to mediate annealing of the displaced DNA strand covered with SSB protein, RecO and SSB–ssDNA(SK+) complex were added in step 2 (Figure 6, lanes 5–8). Increasing amounts of RecO produced the gapped circular product, showing that the RecO partially supported the second-ssDNA capture under these conditions. The

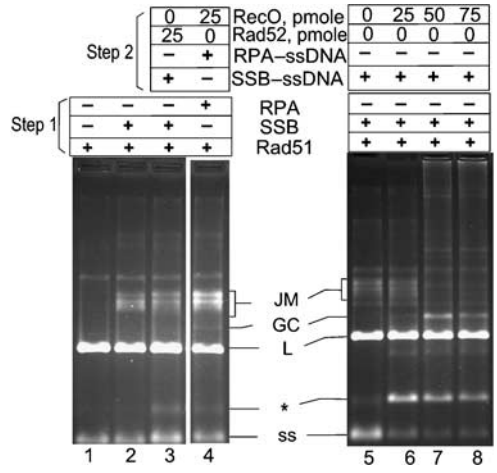

Figure 6 Species-specific interactions between ssDNA-binding protein and recombination mediator are involved in the second-ssDNA capture. DNA strand exchange between partially homologous substrates (Step 1) was performed under the standard conditions except that either *E. coli* SSB protein (58.8 pmoles), RPA (20.6 pmoles), or no ssDNA-binding protein was used as indicated. Then, annealing of the displaced DNA strand (Step 2, lanes 3–8) was performed using ssDNA(SK+) complexed with either SSB or RPA, and the indicated amount of Rad52 or RecO.

product was mixture of the gapped circle and the 'intermediate' nicked circle molecule (Figure 4E) (Supplementary Figure S2A and B). Interestingly, the same mixture was produced by the two-step reaction using Rad52 without RPA in the second step (Supplementary Figure S2C and D). These results suggest that RecO with SSB, and Rad52 with 'naked' ssDNA cannot produce a plasmid-sized dsDNA that is free from the interruption by secondary structure. As RecO-mediated annealing of the displaced DNA strand was not supported by RPA (lane 4), these results confirm that species-specific interaction between RecO and SSB protein was necessary in the reaction, as was the case with Rad52 and RPA. These results further support the idea that RecO is functional homolog of Rad52 with regard to both recombination mediator activity and the second-ssDNA capture.

Recruiting Rad51 recombinase and annealing displaced ssDNA are coordinated by Rad52

Rad52 recruits Rad51 onto RPA–ssDNA complex to form the contiguous Rad51-ssDNA filament (Sung, 1997; New *et al*, 1998; Shinohara and Ogawa, 1998). As presented in this report, Rad52 and RPA can mediate annealing of the displaced DNA strand after DNA strand exchange. We considered that these two activities of Rad52 might be linked: that is, the same Rad52 molecule that recruited Rad51 could also mediate annealing of the second ssDNA. To test this idea, we used a saturated RPA–ssDNA(SK−) complex as a starting substrate for DNA strand exchange and annealing (Figure 7A). The RPA-ssDNA(SK−) complex was incubated with Rad52 and then with Rad51 for 10 min to allow Rad52 to recruit Rad51 onto the ssDNA. Then partially homologous dsDNA was added to start DNA strand exchange ('$T=0$' in Figure 7A). When the reaction continued without the second ssDNA (Figure 7A, no second ssDNA), joint molecules were produced, showing that the Rad51 filament formed on the ssDNA mediated DNA strand exchange (Figure 7B and E 'uncoupled'). To examine if DNA strand exchange and annealing can be coordinated, the RPA–ssDNA(SK+) complex was added to the reaction mixture 10 min after dsDNA was added ('$T=10$ min' in Figure 7A). The reaction produced nicked circular and gapped circular products (Figure 7C and E), indicating that the proteins present in the reaction mediated Rad51 loading, DNA strand exchange, and annealing of the second ssDNA.

Rad52 titration in the coordinated reaction (Figure 7D and F) showed that very little gapped circular product was produced in the absence of Rad52 (lane 1), indicating that the coordinated DNA strand exchange and annealing requires Rad52. The optimum amount of Rad52 is approximately 1.5 μM for circular product formation. This coincides roughly with the amount of RPA on the starting ssDNA(SK−) (1.65 μM). This is consistent with the optimum Rad52/RPA ratio in recombination mediator assays (Sung, 1997), and very close to ratio optimum for the two-step reaction (Figure 2D). These results indicate that no additional Rad52 is needed for subsequent annealing beyond the amount needed for recruitment of Rad51 onto ssDNA in presynaptic step. As Rad52 can bind both Rad51 and RPA (Shinohara *et al*, 1992, 1998; Sung, 1997), Rad52 and RPA presumably bound to the Rad51–ssDNA complex via protein–protein interactions (Figure 7A, 'presynaptic complex'). As the Rad51 filament invades dsDNA, Rad52 and RPA might transfer from the presynaptic complex to the displaced DNA strand and mediate DNA annealing (Figure 7A, 'coordinated DNA strand exchange and annealing').

Discussion

A model for RPA, Rad51, and Rad52 functions in double strand DNA break repair

Rad52 protein has two activities: to recruit Rad51 onto RPA–ssDNA complex and to mediate DNA annealing. The first activity is essential for *in vivo* function of Rad51 and has been well characterized (Sung *et al*, 2003). The second activity of Rad52 is believed to be required for the ssDNA-annealing pathway. However, the role of Rad52-mediated DNA annealing in the Rad51-dependent recombination pathway has not been clearly demonstrated. In this paper, we show that Rad52 can mediate annealing of the displaced DNA strand that is formed by Rad51-mediated DNA strand exchange. This provides biochemical evidence that Rad52 can promote maturation of the DNA strand exchange products by capturing a second ssDNA. Based on these observations, we propose the following model for the functions of RPA, Rad51, and Rad52 in homologous recombination (Figure 7G). After a DSB is introduced and resected to form ssDNA tails, RPA binds to the ssDNA to remove DNA secondary structure. Rad52 then forms a complex with RPA on at least one ssDNA tail and recruits Rad51 via protein–protein interaction to produce the Rad51 filament (presynaptic complex) that is active in DNA strand exchange. During the DNA strand exchange by Rad51, both RPA and Rad52 are released from the presynaptic complex and bind the displaced ssDNA strand, thereby stabilizing the strand exchange intermediate. After DNA synthesis from the invading 3′-end, Rad52 facilitates annealing of the displaced strand with the other end of the DSB, producing double Holliday junctions. This model not only agrees with biochemical characteristics of these proteins but

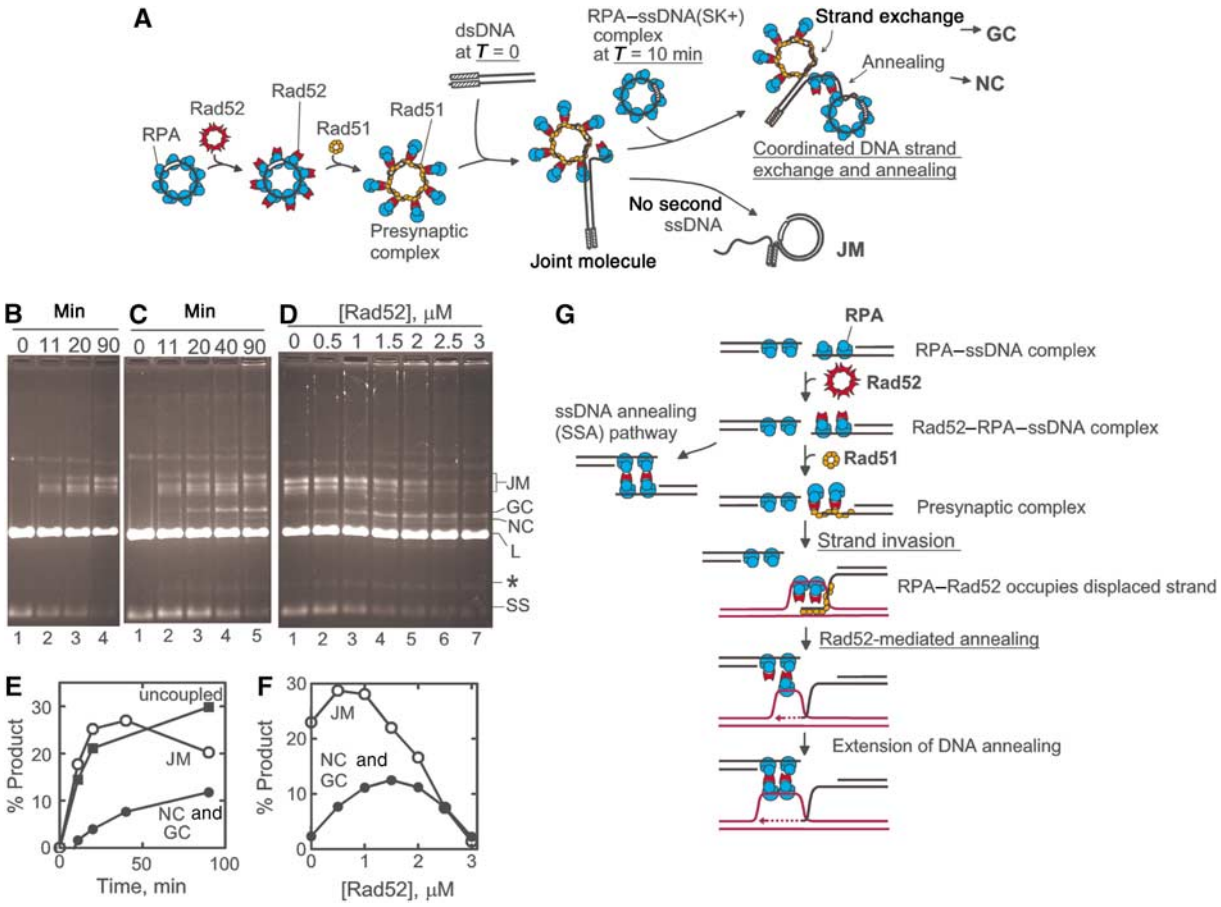

Figure 7 Coordinated DNA strand exchange and annealing. (**A**) Illustration of the reaction scheme. ssDNA(SK−) was first incubated with RPA, followed by Rad52 and Rad51. After 10 min, dsDNA was added to start DNA strand exchange ($T = 0$ min). At $T = 10$ min, the RPA–ssDNA(SK+) complex was added to initiate annealing of the second ssDNA. (**B**) Reaction illustrated in (A) was performed without the second ssDNA, and time course was taken after addition of dsDNA, and products were analyzed by agarose gel electrophoresis. (**C**) Reaction illustrated in (A) was performed with RPA–ssDNA(SK+) complex that was added at $T = 10$ min. (**D**) Coordinated reactions (the same reaction as lane 5 in (C)) were carried out in the presence of different concentrations of Rad52. (**E**) Amounts of joint molecules in (B) (filled square) and (C) (open circle), and the sum of nicked and gapped circular DNA products in (C) (filled circle) were expressed as the percentage to the total amount of DNA. (**F**) Joint molecule (open circle) and the sum of nicked and gapped circular DNA products (filled circle) in (D) were expressed as the percentage to the total amount of DNA. (**G**) Model for action of RPA, Rad52, and Rad51 in DSB repair. See text for details.

also explains *in vivo* observations of the order of protein assembly on DSBs. In vegetative cells, after DSBs are resected to ssDNA, RPA, and then Rad52, Rad51, Rad55/57, and Rad54 are loaded at the site (Lisby *et al*, 2004; Miyazaki *et al*, 2004). In addition, both RPA and Rad52 remain at the DSB site after Rad51 leaves there; this observation is most easily explained if RPA and Rad52 occupy the displaced DNA strand (Miyazaki *et al*, 2004; Wang and Haber, 2004).

Our *in vitro* results also show that the Rad51 recruitment, DNA strand exchange, and the second-ssDNA capture are likely to be carried out in a coordinated manner. This suggests that after Rad52 recruits Rad51 it can subsequently transfer to the displaced DNA strand to mediate DNA annealing. For efficient transfer of Rad52 and RPA from the presynaptic complex to the displaced DNA strand, the presynaptic complex presumably retains Rad52 and RPA. Although an RPA–Rad52–Rad51–ssDNA co-complex has not been identified, such a co-complex would be advantageous because retention of Rad52 and RPA on the presynaptic complex could facilitate subsequent transfer to the displaced DNA strand, while the complex invades dsDNA. However, *in vivo*, those RPA and Rad52 molecules may not directly participate subsequent annealing, as second ssDNA capture occurs after DNA synthesis from the invading end. The RPA and Rad52 molecules on the displaced strand may facilitate DNA synthesis by stabilizing the joint molecule and by recruiting new RPA and Rad52 molecules by cooperative binding, which mediates second-ssDNA capture *in vivo*.

In our model, Rad51 is loaded on one of two ssDNA tails that are produced by a DSB. The other end is coated with RPA, with or without Rad52 (Figure 7G). Alternatively, in the single strand-annealing (SSA) pathway, which can occur without Rad51, neither ssDNA forms presynaptic complex. In this pathway, Rad52 may directly mediate DNA annealing of the two ssDNAs when complementary sequences are exposed by the processing of the break. In meiotic recombination, two recombinases Rad51 and Dmc1 are loaded in separate but closely localized regions (Shinohara *et al*, 2000). Recent studies showed that meiosis-specific proteins Mei5 and Sae3 recruit Dmc1 onto the DSB sites (Hayase *et al*, 2004; Tsubouchi and Roeder, 2004), while Red1 and Hed1 down-regulate Rad51 activity to ensure Dmc1-mediated inter-homolog crossover (Schwacha and Kleckner, 1997; Tsubouchi and Roeder, 2006). The mechanisms that recruit the recombinases

to ssDNA to form presynaptic complexes should play a key regulatory role in multiple pathways of recombination.

Our results (Figure 4) also suggest that extension of Rad52-mediated DNA annealing can promote branch migration. This is consistent with a previous report (Reddy *et al*, 1997), showing that human Rad52 promotes the branch migration that displaces a oligonucleotide annealed to circular ssDNA. This suggests that branch migration may facilitate unwinding of the invaded dsDNA, so that it further stabilizes the structure of the recombination intermediate. Further study is needed to elucidate the nature and significance of the Rad52-mediated branch migration.

Recombination-deficient RPA fails to support both presynaptic and postsynaptic step of recombination

In this paper, we show that RPA(rfa1-t11) mutaint protein is deficient in DNA annealing and second-ssDNA capture by Rad52 *in vitro* (Figure 5). This result is consistent with *in vivo* studies showing that *rfa1-t11* strain has a defect in a later step than DNA strand invasion (Wang and Haber, 2004). The defect in DNA annealing with RPA(rfa1-t11) can also explain the *in vivo* defect in the SSA pathway (Umezu *et al*, 1998). Our *in vitro* assay showed that the defect of RPA(rfa1-t11) was clearly observed only when the reaction contains a near physiological concentration of salt (50 mM KCl). The DNA-binding capability of RPA(rfa1-t11), however, does not show a significant defect in the presence of salt ranging from 0 to 300 mM (Kantake *et al*, 2003). This might suggest that the mutant RPA cannot interact with Rad52 under physiological salt conditions. Analysis of direct protein–protein interaction of RPA(rfa1-t11) and Rad52 may elucidate the mechanisms of recombination defect by *rfa1-t11* mutation.

Importance of the second-ssDNA capture by recombination mediators

In this and a previous report (Kantake *et al*, 2002), we proposed that *E. coli* RecO is a functional homologue of the yeast Rad52. The recombination mediator of T4 phage (UvsY) also has ssDNA annealing activity with its cognate ssDNA-binding protein gp32 (Kantake *et al*, 2002 and our unpublished observation). This functional resemblance suggests the universal importance of the second-ssDNA capture by recombination mediators. This reinforces our model (Figure 7G) in which the mediator protein on the presynaptic complex can be used for the second-ssDNA capture. These findings also suggest the model presented in this paper is conserved from bacteriophage to eukaryotes.

Materials and methods

DNA and proteins

Phagemid Bluescript SK+ and SK− ssDNA (Stratagene) were prepared as described (Sambrook *et al*, 1989). pBluescript SK+ and SK− dsDNA were prepared by equilibrium centrifugation in a CsCl-ethidium bromide gradient (Sambrook *et al*, 1989). The nucleotide concentrations of ssDNA and dsDNA were measured by using extinction coefficients of 8.1×10^3 and 6.5×10^3/M/cm, respectively. For DNA strand exchange and annealing, dsDNA was digested with *Pst*I. To label only '+ strand' of pBluescript SK+ dsDNA with ^{32}P, the dsDNA was digested first with *Eco*RV, treated with T4 polynucleotide kinase and γ-^{32}P-ATP, and then digested with *Pst*I. To label only '− strand' with ^{32}P, pBluescript SK+ dsDNA was digested first with *Pst*I and *Eco*RI, and then treated with Klenow fragment that has no 3′–5′ exonuclease activity (New England Biolabs) with α-^{32}P-dATP and unlabeled dTTP. Labeled dsDNAs were isolated by spin-column filtration through Microspin S-400 (GE Healthcare). All DNA concentrations are expressed in moles of nucleotide. Rad51 (New *et al*, 1998), Rad52 (New *et al*, 1998), wild-type RPA (Kantake *et al*, 2003), RPA(rfa-t11) (Kantake *et al*, 2003), *E. coli* SSB protein (Kowalczykowski *et al*, 1981; LeBowitz, 1985), and *E. coli* RecO (Luisi-DeLuca and Kolodner, 1994) were prepared as described.

Two-step DNA strand exchange and annealing

DNA strand exchange reactions (reactions for Step 1 in Figure 2A and B) were carried out essentially as described (Sugiyama *et al*, 1997). Bluescript SK− circular ssDNA (ssDNA(SK−), 412.5 pmoles) and 100 pmoles of Rad51 were incubated for 15 min in 9 μl of 40 mM Tris–acetate (pH 7.5), 3.3 mM ATP, 66.7 mM KCl, 26.7 mM magnesium acetate, 66.7 μg/ml bovine serum albumin, and 1.33 mM dithiothreitol at 37°C. Then, 20.6 pmoles of RPA (2 μl) was added and incubation was continued for 30 min. Then, 826 pmoles of linear SK+ dsDNA (partially homologous to ssDNA(SK−)) or SK− dsDNA (completely homologous to ssDNA(SK−)) in 1 μl of TE buffer was added and incubated for 90 min. For the annealing of the displaced DNA strand (Step 2 in Figure 2A), 20.6 pmoles of RPA and 412.5 pmoles of ssDNA(SK+) were preincubated for 10–15 min in 3.6 μl of a buffer containing 30 mM Tris–acetate (pH 7.5), 2.5 mM ATP, 50 mM KCl, 20 mM magnesium acetate, 50 μg/ml bovine serum albumin, and 1 mM dithiothreitol, and then added to the DNA strand exchange reaction mixture. Approximately 1 min after addition of RPA-ssDNA(SK+) complex, 25 pmoles of Rad52 (1.08 μl) was added to start DNA annealing. The reaction was incubated for 1 h unless otherwise indicated, and stopped by adding 5 μl of stop buffer (1.6% SDS and 164 μg/ml proteinase K in TAE buffer) and incubating for 10 min at 37°C. Conditions were modified as indicated in each figure legend. For reactions in Figure 5A, 20.6 pmoles of RPA(rfa1-t11) was used in place of RPA, in both Step 1 and Step 2. For reactions in Figure 6, 58.8 pmoles of SSB protein was used in place of RPA in both Step 1 and Step 2. For time course experiments (Figure 4), reactions were performed in a larger volume (90 μl), and 12.5 μl aliquots were withdrawn and mixed with stop buffer at the indicated time points. After the reactions were stopped, products were separated by electrophoresis through a 1.2% agarose gel in TAE buffer and subsequently visualized by staining with ethidium bromide. Band intensities were quantified by using BioRad Quantity One software (version 4.6). Reactions with ^{32}P-labeled dsDNA were performed as above except that the gel was dried on DE81 chromatography paper (Whatman) after visualization by ethidium bromide staining, and the labeled products were visualized and quantified by BioRad Personal FX phosphorimager with the Quantity One software.

Annealing of heat-denatured plasmid DNA

Annealing of linearized and heat-denatured pBluescript(SK−) dsDNA was performed essentially as described (Wu *et al*, 2006). DNA (10 μM) was first incubated for 5 min at 30°C with wild-type RPA or RPA(rfa1-t11) (0.5 μM) in a buffer containing 30 mM Tris–acetate (pH 7.5), 2.5 mM ATP, 20 mM magnesium acetate, 50 μg/ml bovine serum albumin, and 1 mM dithiothreitol, with or without 50 mM KCl. Then, Rad52 (1.59 μM) was added to start annealing. Concentration of proteins and DNA are final concentrations in 100-μl reaction. At the indicated time points, 10 μl aliquots were withdrawn and deproteinized by incubating with 1 μl each of 10% SDS and proteinase K (15.3 mg/ml) for 10 min at 30°C. For the zero-time sample, SDS/proteinase K was added before addition of Rad52. Where indicated, KCl and/or Rad52 was omitted from the reaction. Products were separated by electrophoresis though 1% agarose gel in TAE buffer and visualized by staining afterward with ethidium bromide.

Coordinated DNA strand exchange and annealing

First, 412.5 pmoles of ssDNA(SK−) was incubated with 25 pmoles of RPA for 5 min at 37°C in 9.74 μl of 38.5 mM Tris–acetate (pH 7.5), 3.21 mM ATP, 64.2 mM KCl, 25.7 mM magnesium acetate, 64.2 μg/ml bovine serum albumin, and 1.28 mM dithiothreitol. Then, 25 pmoles of Rad52 (1.08 μl, unless otherwise indicated) and 100 pmoles of Rad51 (0.68 μl) were added in this order. After 10 min, 825 pmoles of linear dsDNA(SK+) in 1 μl TE buffer was added to start the DNA strand exchange reaction (time = 0 min). After 10 min (time = 10 min), 412.5 pmoles of ssDNA(SK+) and 25 pmoles of

RPA preincubated in 3.6 µl of 30 mM Tris–acetate (pH 7.5), 2.5 mM ATP, 50 mM KCl, 20 mM magnesium acetate, 50 µg/ml bovine serum albumin, and 1 mM dithiothreitol was added to start annealing of the displaced DNA strand. The reaction was stopped at time = 90 min as described above. For time course experiments (Figure 7B and C), reactions were performed in a larger volume (62.5 µl), and 12.5 µl aliquots were withdrawn and mixed with stop buffer at the indicated time points. Products were separated by agarose gel electrophoresis, visualized by ethidium bromide staining, and quantified as above.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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