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The Recombination-deficient Mutant RPA (rfa1-t11) Is Displaced Slowly from Single-stranded DNA by Rad51 Protein*

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Replication protein-A (RPA) is involved in many processes of DNA metabolism, including DNA replication, repair, and recombination. Cells carrying a mutation in the largest subunit of RPA (*rfa1-t11*: K45E) have defects in meiotic recombination, mating-type switching, and survival after DNA damage caused by UV and methyl methanesulfonate, as well as increased genome instability; however, this mutant has no significant defect in DNA replication. We purified the RPA heterotrimer containing the *rfa1-t11* substitution (RPA(rfa1-t11)). This mutant RPA binds single-stranded DNA (ssDNA) with the same site size, and the RPA(rfa1-t11)·ssDNA complex shows a similar sensitivity to disruption by salt as the wild-type RPA·ssDNA complex. RPA(rfa1-t11) stimulates DNA strand exchange, provided that the Rad51 protein·ssDNA nucleoprotein complex is assembled prior to introduction of the mutant RPA. However, RPA(rfa1-t11) is displaced from ssDNA by Rad51 protein more slowly than wild-type RPA and, as a consequence, Rad51 protein-mediated DNA strand exchange is inhibited when the ssDNA is in a complex with RPA(rfa1-t11). Rad52 protein can stimulate displacement of RPA(rfa1-t11) from ssDNA by Rad51 protein, but the rate of displacement remains slow compared with wild-type RPA. These *in vitro* results suggest that, *in vivo*, RPA is bound to ssDNA prior to Rad51 protein and that RPA displacement by Rad51 protein is a critical step in homologous recombination, which is impaired in the *rfa1-t11* mutation.

Homologous recombination is necessary for the repair of broken chromosomes, maintenance of genome integrity, and production of genome diversity in all organisms. The double strand break (DSB)[1] repair model (1, 2) provides a prototypic molecular mechanism for homologous recombination. It consists of 1) introduction of the DSB, 2) processing of the DSB to produce tailed DNA with 3′-ssDNA overhang, 3) invasion of one ssDNA end into homologous dsDNA, 4) subsequent invasion or annealing of the other processed end, 5) DNA synthesis and ligation to form double Holliday junctions, and 6) branch migration and resolution of the double Holliday junctions (for review, see Ref. 3). For one of these steps, namely, invasion of the 3′-ssDNA tail into homologous dsDNA, a nucleoprotein complex comprising the DNA strand exchange protein and ssDNA (referred to as the presynaptic complex) is required. In the budding yeast, *Saccharomyces cerevisiae*, at least five proteins are involved in the formation of the presynaptic complex: Rad51, Rad52, Rad55, and Rad57 proteins and replication protein-A (RPA) (for review, see Ref. 4). Rad51 protein, a yeast homologue of *Escherichia coli* RecA protein, catalyzes DNA strand exchange in the presence of ATP (5, 6). RPA is the ssDNA-binding protein of eukaryotes (7, 8).

Biochemical studies have suggested that there are two possible paths for formation of the presynaptic complex (Fig. 1). When Rad51 protein is allowed to bind the ssDNA produced by resection of the DSB prior to RPA, it binds to both ssDNA and DNA secondary structure, because Rad51 protein binds dsDNA as effectively as ssDNA (9–11). Such binding prevents it from forming a uniform presynaptic complex. In this pathway, RPA facilitates presynaptic complex formation by removing DNA secondary structure (6), due to its helix-destabilizing activity (12) (Fig. 1, *left*). Assembly of a functional presynaptic filament proceeds very rapidly and, in this report, this pathway is referred to as the "Rad51-first" pathway. In the second pathway, RPA binds the ssDNA prior to Rad51 protein, and then Rad51 protein displaces RPA to form a uniform Rad51 nucleoprotein complex (Fig. 1, *right*). In this report, this pathway is referred to as the "RPA-first" pathway. Due to the high affinity of RPA for ssDNA (7), the displacement of RPA by Rad51 protein is slow (13) and, hence, DNA strand exchange is also slow.

Rad52 protein accelerates the displacement of RPA for Rad51 protein and, thereby, facilitates the rate of presynaptic complex formation, which, in turn, accelerates DNA strand exchange (13–15). This activity of Rad52 protein is called its recombination mediator activity (14, 16). The Rad55-Rad57 heterodimer also acts as a recombination mediator (17). Stimulation of Rad51 protein-mediated DNA strand exchange by Rad52 protein is species-specific: the *E. coli* nor human counterparts can substitute for the yeast proteins (13). Therefore, it is believed that species-specific interactions between RPA and Rad52 protein, and between Rad52 and Rad51 proteins facilitate nucleation of Rad51 protein onto the RPA-complexed ssDNA (18). Furthermore, cytological analysis of meiotic chromosomes showed that RPA and Rad52 protein colocalize extensively and that Rad52 protein is necessary for Rad51 foci formation (19). Based on the above observations, it was suggested

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[1] The abbreviations used are: DSB, double-strand break; ssDNA, single-stranded DNA; RPA, replication protein-A; SSA, single-strand annealing; BIR, break-induced replication; MMS, methyl methanesulfonate; ϵDNA, etheno DNA; DTT, dithiothreitol; BSA, bovine serum albumin; MOPS, 4-morpholinepropanesulfonic acid; SSB, single-stranded DNA binding protein.

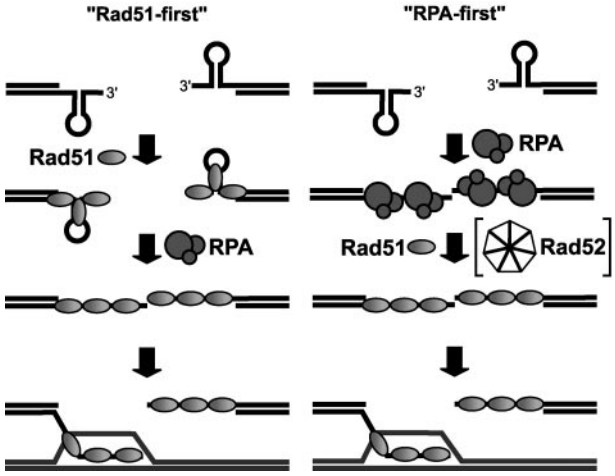

FIG. 1. **Two possible pathways for presynaptic complex formation.** *Left*: the Rad51-first pathway. When Rad51 protein is assembled on ssDNA before addition of RPA, Rad51 protein binds the processed DSB, binding to both the ssDNA and the regions of DNA secondary structure. RPA removes DNA secondary structure so that Rad51 protein can assemble *uniformly* on ssDNA to form the presynaptic complex needed for subsequent DNA strand invasion. Rad52 protein has no effect on this pathway. *Right*: the RPA-first pathway. When RPA is bound to ssDNA prior to Rad51 protein, RPA removes DNA secondary structure; however, then Rad51 protein must displace RPA to form the presynaptic complex. Because RPA binds to ssDNA tightly, Rad51 protein displaces RPA slowly. Rad52 protein facilitates displacement of RPA from ssDNA by Rad51 protein, so that a contiguous presynaptic complex forms more rapidly.

that "RPA-first" is the *in vivo* pathway for presynaptic complex formation.

In addition to its recombination mediator activity, Rad52 protein also has DNA-annealing activity (20). Rad52 protein can anneal both free ssDNA and ssDNA that is complexed with its cognate RPA (21, 22). The annealing activity of Rad52 protein is very important, because an annealing step is crucial for many recombination pathways in which *RAD52* is required (3). Annealing can follow the DNA strand invasion step of DSBR (23), and it is essential for both single-strand annealing (SSA) (24, 25) and break-induced replication (BIR) (26) (for review, see Ref. 3). In SSA, a DSB between tandem repeat sequence is processed so that complementary sequences are exposed and annealed; in BIR, strand invasion is believed to be mediated by annealing activity of Rad52 protein, then the invading strand can serve as a primer for DNA replication.

RPA consists of three subunits, RPA1, RPA2, and RPA3, with approximate molecular masses of 70,000, 32,000, and 14,000 Da, respectively (7, 8). Among them, the largest subunit is the most studied. RPA1 has three major domains: an N-terminal domain involved in DNA polymerase α stimulation (27), two ssDNA-binding domains in the middle of the protein (28), and a C-terminal domain with a putative zinc finger involved in subunit interaction and ssDNA binding (29–31). RPA1 is phosphorylated in a DNA damage-induced manner (32). In addition to its ssDNA-binding activity, RPA interacts with many proteins, including DNA polymerase α, XPA, p53, and Rad52 protein (for review, see Refs. 7 and 8).

In the budding yeast *S. cerevisiae*, RPA1 is encoded by the *RFA1* gene (33, 34). Genetic analysis showed that *RFA1* belongs to the *RAD52* epistasis group of recombinational repair genes (35, 36). Umezu *et al.* (37) systematically isolated 21 *rfa1* mutants: five of them were temperature-sensitive for growth, and 19 of them (including some temperature-sensitive mutants) were sensitive to UV and methyl methanesulfonate (MMS). A subset of the 21 mutants also shows increased genome instability (38). One of the mutants is *rfa1-t11* (K45E); the *rfa1-t11* mutant is about 1000-fold more sensitive to both UV and MMS than wild-type. Its defects in mating-type switching, the single-strand annealing (SSA) pathway of DSB repair (37), and meiotic recombination (39) indicate that the *rfa1-t11* mutant is recombination-defective. The *rfa1-t11* mutation also rescues the "terminal arrest" phenotype of Δ*yku70* cells and Δ*tid1* cells in the presence of irreparable DSB (40, 41). Despite severe defects in DNA recombination and repair, the *rfa1-t11* mutant shows limited deficiency, if any, in DNA replication, suggesting that the *rfa1-t11* mutation affects only the recombination and repair functions of RPA.

In this report, we show the biochemical properties of the RPA heterotrimer containing the *rfa1-t11* mutation (RPA(rfa1-t11)). RPA(rfa1-t11) binds ssDNA as efficiently as wild-type RPA and stimulates presynaptic complex formation when added to preformed Rad51 protein·ssDNA complexes. However, RPA(rfa1-t11) is defective in presynaptic complex formation when RPA and ssDNA are complexed prior to addition of Rad51 protein. These results provide additional evidence that presynaptic complex formation occurs by the prior binding of RPA to the processed DSB and that failure by Rad51 protein to displace the bound RPA results in recombination deficiency.

EXPERIMENTAL PROCEDURES

DNA and Proteins—ϕX174 ssDNA, dsDNA, restriction endonucleases, and Vent DNA polymerase were purchased from New England BioLabs. Proteinase K was purchased from Roche Applied Science. ϕX174 dsDNA was digested with *Pst*I. Poly(dT) was purchased from Amersham Biosciences. The concentrations of linearized ϕX174 dsDNA, ϕX174 ssDNA, M13 ssDNA, and poly(dT) were determined using molar extinction coefficients of 6500, 8125, 8125, and 7300 $M^{-1}\cdot cm^{-1}$, respectively, at 260 nm. Etheno DNA (ϵDNA) was prepared as described (42). The concentration of ϵDNA was determined using a Malachite green-ammonium molybdate assay (43). All DNA concentrations are expressed as moles of nucleotides.

The plasmid for overexpression of Rfa1-t11 subunit (pYES-*rfa1-t11*) was constructed by cloning the PCR-amplified *rfa1-t11* open reading frame into pYES2 (Invitrogen) plasmid under the GAL promoter. The sequence of the open reading frame was confirmed by the sequencing facility at University of California, Davis.

Wild-type RPA was purified from BJ5464 (*MATα ura3–52 trp1 leu2Δ1 his3Δ200 pep4*::*HIS3 prb1Δ1.6R can1* GAL) containing three plasmids that express RPA1, RPA2, and RPA3 under control of the GAL promoter (44), essentially as described (45), except for the omission of Affi-Gel blue column chromatography and use of Resource Q chromatography instead of DEAE-cellulose chromatography. To overproduce RPA(rfa1-t11), BJ5464 with the genomic *rfa1-t11* mutation was constructed by the "pop-in pop-out" method as described (56), using pKU2 *rfa1-t11* linearized by *Nhe*I (37). The genomic DNA sequence was confirmed by sequencing of PCR-amplified *rfa1-t11* fragment. RPA(rfa1-t11) was overexpressed in BJ5464 *rfa1-t11* with pYES-*rfa1-t11* and RPA2 and RPA3 overexpressing plasmids that were used for wild-type RPA expression. RPA(rfa1-t11) was purified by the same method used for wild-type RPA, except that the second wash in ssDNA-cellulose chromatography was done at 1 M NaCl instead of 0.75 M NaCl. Rad51 and Rad52 proteins were purified from *E. coli* as described (11, 13). RecA protein was purified as described (46). Concentrations of RPA, Rad51, Rad52, and RecA proteins were determined using extinction coefficients of 8.8×10^4, 1.29×10^4, 2.4×10^4, and 2.7×10^4 $M^{-1}\cdot cm^{-1}$, respectively, at 280 nm.

ssDNA Binding Assays—Binding to ssDNA was monitored by two procedures. One took advantage of the quenching of RPA intrinsic fluorescence upon ssDNA binding, using an SLM 8000 spectrofluorometer. The excitation and emission wavelengths were 290 and 345 nm, respectively, and the excitation and emission band widths were 2 and 8 nm, respectively. The second assay measured the fluorescence change in ϵDNA fluorescence upon RPA binding, using an excitation wavelength of 300 nm and an emission wavelength of 405 nm. The reaction buffer contained 30 mM Tris acetate (pH 7.5), 20 mM magnesium acetate, 50 mM NaCl, and 1 mM dithiothreitol (DTT) unless otherwise indicated. DNA and protein concentrations are indicated in the figure legends. To prevent binding of RPA to the cuvette surface, a methyl acrylate cuvette (Perfector Scientific) was coated with bovine serum albumin (BSA) by incubating the cuvette with 4 ml of 250 μg/ml BSA

(Fraction V, Sigma) for at least 20 min at room temperature, and then washed with water only and dried.

DNA Strand Exchange Assays—DNA strand exchange reactions were performed as described (6, 13). Briefly, ϕX174 ssDNA, RPA, Rad51, and Rad52 proteins were mixed at 37 °C in the indicated order and time in 12.5 μl of buffer containing 42 mM MOPS (pH 7.4), 3 mM magnesium acetate, 1 mM DTT, 20 mM NaCl, 25 μg/ml BSA, and 2.5 mM ATP. DNA strand exchange was initiated by addition of 33 μM linearized ϕX174 dsDNA and 4 mM spermidine acetate and then incubated at 37 °C for 90 min. Joint molecules are formed as an intermediate product, and nicked circular dsDNA is formed as a final product in this assay. The reaction mixture was deproteinized by addition of 0.67% SDS and 1.1 mg/ml of Proteinase K and incubation for 10 min at 37 °C. The reaction mixtures were analyzed by electrophoresis through 1% agarose gel in TAE buffer at 40 V for 14 h, and the gel was stained in 1 μg/ml ethidium bromide in TAE buffer. Both the order of addition, and the concentration of proteins are described in the figure legends.

ATP Hydrolysis Assays—The ATPase activity of Rad51 and RecA proteins was measured in 120 μl of buffer at 37 °C essentially as described (6, 47, 48). Both the order of addition, and the concentration of DNA and proteins are described in the figure legends. For reactions with Rad51 protein, the buffer contained 30 mM Tris acetate (pH 7.5), 5 mM magnesium acetate, 50 mM KCl, 1 mM DTT, 2.5 mM ATP, 0.3 mM phosphoenolpyruvate, 512 μM reduced form β-nicotinamide adenine dinucleotide (NADH), 50 μg/ml BSA, 10 units/ml pyruvate kinase, and 10 units/ml lactate dehydrogenase. For reactions with RecA protein, the buffer contained 30 mM Tris acetate (pH 7.5), 5 mM magnesium acetate, 1 mM DTT, 2.5 mM ATP, 1.5 mM phosphoenolpyruvate, 512 μM NADH, 50 μg/ml BSA, 15 units/ml pyruvate kinase, and 15 units/ml lactate dehydrogenase. In this assay system, ATP hydrolysis is coupled to the oxidation of NADH; therefore, ATP hydrolysis is monitored as decrease of absorbance at 340 nm. Absorbance at 340 nm was monitored using an HP 8452A spectrophotometer. The ATP hydrolysis rate (micromolar/min) was calculated by the following formula: rate of A340 decrease $\times$ 9880. The amount of ATP hydrolyzed (micromolar) was calculated by A340 decrease $\times$ 6.22 $\times$ 10^{-3}.

Complementary ssDNA Annealing Assays—Annealing of RPA·ssDNA complexes by Rad52 protein was performed as described (21). Briefly, heat-denatured pBluescript II SK$^-$ (Stratagene) linearized by *Pst*I (600 nM) and various concentrations of RPA were incubated at 30 °C in buffer (400 μl) containing 30 mM Tris acetate (pH 7.5), 5 mM magnesium acetate, 1 mM DTT, and 0.2 μM 4′,6-diamidino-2-phenylindole, Rad52 protein (75 nM) was added to start annealing. The fluorescence of 4′,6-diamidino-2-phenylindole was monitored using an SLM 8000 spectrofluorometer with excitation and emission wavelengths of 345 and 467 nm, and bandwidths of 2 and 8 nm, respectively.

RESULTS

RPA(rfa1-t11) Has ssDNA-binding Properties That Are Similar to Wild-type RPA—To understand the nature of *rfa1-t11* mutation, we purified the RPA heterotrimer containing the *rfa1-t11* (K45E) mutation to homogeneity (data not shown). We designate the mutant RPA heterotrimer as RPA(rfa1-t11). First, we compared the ssDNA-binding activities of wild-type RPA and RPA(rfa1-t11). To determine the occluded binding site size of RPA(rfa1-t11), two different fluorometric methods were employed. One method was to monitor the intrinsic fluorescence of RPA (Fig. 2*A*); the intrinsic fluorescence of RPA is quenched up to 40% when bound to ssDNA (49). Therefore, as RPA or RPA(rfa1-t11) is added in to a fixed amount of ssDNA under stoichiometric binding conditions, the fluorescence increase per amount of protein added remains low until ssDNA is saturated; at that point, the slope of the titration curve changes. Using this method, the occluded binding site size of both wild-type RPA and RPA(rfa1-t11) was determined to be 26 nucleotides.

The second method used to measure ssDNA binding was to monitor changes in the fluorescence of etheno DNA (ϵDNA) upon RPA binding (42). ϵDNA contains ethenoadenosine and ethenocytosine residues made by modification with chloroacetaldehyde; ethenoadenosine has strong fluorescence at 405 nm when excited at 300 nm. The fluorescence of ϵDNA increases up to 4-fold when RPA is bound to it (45). Using this method, we determined the protein to DNA ratio at which DNA binding was saturated for both wild-type RPA and RPA(rfa1-t11) (Fig. 2*B*). In agreement with the results of the intrinsic protein fluorescence quenching experiments, the ϵDNA results show that both wild-type RPA and RPA(rfa1-t11) have the same occluded site size of ~26 nucleotides per RPA heterotrimer.

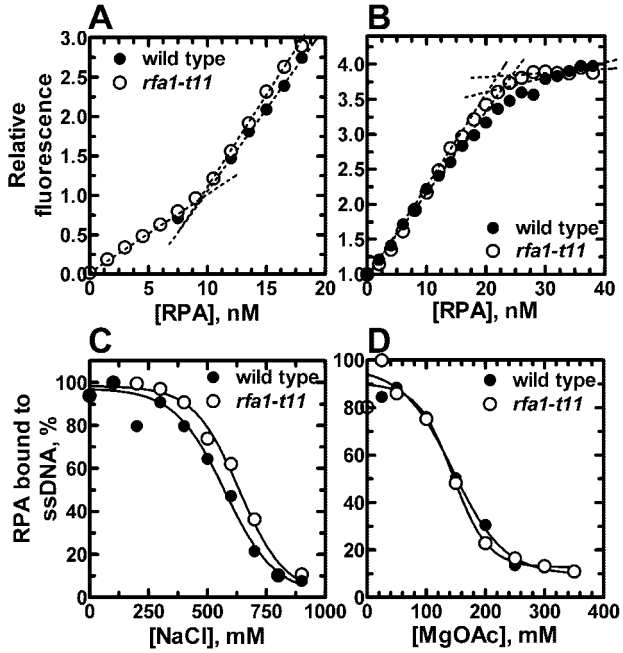

FIG. 2. **DNA binding by RPA(rfa1-t11) displays the same site size, and a similar salt sensitivity as wild-type RPA.** *A*, the quenching of the intrinsic fluorescence of wild-type RPA (●) and RPA(rfa1-t11) (○) upon binding to poly(dT) (225 nM) was monitored as described under "Experimental Procedures." The estimated binding site size for both wild-type RPA and RPA(rfa1-t11) is ~26 nucleotides per RPA heterotrimer (*dashed lines*). *B*, the binding to ϵDNA (600 nM) by wild-type RPA (●) and RPA(rfa1-t11) (○) was monitored as described under "Experimental Procedures." The binding site size for both wild-type RPA and RPA(rfa1-t11) to ϵDNA is ~26 nucleotides per RPA heterotrimer (*dashed lines*). *C*, the sensitivity of the RPA·ssDNA complexes to increasing NaCl concentration was measured. Excess ssDNA was added to the RPA in buffer containing various NaCl concentrations, and the fluorescence was measured. M13mp19 ssDNA (375 nM) and RPA (7.5 nM) were used. The midpoint for dissociation was at 575 ± 15 mM NaCl for wild-type RPA (●), and 635 ± 25 mM NaCl for RPA(rfa1-t11) (○). *D*, the sensitivity of the RPA·ssDNA complexes to increasing magnesium acetate concentration was also measured. Excess ssDNA was added to the RPA in buffer containing various magnesium acetate concentrations, and the fluorescence was measured. M13mp19 ssDNA (450 nM) and RPA (7.3 nM) were used. The midpoint for dissociation for both wild-type RPA (●) and RPA(rfa1-t11) (○) was 145 ± 10 mM magnesium acetate.

To determine the relative binding affinity of the two RPA proteins, the stability of the protein·DNA complexes to disruption by salt was compared at various sodium chloride and magnesium acetate concentrations. When the sodium chloride concentration was varied (Fig. 2*C*), RPA(rfa1-t11) showed a slightly greater resistance to NaCl concentration than wild-type; the midpoint for dissociation was 635 ± 25 mM sodium chloride for RPA(rfa1-t11), and 575 ± 15 mM sodium chloride for wild-type RPA. Fig. 2*D* shows the sensitivity of ssDNA binding of RPA to various magnesium acetate concentrations. The midpoint for dissociation of both wild-type and RPA(rfa1-t11) heterotrimers was ~145 ± 10 mM magnesium acetate, indicating that RPA(rfa1-t11) is as sensitive to the disruption by magnesium acetate as wild-type RPA. The ssDNA binding experiments indicate that RPA(rfa1-t11) has an ssDNA-binding activity that is similar to, or even slightly greater than, wild-type RPA.

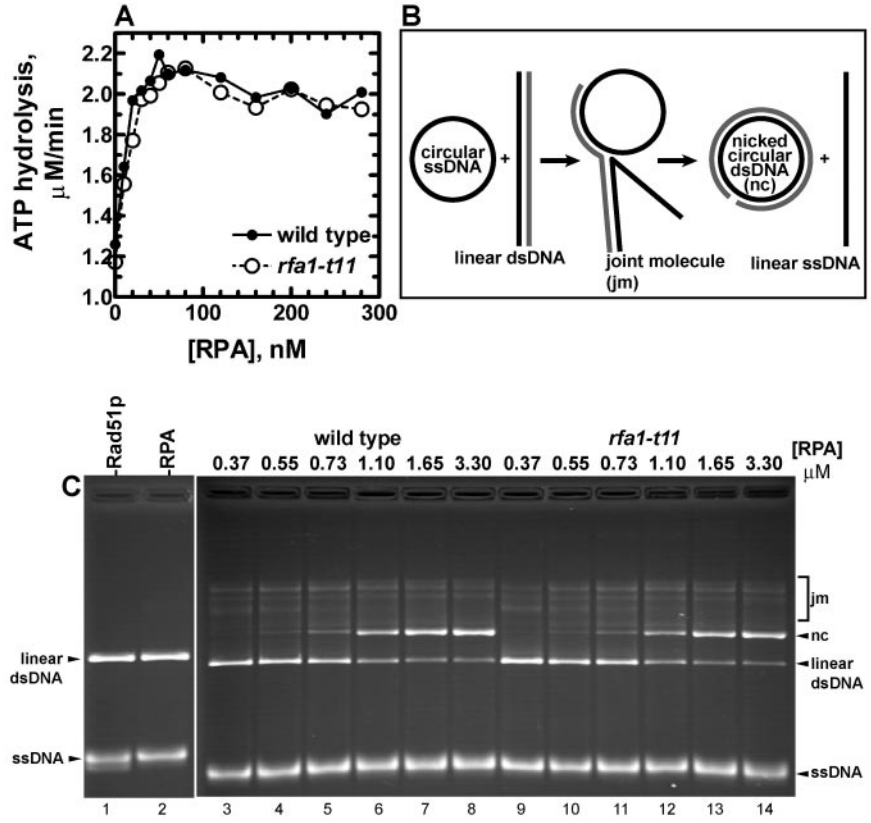

FIG. 3. **When Rad51 protein is pre-assembled on ssDNA, RPA(rfa1-t11) stimulates presynaptic complex formation as efficiently as wild-type RPA.** *A*, the ssDNA-dependent ATPase activity of Rad51 protein is stimulated by both wild-type RPA and RPA(rfa1-t11). Rad51 protein (5 μM) was incubated with ϕX174 ssDNA (10 μM) for ~5 min, and then either wild-type RPA (●) or RPA(rfa1-t11) (○) was added. ATP hydrolysis was monitored as described under "Experimental Procedures." *B*, schematic illustration of the DNA strand exchange assay. *C*, ethidium bromide-stained gel of DNA strand exchange reactions. ϕX174 ssDNA (33 μM) was incubated with Rad51 protein (11 μM) for 15 min, then various concentrations of RPA were added and incubated for 5 min. DNA strand exchange was initiated by addition of linear ϕX174 dsDNA (33 μM) and spermidine (4 mM); incubation was for 90 min. The concentrations of RPA or RPA(rfa1-t11) are indicated in the figure. *Lanes 1* and *2* represent control reactions lacking Rad51 protein and RPA, respectively.

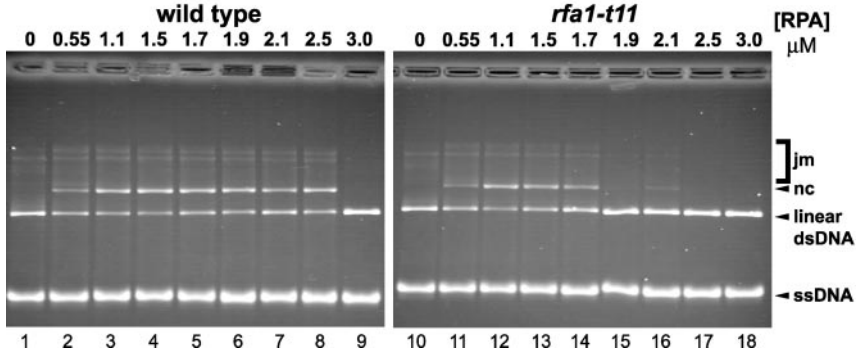

FIG. 4. **RPA(rfa1-t11) inhibits DNA strand exchange when an RPA·ssDNA complex is pre-formed.** Ethidium bromide-stained gel of DNA strand exchange reactions. ϕX174 ssDNA (33 μM) was incubated with various concentrations of either wild-type RPA or RPA(rfa1-t11) for 5 min, then Rad51 protein (11 μM) was added and incubation was for 5 min. Rad52 protein (3.3 μM) was added subsequently and incubation was for 5 min. DNA strand exchange was initiated by addition of linear ϕX174 dsDNA (33 μM) and spermidine (4 mM) and incubation was for 90 min: *lanes 1–9*, wild-type RPA; *lanes 10–18*, RPA(rfa1-t11). RPA concentrations are indicated in the figure.

Subsequent Introduction of RPA(rfa1-t11) Stimulates Presynaptic Complex Formation by Rad51 Protein—RPA stimulates presynaptic complex formation by Rad51 protein when Rad51 protein is first assembled on ssDNA (see Fig. 1, *Rad51-first*) (5, 6). Because *E. coli* SSB protein can substitute for RPA in this activity (6), it is inferred that only ssDNA-binding activity, and not species-specific protein interaction, is required for this stimulation (which is not the case for Rad52-stimulated reactions (13)). Thus, stimulation results from removal of DNA secondary structure, allowing Rad51 protein to form a uniform presynaptic complex. Because RPA(rfa1-t11) binds ssDNA at least as efficiently as wild-type RPA, we expected that RPA(rfa1-t11) should stimulate presynaptic complex formation and DNA strand exchange by Rad51 protein as effectively as wild-type RPA.

To monitor presynaptic complex formation by Rad51 protein and ssDNA directly, the ssDNA-dependent ATPase activity of Rad51 protein was monitored (Fig. 3*A*). ATP hydrolysis by Rad51 protein reflects formation of an ssDNA-Rad51 complex and, thus, presynaptic complex formation. RPA (or *E. coli* SSB protein) stimulates the ssDNA-dependent ATPase activity of Rad51 protein when Rad51 protein and ssDNA containing secondary structure are mixed prior to addition of RPA (6). Rad51 protein was first incubated with ssDNA, then various amounts of either wild-type or RPA(rfa1-t11) was added: the ATPase activity of Rad51 protein was stimulated to the same extent by either RPA. Optimum stimulation was achieved at about 50 nM RPA, corresponding to 100 nucleotides of ssDNA per one RPA heterotrimer. This result is consistent with previous observations (6). Therefore, we conclude that RPA(rfa1-t11) is as good as wild-type RPA in stimulation of presynaptic complex formation when ssDNA and Rad51 protein are complexed before the addition of RPA.

RPA(rfa1-t11) Stimulates DNA Strand Exchange by Rad51

Protein When Added Subsequent to Rad51·ssDNA Complex Formation—In addition to presynaptic complex formation, in the bacterial system, SSB protein has another role in DNA strand exchange; it binds to the displaced strand so that re-invasion is prevented. This function of SSB protein is its post-synaptic role (50). A post-synaptic function for RPA in Rad51 protein-mediated DNA strand exchange was also reported recently (51). To analyze both presynaptic and post-synaptic capabilities of RPA(rfa1-t11), we examined DNA strand exchange in the presence of RPA(rfa1-t11).

Fig. 3*B* illustrates the DNA strand exchange assay: the circular ssDNA·Rad51 protein complex pairs with homologous linear dsDNA, and strands are exchanged to form an intermediate called the joint molecule; completion of DNA strand exchange results in nicked circular dsDNA and linear ssDNA products.

First, we tested if RPA(rfa1-t11) stimulated DNA strand exchange when Rad51 protein was incubated with ssDNA before addition of RPA. Both wild-type RPA and RPA(rfa1-t11) stimulated product formation (Fig. 3*C*); there was no significant difference between wild-type RPA and RPA(rfa1-t11). For both proteins, optimal DNA strand exchange occurred at 1.65 μM RPA, which translates into 20 nucleotides of ssDNA per RPA heterotrimer. More RPA (wild-type or mutant) is needed for DNA strand exchange than for stimulation of presynaptic complex formation, as analyzed by ATPase assays, a result that is consistent with previous observations (6). This difference results from the facts that a stoichiometric amount of RPA is needed in its post-synaptic role to bind the displaced strand to prevent re-invasion of the displaced strand, whereas a less than stoichiometric amount of RPA is needed in its presynaptic role to remove the limited amount of DNA secondary structure.

Excess RPA(rfa1-t11) Inhibits DNA Strand Exchange When Bound to ssDNA before Rad51 Protein—RPA inhibits DNA strand exchange by Rad51 protein when RPA is incubated with ssDNA prior to or simultaneously with Rad51 protein (Fig. 1, *RPA-first*). Therefore, we tested whether DNA strand exchange was affected when RPA(rfa1-t11) was complexed with ssDNA before addition of Rad51 protein (Fig. 4). In this assay, various amounts of either wild-type RPA or RPA(rfa1-t11) were bound to ϕX174 ssDNA, then Rad51 protein was added. However, as described in the introduction, Rad51 protein displaces RPA very slowly, resulting in a very low yield of DNA strand exchange product (13) and making a comparison difficult. To overcome this limitation, Rad52 protein was added to accelerate RPA displacement from ssDNA by Rad51 protein.

When RPA(rfa1-t11) was used, subsaturating RPA stimulated DNA strand exchange. However, RPA(rfa1-t11) at higher concentrations than saturation (relative to the ssDNA, 1.9 μM) strongly inhibited DNA strand exchange (*lanes 15–18*). On the other hand, wild-type RPA did not show such a strong inhibition, and a 2-fold higher concentration (3 μM) was required for inhibition. Because free RPA and Rad52 protein interact (36, 52) the inhibition of DNA strand exchange by excess wild-type RPA (one RPA heterotrimer per 11 nucleotides of ssDNA) is thought to result from titration of Rad52 protein by RPA thereby preventing Rad52 protein from binding the RPA·ssDNA complex and preventing stimulation of Rad51 protein nucleation. This suggests that one possible explanation for the almost immediate inhibition seen for RPA(rfa1-t11) at concentrations just beyond saturation of the ssDNA is that the free mutant RPA might have a higher affinity for Rad52 protein. Thus, the excess RPA(rfa1-t11) might titrate out Rad52 protein, making it unavailable for interaction with the RPA(rfa1-t11)·ssDNA complex. If this were the case, then adding more Rad52 protein should restore DNA strand exchange. However,

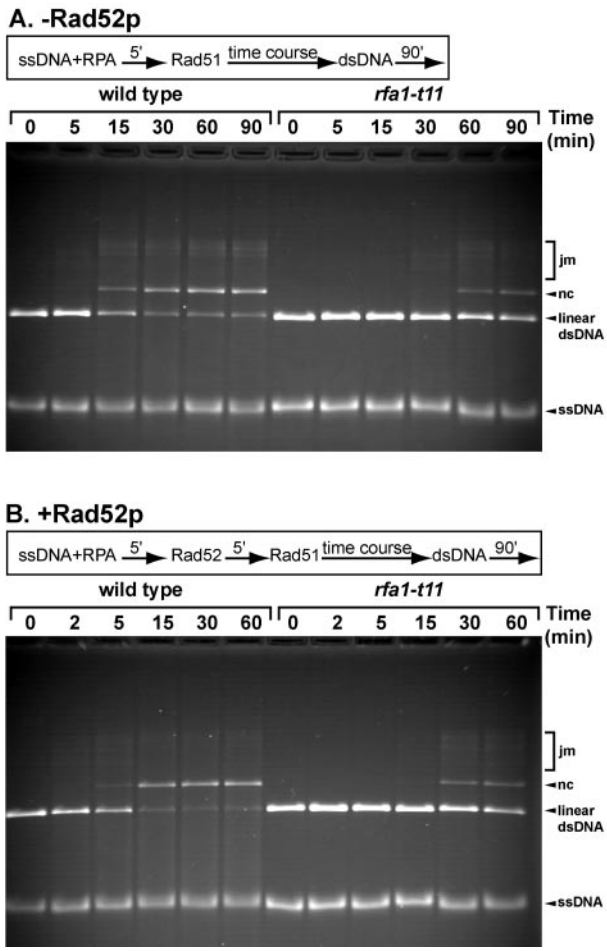

FIG. 5. **RPA(rfa1-t11) is displaced by Rad51 protein more slowly than wild-type RPA in DNA strand exchange reactions, both in the presence and absence of Rad52 protein.** *A*, time course of DNA strand exchange in the absence of Rad52 protein. ϕX174 ssDNA (33 μM) was incubated with 2.2 μM of either wild-type RPA or RPA(rfa1-t11) for 5 min. Rad51 protein (11 μM) was added and incubated for the time indicated. DNA strand exchange was initiated by addition of linear ϕX174 dsDNA (33 μM) and spermidine (4 mM) and proceeded for a fixed time of 90 min. *B*, time course of DNA strand exchange in the presence of Rad52 protein. ϕX174 ssDNA (33 μM) was incubated with either wild-type RPA or RPA(rfa1-t11) (2.2 μM) for 5 min. Rad52 protein (3.3 μM) was added and incubation was for 5 min. Rad51 protein (11 μM) was then added and incubation was for the time indicated. DNA strand exchange was initiated by addition of ϕX174 dsDNA (33 μM) and spermidine (4 mM), and proceeded for a fixed time of 90 min.

we found that excess Rad52 protein did not overcome the inhibition seen with RPA(rfa1-t11) (data not shown). Therefore, we conclude that the defect of RPA(rfa1-t11) in DNA strand exchange is not due to a stronger interaction with Rad52 protein.

Rad51 Protein Displaces RPA(rfa1-t11) from ssDNA More Slowly Than Wild-type RPA—When RPA is complexed with ssDNA prior to Rad51 protein, its displacement from ssDNA by Rad51 protein is slow due to the high affinity of RPA for ssDNA (13). With sufficient time, Rad51 protein can eventually displace RPA without the help of Rad52 protein, to form a fully functional presynaptic complex (13) (see also Fig. 5).

If the inhibition of DNA strand exchange by RPA(rfa1-t11) was caused by a diminished RPA-Rad52 protein interaction, then RPA displacement by Rad51 protein in the absence of Rad52 protein should not be affected by the *rfa1-t11* mutation. Therefore, we examined the time course of RPA displacement by Rad51 protein in the presence and absence of Rad52 protein (Fig. 5). In the absence of Rad52 protein, RPA(rfa1-t11) is displaced by Rad51 protein more slowly than wild-type RPA,

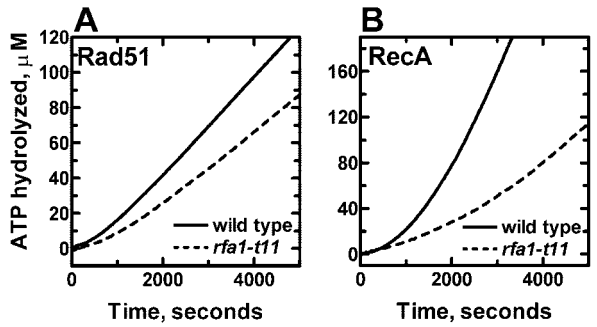

FIG. 6. **RPA(rfa1-t11) is displaced by both Rad51 and RecA proteins more slowly than is wild-type RPA.** ϕX174 ssDNA (10 μM) was incubated with 1 μM of either wild-type RPA or RPA(rfa1-t11) at 37 °C for 5 min. Then, 3.3 μM of either Rad51 (*A*) or RecA (*B*) protein was added, and ATP hydrolysis was monitored as described under "Experimental Procedures."

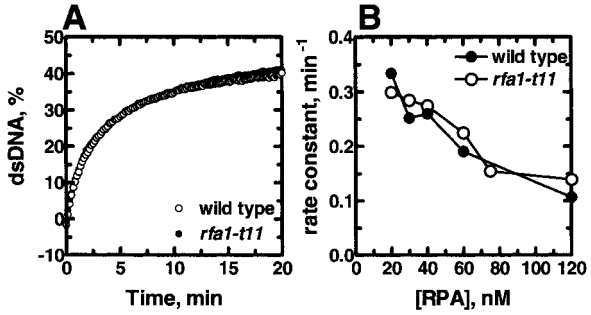

FIG. 7. **RPA(rfa1-t11) supports DNA annealing by Rad52 protein.** Heat-denatured plasmid DNA (600 nM) was incubated with various concentrations of either wild-type RPA (*closed circle*) or RPA(rfa1-t11) (*open circle*) for 100 s. Then Rad52 protein (75 μM) was added to start DNA annealing as described under "Experimental Procedures." Rate constants were calculated by curve-fitting to a single-phase exponential function using the GraphPad Prism program. *A*, time course of DNA annealing using 40 nM RPA. *B*, rate constant for DNA annealing as a function of RPA concentration.

resulting in negligible amounts of DNA strand exchange products for up to 30 min (Fig. 5*A*). However, DNA strand exchange products did appear at the later times; therefore, RPA(rfa1-t11) *could* be displaced by Rad51 protein, but only much more slowly than wild-type RPA. Thus, at least part of the defect of RPA(rfa1-t11) in DNA strand exchange is not related to its interaction with Rad52 protein but, rather, to its resistance to displacement by Rad51 protein.

In the presence of Rad52 protein, DNA strand exchange products in the RPA(rfa1-t11)-containing reaction also appear more slowly than those in the wild-type RPA reactions (Fig. 5*B*). However, Rad52 protein nevertheless stimulates DNA strand exchange in the RPA(rfa1-t11)-containing reaction (compare the 30 and 60 min time points in Fig. 5, *A* and *B*). The results in Fig. 5 (*A* and *B*) suggest that RPA(rfa1-t11) is displaced from ssDNA by Rad51 protein more slowly than wild-type RPA, regardless of the presence of Rad52 protein.

Both Rad51 and RecA Proteins Displace RPA(rfa1-t11) from ssDNA More Slowly Than Wild-type RPA—Rad51 protein is believed to displace RPA by binding to ssDNA where RPA dissociates. However, it is also possible that Rad51 protein actively displaces RPA from ssDNA through a direct interaction with RPA. Although an interaction between yeast Rad51 protein and RPA has not been reported, human Rad51 protein and human RPA do physically interact (53). Therefore, we cannot exclude the possibility that RPA(rfa1-t11) is defective in an, as yet, unreported specific interaction between Rad51 protein and RPA, resulting in slower displacement of RPA(rfa1-t11) by Rad51 protein during presynaptic complex formation. If the loss of such a potential interaction were responsible for the *rfa1-t11* defect in presynaptic complex formation, then the defect would likely be species-specific. To test this possibility, displacement of RPA by *E. coli* RecA protein was examined. In these experiments, the ATPase activity of the DNA strand exchange proteins was monitored because this activity reflects only presynaptic complex formation, unlike the DNA strand exchange assay, which reflects both presynaptic and post-synaptic effects.

When Rad51 or RecA protein is added to a pre-formed RPA·ssDNA complex, RPA is displaced and either Rad51 or RecA protein binds ssDNA. Consequently, the observed ssDNA-dependent ATP hydrolysis by either Rad51 or RecA protein accelerates: a steeper increase in ATP hydrolysis indicates faster displacement of RPA. Fig. 6 shows that both Rad51 and RecA proteins displace RPA(rfa1-t11) more slowly than wild-type RPA. These data suggest that RPA(rfa1-t11) is intrinsically more resistant to displacement regardless of the species of the displacing DNA strand exchange protein, suggesting that it has a greater kinetic lifetime on ssDNA than wild-type RPA.

Rad52 Protein Can Anneal ssDNA Complexed with RPA (rfa1-t11)—The *rfa1-t11* mutant is defective in the single-strand annealing (SSA) pathway of homologous recombination (37). SSA requires Rad52 protein and Rad1-Rad10 endonuclease, and is independent of Rad51 protein. Rad52 protein presumably functions in SSA by virtue of its annealing activity. In addition, in the canonical double-strand break repair pathway, the annealing activity of Rad52 protein could be important for annealing of the displaced DNA strand in the D-loop and the second processed DNA end (23). To determine whether ssDNA annealing by Rad52 protein is affected by the *rfa1-t11* mutation, we analyzed annealing of plasmid-sized ssDNA complexed with either wild-type RPA or RPA(rfa1-t11) by Rad52 protein. We used a fluorometric assay previously used to monitor ssDNA-annealing by Rad52 protein (21). Fig. 7 summarizes the results obtained from annealing time courses for ssDNA that is complexed with various amounts of either wild-type RPA or RPA(rfa1-t11). For either RPA, the rate constant decreased as RPA concentration increased; most importantly, there is no significant difference between wild-type RPA and RPA(rfa1-t11) in the annealing of ssDNA complexed with various amounts of RPA by Rad52 protein. The extent of annealing was also not affected by the *rfa1-t11* mutation (data not shown). These results show that RPA(rfa1-t11) is capable of supporting ssDNA annealing by Rad52 protein. Therefore, it is more likely that the SSA defect of *rfa1-t11* is unrelated to the annealing function of Rad52 protein.

DISCUSSION

In this report, we describe the behavior of a mutant RPA (RPA(rfa1-t11)), which is defective in genetic recombination. RPA(rfa1-t11) has ssDNA-binding ability that is as good as (or slightly greater than) wild-type RPA. Both wild-type and RPA(rfa1-t11) have an occluded site size of ~26 nucleotides (Fig. 2). In addition to the two methods we employed here (intrinsic fluorescence quenching and ϵDNA fluorescence), electrophoretic mobility band shift assays yielded a similar occluded site size (data not shown). This site size is smaller than either of those previously reported (90–100 nucleotides (45) and 40 nucleotides (49)). Our recent analysis revealed that the RPA preparation used by Alani *et al.* (45), contained some contaminating Rim1p, the mitochondrial SSB protein that is the same size as the RPA3 subunit.[2] This contamination is

[2] N. Kantake, S. C. Kowalczykowski, and E. Alani, unpublished results.

likely to have caused the discrepancies in the binding site sizes reported.

Consistent with this efficient ssDNA-binding activity, RPA(rfa1-t11) stimulates DNA strand exchange by Rad51 protein, provided that Rad51 protein is first assembled on ssDNA before introduction of RPA (Fig. 3, *Rad51-first*, and Fig. 1). In this Rad51-first pathway of presynaptic complex formation, only the ssDNA-binding activity of RPA is required for stimulation (6).

However, RPA(rfa1-t11) inhibits DNA strand exchange when RPA is first bound to the ssDNA (Fig. 4, *RPA-first*, and Fig. 1). Time course analysis of both presynaptic complex formation and DNA strand exchange show that RPA(rfa1-t11) is displaced from ssDNA by Rad51 protein more slowly than is wild-type RPA (Fig. 5). Therefore, slower presynaptic complex formation due to slower RPA(rfa1-t11) displacement results in less efficient DNA strand exchange. Rad52 protein stimulates RPA(rfa1-t11) displacement from ssDNA by Rad51 protein but not to the extent that it does for displacement of wild-type RPA. Both yeast Rad51 and *E. coli* RecA proteins displace RPA(rfa1-t11) more slowly than wild-type RPA (Fig. 6). Therefore, the slow displacement phenotype of RPA(rfa1-t11) is not species-specific; rather, RPA(rfa1-t11) intrinsically remains bound to ssDNA longer than does wild-type RPA.

The defect of RPA(rfa1-t11) *in vitro* is observed only in recombination reactions where RPA is first complexed with ssDNA. Rad52 protein, the recombination mediator protein, stimulates DNA strand exchange *in vitro* only when RPA is first complex with ssDNA (13–15). *In vivo*, RPA and Rad52 protein colocalize extensively prior to Rad51 foci formation, and Rad52 protein is necessary for Rad51 foci formation (19). Taken together, these findings suggest that, *in vivo*, presynaptic complex formation occurs by the RPA-first pathway (Fig. 1) rather than the Rad51-first pathway. Therefore, the defect of the *rfa1-t11* mutation in DSBR stems from the slow displacement of the mutant RPA from resected DSBs. In *rfa1-t11* cells, this RPA displacement is reduced even in the presence of Rad52 protein, due to the intrinsic tendency of RPA(rfa1-t11) to remain bound to ssDNA longer than wild-type RPA. This property of RPA(rfa1-t11) is independent of Rad52 protein. As a result, in *rfa1-t11* cells, presynaptic complex formation is blocked or slowed, so that DSB repair cannot be completed. These characteristics of RPA(rfa1-t11) can explain the deficiency of the *rfa1-t11* mutant in Rad51 protein-dependent processes: the repair of DNA damage caused by MMS, HO endonuclease-induced mating-type switching (37) and meiotic recombination (39).

The *rfa1-t11* mutant is also defective in SSA (37). One possible cause of the SSA defect is defective annealing by Rad52 protein. However, RPA(rfa1-t11) supported annealing by Rad52 protein as well as wild-type RPA (Fig. 7). Therefore, the SSA defect of the *rfa-t11* mutant may not be the direct consequence of defective annealing. An alternative is that RPA(rfa1-t11) may block access of the Rad1-Rad10 endonuclease so that the SSA pathway remains incomplete. The cleavage of 3′-flaps by Rad1-Rad10 endonuclease is an important step of the SSA pathway (54, 55). Because RPA(rfa1-t11) has a tendency to remain bound to ssDNA longer, RPA(rfa1-t11) may slow the access of Rad1-Rad10 endonuclease to ssDNA in a mechanism similar to the one that we discovered here. By the same token, other phenotypes of the *rfa1-t11* mutant such as UV sensitivity and slow growth (37), which indicate defects in excision repair and possibly in DNA replication, can be explained similarly. These collective studies suggest that both BIR and SSA require Rad52-mediated annealing but, that in SSA, RPA(rfa1-t11) blocks processes other than Rad52 protein-mediated annealing, possibly the processing of the 3′-ssDNA flaps.

Lee *et al.* (40) observed that Δ*yku70* (*YKU70* encodes yeast Ku70 protein) cells are unable to "adapt" so that they are "terminally arrested" after an irreparable DSB is induced, due to extensive degradation at the DSB. Adaptation is the progression of the cell cycle after arrest at G_2 phase but without repairing the damage. This defect of Δ*yku70* is overcome by the *rfa1-t11* mutation. Although the *rfa1-t11* mutation alone has no effect on the adaptation, physical analysis of DSB repair showed a greater accumulation of ssDNA in both *rfa1-t11* and Δ*yku70 rfa1-t11* cells than isogenic Δ*yku70 RFA1* cells. Greater accumulation of ssDNA in the *rfa1-t11* cells is consistent with our observation that RPA(rfa1-t11) remains bound to ssDNA more tightly and possibly longer than wild-type. Perhaps the *rfa1-t11* mutation rescues the terminal arrest phenotype of Δ*yku70* cells by protecting ssDNA; such protected ssDNA may send a signal for adaptation either directly or via a bound protein. We could imagine that in a Ku mutant, there is continual resection, continued binding of RPA, and constant displacement of RPA by Rad51 and, hence, continued generation of a ssDNA signal. However, in the *rfa1-t11* mutant, there is continual resection, continued binding of RPA, but less or no displacement by Rad51 protein, and hence less or no ssDNA signal. The latter case would allow for adaptation. Another explanation is that wild-type RPA·ssDNA complex signals to maintain arrest and that the *rfa1-t11* mutation cannot signal. Finally, the last alternative is that the RPA·ssDNA complex can be used for rescue by a pathway that does not use the Rad51 protein·ssDNA complex.

Thus, in summary, the results in this report provide additional evidence that presynaptic complex formation proceeds by the binding of RPA to ssDNA prior to Rad51 protein. The long-lived RPA(rfa1-t11)·ssDNA complex provides an explanation for most of the *rfa1-t11* phenotypes that are observed *in vivo*.

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