Molecular Cell, Vol. 11, 1337–1347, May, 2003, Copyright ©2003 by Cell Press

RecFOR Proteins Load RecA Protein onto Gapped DNA to Accelerate DNA Strand Exchange: A Universal Step of Recombinational Repair

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Summary

Genetic evidence suggests that the RecF, RecO, and RecR (RecFOR) proteins participate in a common step of DNA recombination and repair, yet the biochemical event requiring collaboration of all three proteins is unknown. Here, we show that the concerted action of the RecFOR complex directs the loading of RecA protein specifically onto gapped DNA that is coated with single-stranded DNA binding (SSB) protein, thereby accelerating DNA strand exchange. The RecFOR complex recognizes the junction between the ssDNA and dsDNA regions and requires a base-paired 5′ terminus at the junction. Thus, the RecFOR complex is a structure-specific mediator that targets recombinational repair to ssDNA-dsDNA junctions. This reaction reconstitutes the initial steps of recombinational gapped DNA repair and uncovers an event also common to the repair of ssDNA-tailed intermediates of dsDNA-break repair. We propose that the behavior of the RecFOR proteins is mimicked by functional counterparts that exist in all organisms.

Introduction

DNA lesions are produced by ultraviolet radiation, γ-ray radiation, or chemical mutagens; if left unrepaired, they are lethal for all organisms. These unrepaired lesions, directly or indirectly, produce double-stranded DNA breaks (DSBs) or single-stranded DNA (ssDNA) gaps (Michel et al., 1997). Many breaks are produced as a consequence of DNA replication: for example, if the lesion is a single-strand break, then replication through the break produces a DSB; if the lesion blocks progression of the DNA polymerase, then an ssDNA gap results (Kowalczykowski, 2000). In *Escherichia coli*, the RecBCD pathway of genetic recombination is responsible for the repair of DSBs. The DSB end is recognized by the RecBCD enzyme, and its helicase and nuclease activities process the DSB to produce the ssDNA required for homologous pairing by RecA protein (Kowalczykowski, 2000). An ssDNA gap, on the other hand, is repaired by another recombination pathway, the RecF pathway (Horii and Clark, 1973; Wang and Smith, 1984; Tseng et al., 1994).

The RecF pathway involves the functions of RecA, RecF, RecG, RecJ, RecN, RecO, RecQ, RecR, RuvA, RuvB, RuvC, and SSB proteins (Kowalczykowski et al., 1994). Although the RecBCD pathway is involved in more than 99% of conjugational recombination events in a wild-type cell (Horii and Clark, 1973), this apparently strong bias for recombination results from the fact that conjugational integration occurs at the ends of the DNA, which are DSBs. However, there is ample evidence that the RecF pathway is essential for nearly all non-DSB recombination in cells: (1) the repair of UV-induced lesions, which produce both DSBs and ssDNA gaps, requires both the RecBCD- and RecF pathways (Smith et al., 1987); (2) plasmid recombination requires the RecF pathway (Kolodner et al., 1985); (3) recombination during conjugation at sites away from the DSB ends occurs by the RecF pathway (Smith, 1991). Furthermore, if the RecBCD pathway is inactivated by mutation, the RecF pathway can be activated to promote DSB repair by suppressor mutations in the *sbcB* and *sbcC* (or *sbcD*) genes (Kushner et al., 1971; Lloyd and Buckman, 1985). Remarkably, the efficiency of conjugational recombination by the RecF pathway in these *recBC sbcBC* cells is similar to that of the RecBCD pathway in wild-type cells, demonstrating that the machinery of the RecF pathway is as capable as the RecBCD pathway of DSB recombinational repair. Thus, in addition to the RecBCD pathway, the RecF pathway of recombination is important for cellular function and, indeed, is especially necessary for the repair of ssDNA gaps.

The mechanism of recombinational repair mediated by the RecF pathway is, relative to the RecBCD pathway, poorly defined. In the RecF pathway, RecQ helicase initiates recombination by unwinding at DNA breaks (Harmon and Kowalczykowski, 1998) and RecJ protein, a 5′ to 3′ nuclease, is likely needed to exonucleolytically process the unwound DNA (Lovett and Kolodner, 1989). The role of the unique components of this pathway, the RecF, RecO, and RecR proteins, is not entirely clear. Genetic studies argue that the RecFOR proteins are involved in a common step of the recombination process. Several *recA* mutations (e.g., *recA803*) that were isolated as suppressors of *recF* mutations (Volkert and Hartke, 1984; Wang and Smith, 1986) not only suppress the *recF* mutation but also suppress *recO* and *recR* mutations (Wang et al., 1993). The behavior of RecA803 protein provides insight into the possible function of the RecFOR proteins: RecA803 protein can displace SSB protein from ssDNA more efficiently than wild-type RecA protein (Madiraju et al., 1988, 1992; Lavery and Kowalczykowski, 1992), suggesting that the role of RecF protein is the loading of wild-type RecA protein onto SSB-coated ssDNA. Thus, the RecFOR proteins may be involved in the loading of RecA protein onto ssDNA. Additional evidence for a single function for each of these proteins came from studies of the bacteriophage λ *orf* gene which showed that this single gene product suppresses *recF*, *recO*, and *recR* mutations (Sawitzke and Stahl, 1992).

Biochemical analyses have defined several functions for the individual proteins. The RecO protein has two activities in vitro: (1) the annealing of complementary ssDNA (Luisi-DeLuca and Kolodner, 1994; Kantake et al., 2002) and (2) the loading of RecA protein onto SSB-

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coated ssDNA (Umezu et al., 1993; Bork et al., 2001). The latter activity is consistent with the biochemical behavior of the *recF* suppressor, RecA803 protein. RecF protein binds to ssDNA and dsDNA (Griffin and Kolodner, 1990; Madiraju and Clark, 1992; Webb et al., 1999) and shows preferential binding to gapped DNA (Hegde et al., 1996b). RecF protein also binds (Madiraju and Clark, 1992) and hydrolyzes ATP (Webb et al., 1999). RecF protein interacts with RecO (Umezu and Kolodner, 1994; Hegde et al., 1996a) and RecR (Umezu and Kolodner, 1994; Webb et al., 1995) and the RecFR complex limits the extension of RecA filament beyond the ssDNA gap (Webb et al., 1997). Despite the extensive genetic evidence suggesting that RecF protein acts in conjunction with the RecOR proteins, all attempts to reconstitute such collaboration in vitro have been negative: the RecF protein was found to inhibit the RecA-loading activity by the RecOR proteins (Umezu et al., 1993; Bork et al., 2001). Nevertheless, both genetic analyses and the interaction of RecF protein with either RecO or RecR protein (Umezu and Kolodner, 1994; Webb et al., 1995; Hegde et al., 1996a) strongly suggests that the RecF, RecO, and RecR proteins can act in a concerted manner. Here, we demonstrate that the coordinated action of RecF, RecO, and RecR proteins mediates the loading of RecA protein specifically onto SSB-coated gapped DNA. We suggest that this proposed function of RecFOR proteins can represent a common feature of DNA recombination in all organisms.

Results

The RecFOR Proteins Load RecA Protein onto SSB-Coated Gapped DNA but Not onto SSB-Coated ssDNA

Prior studies suggested that the RecFOR proteins could function at the level of RecA protein filament formation. To monitor the kinetics of RecA nucleoprotein filament assembly, the ATP hydrolysis activity of RecA protein that results from filament formation was followed. Because RecA protein hydrolyzes ATP when bound to DNA (k_{cat} = 25–30/min; Kowalczykowski and Krupp, 1987; Brenner et al., 1987), whereas RecF protein hydrolyzes negligible amounts of ATP (k_{cat} <0.5/min; Webb et al., 1995), the observed ATPase activity predominantly reflects the extent of RecA nucleoprotein assembly. Our general strategy was to pre-bind SSB protein to a variety of gDNA substrates and then to monitor the ATPase activity resulting from RecA nucleoprotein filament formation, in the presence and absence of RecF, RecO, or RecR protein. The gDNA substrates, containing large gapped and widely spaced duplex regions, were produced by annealing short synthetic oligonucleotides to circular M13 ssDNA (Figure 1A).

We first compared the kinetics of RecA protein assembly onto SSB-coated ssDNA and onto SSB-coated gDNA7200 (Figures 1B–1E, thin dashed and thin continuous lines). In this and all following figures showing ATPase activity, the *rate* of ATP hydrolysis is plotted. In the absence of the RecFOR proteins, the binding of RecA protein onto SSB-free ssDNA was rapid (Figure 1E), as previously reported (Kowalczykowski and Krupp, 1987); in contrast, and as expected, the binding of RecA protein onto ssDNA coated with SSB protein was very slow (Figure 1B, thin dashed line, and Figure 1E) but ultimately reached the same plateau value for ATPase activity (Kowalczykowski and Krupp, 1987; Lavery and Kowalczykowski, 1992; Madiraju et al., 1992). The RecOR proteins somewhat stimulated the binding of RecA protein onto the SSB-ssDNA complex (compare Figure 1B versus 1C, thin dashed lines, and Figure 1E) as reported (Umezu et al., 1993; Bork et al., 2001) and also onto the gDNA7200 (compare Figures 1B versus 1C, thin continuous line, and Figure 1E). When RecF protein was added together with RecOR proteins, it inhibited the binding of RecA onto the ssDNA lacking an ssDNA-dsDNA junction, as previously reported (compare Figure 1C versus 1D, thin dashed lines; Umezu et al., 1993; Bork et al., 2001). However, unexpectedly, rather than inhibiting binding, the RecFOR proteins stimulated RecA nucleoprotein assembly when the gapped (gDNA7200) substrate was used (compare Figure 1D to both 1B and 1C, thin continuous lines, and Figure 1E).

The ssDNA-dsDNA Junction Is the Target of RecFOR Protein Action

The only structural difference between the gDNA7200 and circular ssDNA substrates is the presence of a dsDNA region and the dsDNA-ssDNA junctions in gDNA7200. To determine whether this structure is the site for RecFOR protein action, the number of dsDNA regions and junctions was increased by annealing additional oligonucleotides: one for gDNA7200, two for gDNA3600, and four for gDNA1800. In the presence of RecFOR proteins, RecA protein assembled onto SSB-coated gDNA3600 or gDNA1800 more rapidly than to the gDNA7200 (Figures 1D and 1E). Furthermore, the kinetics of RecA protein assembly onto the SSB-coated gDNA1800 in the presence of RecFOR proteins (Figure 1D, thick continuous line) were the same as for SSB-free gDNA1800 (data not shown; Figure 1E), indicating that the rate of SSB-displacement was no longer limiting. When RecF protein was omitted, changing the number of duplex junction regions only slightly affected the kinetics of RecA protein binding in the presence of RecOR proteins (Figure 1C); similarly, in the absence of RecFOR proteins, varying the number of duplex junction regions had only a very small effect (Figure 1B). Finally, omission of either RecO or RecR protein also eliminated the stimulatory effect (Figure 1E). These results suggest that the RecFOR proteins function in a concerted manner at the ssDNA-dsDNA junction in the gDNA to facilitate the assembly of RecA protein onto SSB-coated ssDNA.

RecFOR Complex Function Requires a Base-Paired 5′ Terminus at the ssDNA-dsDNA Junction in the gDNA

To determine which region in the gDNA is essential for RecA-loading activity, an unpaired region was introduced into the 5′- or 3′ end of the dsDNA region by annealing an oligonucleotide containing heterologous sequences at either end, to produce gDNA with ssDNA tails (gDNA7200-5′T or gDNA7200-3′T). The ability of RecFOR to stimulate the loading of RecA protein on these unpaired gDNA substrates was examined (Figure 2). RecFOR proteins loaded RecA protein onto gDNA7200-5′T less efficiently than onto gDNA7200 (Figure 2A); the

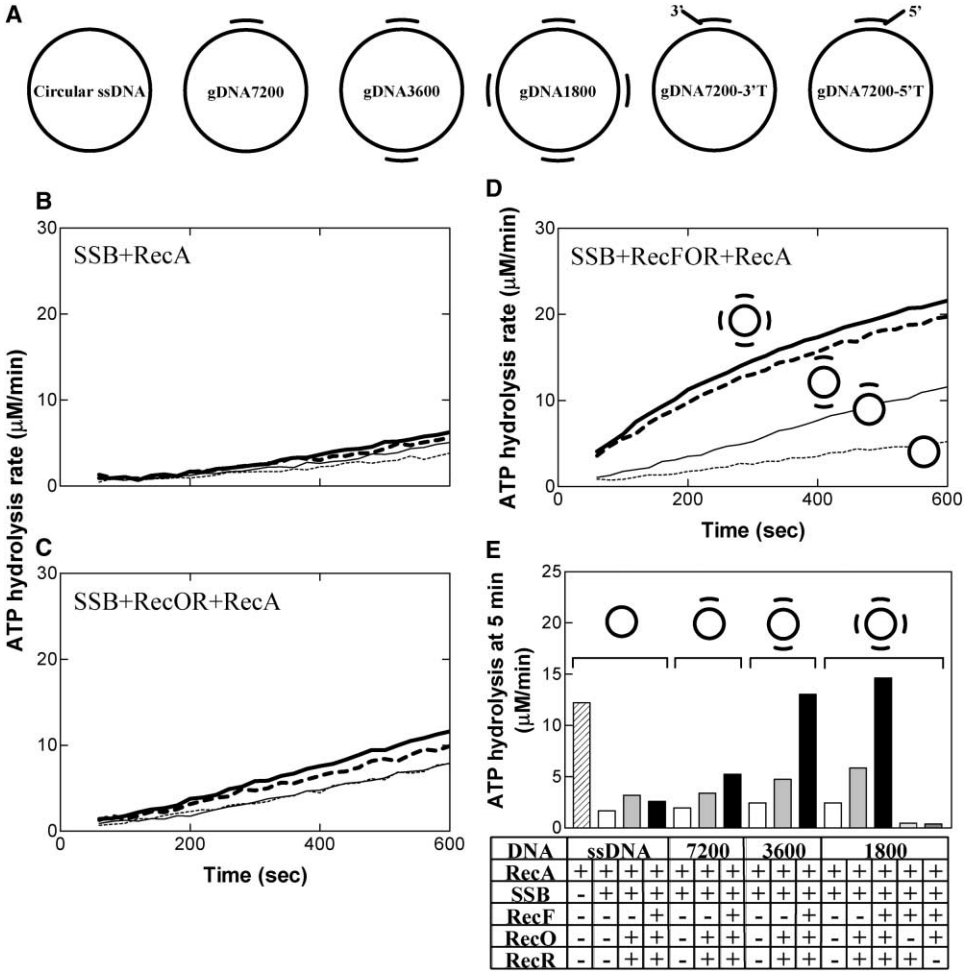


Figure 1. RecFOR Proteins Stimulate Assembly of the RecA Protein Filament onto Gapped DNA

The DNA substrates used in this study are illustrated in (A). M13mp7 viral ssDNA was used as the circular ssDNA. The gapped DNA, gDNA7200, contains an oligonucleotide annealed to the M13mp7 ssDNA to produce a gapped region of 7.2 kb. Similarly, gDNA3600 and gDNA1800 contain two or four oligonucleotides, respectively, that are complementary to the circular ssDNA to produce gDNAs having two 3.2 kb gapped regions, or four 1.6 kb gapped regions, respectively. The oligonucleotides used for gDNA7200-3′T and gDNA7200-5′T have a 10 nucleotide noncomplementary sequence at the 3′- or 5′ end, respectively.

The kinetics of RecA protein binding onto the DNA substrates were followed by measuring DNA-dependent ATP hydrolysis by RecA protein. M13 circular ssDNA (thin dashed line), gDNA7200 (thin continuous line), gDNA3600 (thick dashed line), gDNA1800 (thick continuous line) were used as the DNA substrates. The reactions also contained SSB and RecA proteins (B); SSB, RecOR, and RecA proteins (C); or SSB, RecFOR, and RecA proteins (D). The concentration of RecF protein was 0.1 μM, when present. The reaction requirements are summarized in (E), where the ATP hydrolysis rate obtained 5 min after addition of RecA protein is presented.

binding kinetics were similar to those on circular ssDNA. On the other hand, the RecFOR proteins loaded RecA protein onto gDNA7200-3′T more efficiently than onto gDNA7200, suggesting that not only was the base-paired 5′ end important but also that the base-paired 3′ end is inhibitory, probably due to binding of the RecFR complex to this end (Webb et al., 1997). Since the heterologous sequence attached to the 5′ terminus of the ssDNA-dsDNA junction inhibited RecFOR function whereas 3′-heterology did not, these observations indicate that the RecFOR proteins recognize and require the 5′ terminus of a junction for RecA-loading activity.

RecF Protein Binds to the ssDNA-dsDNA Junction

Our previous results showing that RecF protein is required for the recognition of gDNA suggest a direct interaction between RecF protein and the gDNA. To determine whether this was indeed the case, the concentration of RecF protein was varied in the presence of several fixed gDNA concentrations. To simplify analysis, the gDNA7200 substrate carrying a single gap was used. When the gDNA concentration is 0.7 nM (molecule), approximately 10 nM of RecF protein is required for maximum stimulation (Figure 3A) When the concentration of gDNA is reduced, the RecF protein concentration required to achieve saturation is reduced concomitantly and proportionally. These results suggest a stoichiometric relationship between the gDNA and RecF protein. This conclusion is supported by the reciprocal titration in which the RecF protein concentration is fixed but the gDNA concentration is varied: Figure 3B shows that maximal stimulation occurs at approximately 0.7 nM gDNA when the RecF concentration is 10 nM. On the other hand, no stoichiometric relationship between the

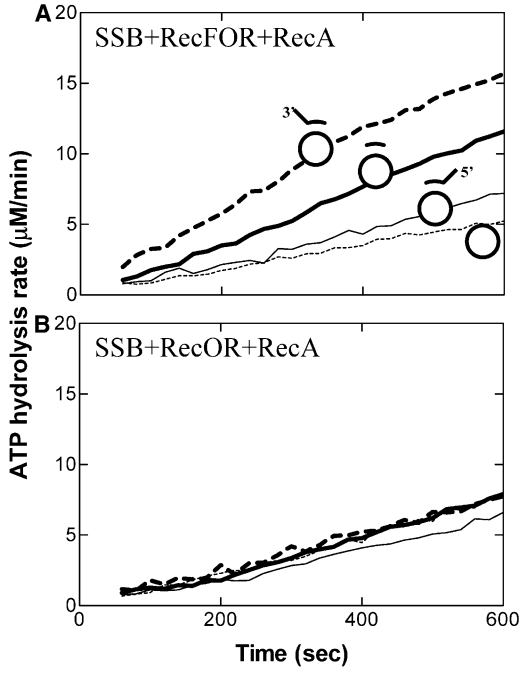


Figure 2. RecF Protein Requires Gapped DNA that Is Base Paired at the 5′ End of the dsDNA-ssDNA Junction

The kinetics of RecA protein filament assembly were followed on the following substrates: M13 circular ssDNA (thin dashed line), gDNA7200-5′T (thin continuous line), gDNA7200 (thick continuous line), and gDNA7200-3′T (thick dashed line). The reaction also contained: SSB, RecFOR, and RecA proteins (A) or SSB, RecOR, and RecA proteins (B). The concentration of RecF protein was 0.1 μM, when present.

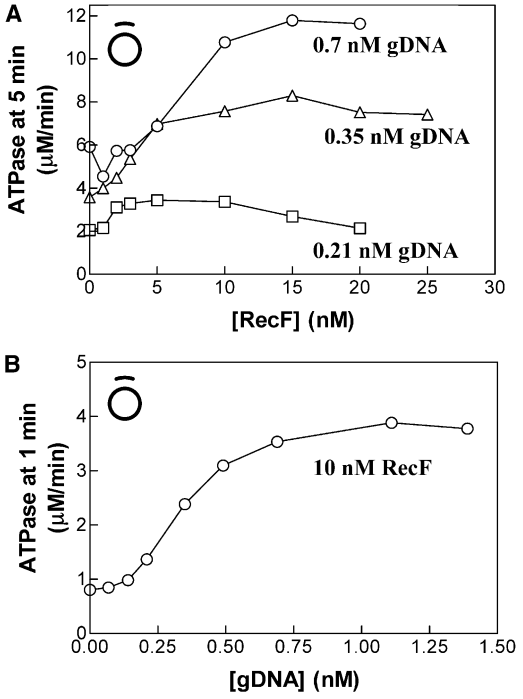


Figure 3. RecF Protein Forms a Stoichiometric Complex with the Gapped DNA

Reactions were performed with RecA, RecFOR, and SSB proteins, and gDNA7200. (A) The ATP hydrolysis rate 5 min after addition of RecA protein is presented. The concentration of RecF protein was varied in the presence of 0.7 nM (circle), 0.35 nM (triangle), or 0.21 nM (square) gDNA (molecule concentration). The concentrations of RecO and RecR proteins were fixed at 0.1 μM and 1 μM, respectively.

(B) The ATP hydrolysis rate obtained 1 min after addition of RecA protein is presented. The molecule concentration of gDNA was varied in the presence of 10 nM RecF protein. The concentrations of RecO and RecR proteins were fixed at 0.1 μM and 1 μM, respectively.

gDNA and RecO protein concentrations is observed (data not shown), suggesting that RecF protein binds at the ssDNA-dsDNA junction but RecO protein does not.

RecF Protein Interacts Stoichiometrically with RecR Protein but Not with RecO Protein

To determine whether a stoichiometric interaction between RecFOR proteins is required for optimal RecA-loading activity, protein titrations were performed using various concentrations of the other proteins. In Figure 4A, the concentration of RecR protein is varied in the presence of two different fixed RecF concentrations (open symbols) and at a constant RecO protein concentration of 0.1 μM. In the presence of 0.015 μM RecF protein, 0.5 μM RecR is required for maximal loading activity, whereas when the RecF concentration is increased to 0.1 μM, a higher concentration of RecR protein (1 μM) is required. These results suggest that an interaction between RecF and RecR proteins is required for RecA-loading function. On the other hand, when the concentration of RecO protein is varied at several different fixed RecF concentrations (in the presence of 1.0 μM RecR protein; Figure 4B), reducing the RecF protein concentration from 0.015 μM (open circle) to 0.004 μM (open triangle) did not significantly affect the concentration of RecO protein required for plateau behavior but (because of the lowered RecF protein concentration) did reduce the ATP hydrolysis rate at each RecO concentration. Therefore, there is no stoichiometric relationship between RecF and RecO; rather, as expected based on prior results (Umezu and Kolodner, 1994), RecO is likely interacting with SSB protein, whose concentration is fixed in these experiments. Thus, RecF protein interacts with RecR protein but not with RecO protein to promote loading of RecA protein onto gDNA.

As a control, circular ssDNA was used instead of gDNA (Figures 4A and 4B, filled circles). Even in the presence of a stimulatory concentration of RecF protein (0.015 μM), increasing amounts of RecR and RecO proteins only slightly facilitate RecA-loading onto this DNA that lacks an ssDNA-dsDNA junction. The slight stimulation observed likely corresponds to the RecOR-facilitated reaction that was previously reported (Umezu et al., 1993; Bork et al., 2001).

RecO Protein Interacts Stoichiometrically with RecR Protein but Not with RecF Protein

To more carefully define the role of RecO protein in this reaction, the interaction of RecO protein with RecF and RecR proteins was investigated. Figure 4C shows that approximately 0.5 μM of RecR protein is required for

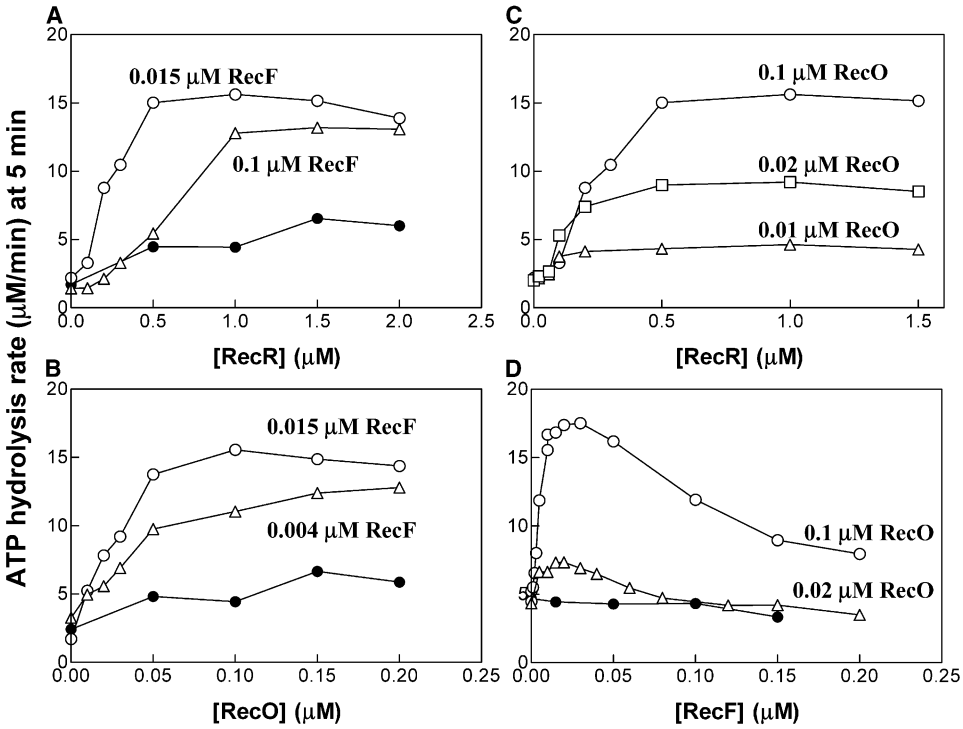


Figure 4. RecF Protein Interacts Stoichiometrically with RecR but Not RecO Protein, and RecO Protein Interacts Stoichiometrically with RecR but Not RecF Protein

Reactions were performed with RecA, RecFOR, and SSB proteins, and with gDNA1800. The ATP hydrolysis rate obtained 5 min after addition of RecA protein is presented.

(A) The concentration of RecR protein was varied in the presence of either 0.015 μM (open circle) or 0.1 μM (open triangle) RecF protein. The concentration of RecO protein was fixed at 0.1 μM. A control experiment using circular ssDNA instead of gDNA in the presence of 0.015 μM RecF protein is also included (filled circle).

(B) The concentration of RecO protein was varied in the presence of either 0.015 μM (open circle) or 0.004 μM (open triangle) RecF protein. The concentration of RecR protein was fixed at 1 μM. A control experiment using circular ssDNA instead of gDNA in the presence of 0.015 μM RecF protein is also included (filled circle).

(C) The concentration of RecR protein was varied in the presence of 0.1 μM (open circle), 0.02 μM (open square), or 0.01 μM (open triangle) RecO protein. The concentration of RecF protein was fixed at 0.015 μM.

(D) The concentration of RecF protein was varied in the presence of 0.1 μM (open circle) or 0.02 μM (open triangle) RecO protein. The concentration of RecR protein was fixed at 1 μM. A control experiment using circular ssDNA instead of gDNA in the presence of 0.1 μM RecO protein is also included (filled circle).

maximal RecA-loading when the RecO protein concentration is 0.1 μM (open circles); when the concentration of RecO protein is reduced to 0.02 μM or less, the RecR protein concentration required is reduced concomitantly (open squares and open triangles). This result implies a stoichiometric interaction between the RecO and RecR proteins.

We also performed a RecF protein titration experiment with different concentrations of RecO protein (Figure 4D, open symbols). The RecF protein concentration required to reach the optimum shifted only slightly when the concentration of RecO protein was changed. The inhibition by the higher concentration of RecF protein likely corresponds to the inhibition of RecA protein binding to ssDNA that was seen previously (Umezu et al., 1993; Bork et al., 2001) and probably reflects the competitive binding of the RecFR complex to ssDNA (Webb et al., 1995, 1997). These observations suggest that RecO protein interacts with RecR protein when the RecFOR proteins load RecA protein onto gDNA, and support the previous conclusion that RecO protein does not interact with RecF protein. Therefore, optimal RecA loading behavior requires both RecO-RecR and RecF-RecR interactions.

When circular ssDNA was used instead of gDNA (Figure 4D, filled circles) and even in the presence of sufficient RecO and RecR proteins, RecF protein had no effect on the ability of RecA protein to bind to ssDNA. This result again indicates that the ssDNA-dsDNA junction in the gDNA is essential for the RecFOR-facilitated RecA-loading reaction.

RecFOR Proteins Stabilize the RecA Nucleoprotein Filament Assembled on gDNA against Disruption by SSB Protein

To test the effect of SSB protein on RecA filament formation on gapped DNA, the concentration of SSB protein was varied in the presence and absence of RecFOR proteins (Figure 5). In the absence of RecFOR proteins, increasing amounts of SSB protein decreased the amount of RecA protein bound to the gDNA. When only the RecOR proteins were present, the amount of RecA

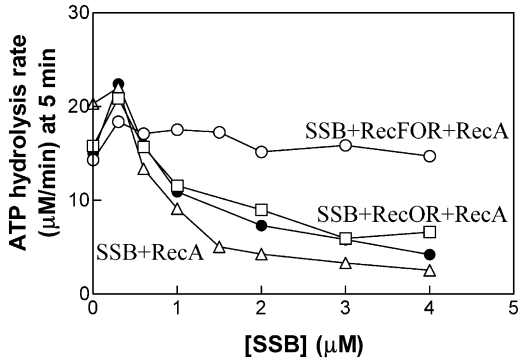


Figure 5. RecFOR Proteins Stabilize the RecA Nucleoprotein Filament against Dissociation by SSB Protein

Either gDNA1800 (open symbols) or M13 circular ssDNA (filled circle) was used as the DNA substrate. The ATP hydrolysis rate 5 min after addition of RecA protein is presented. The reaction was performed with RecA, RecFOR, and SSB proteins (circles), RecA, RecOR, and SSB proteins (squares), or RecA and SSB proteins (triangles). A control experiment using circular ssDNA instead of gDNA in the presence of RecA, RecFOR, and SSB proteins is also shown (filled circle).

protein bound to DNA was slightly greater than in their absence, but the amount of RecA protein assembled on the DNA nevertheless decreased when the SSB protein concentration was increased. On the other hand, when the reaction contained RecF, RecO, and RecR proteins, the binding of RecA protein to the gDNA was negligibly inhibited by SSB protein. In addition, when ssDNA was used, this stabilizing effect was not observed (Figure 5, filled circles). These results indicate that the RecFOR proteins provide a substantial stabilizing function to the RecA nucleoprotein filament against the inhibitory effect of SSB protein, but only when the substrate is gapped DNA.

The Loading Activity of RecFOR Proteins Is Specific to RecA Protein

To investigate the species-specificity of RecA loading onto gDNA by the RecFOR proteins, RecA protein was replaced by other RecA-like proteins, either Rad51 protein from *Saccharomyces cerevisiae* or UvsX protein from bacteriophage T4 (data not shown; see Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/11/5/1337/DC1). SSB protein lowered the rate of Rad51 protein assembly on gDNA, as expected (Sugiyama et al., 1997). RecFOR proteins did not stimulate Rad51 filament assembly onto the SSB-gDNA complex, however, and the assembly kinetics were slower than that seen for Rad51 protein alone.

RecFOR proteins also failed to stimulate UvsX nucleoprotein filament assembly onto SSB-gDNA (data not shown; see Supplemental Figure S1). SSB protein inhibited the binding of UvsX protein to gDNA, and neither RecFOR nor RecOR protein complexes overcame this inhibition substantially.

Our findings are consistent with published reports showing that the loading of Rad51 and UvsX proteins onto ssDNA complexed with the cognate ssDNA binding proteins requires the species-specific loading proteins Rad52 and UvsY, respectively (Sung, 1997a; New et al., 1998; Shinohara and Ogawa, 1998; Yonesaki and Minagawa, 1989; Harris and Griffith, 1989). Thus, the loading by RecFOR proteins onto SSB-gDNA is specific to RecA protein.

RecFOR Proteins Accelerate DNA Strand Exchange Mediated by RecA Protein between SSB-gDNA Complexes and Homologous dsDNA

The previous results showed that the RecFOR proteins facilitate the loading of RecA protein specifically onto gDNA to form a nucleoprotein filament. We therefore determined whether the resultant RecA filament has the capacity to promote strand exchange between gDNA and homologous dsDNA (Figure 6A). As in the ATPase assays, gDNA complexed with SSB protein and the RecFOR proteins were present in the complete reactions. To measure DNA strand exchange, ^{32}P-labeled homologous dsDNA (35 bp) was included. Upon addition of RecA protein, DNA strand exchange ensued; however, due to the need to displace SSB protein from the gDNA, the kinetics of DNA heteroduplex product appearance depended on whether RecF, RecO, and/or RecR protein was present (Figure 6B). The fastest rate of product appearance occurred in the complete reaction that included the RecFOR complex (Figure 6B, left-most time course): DNA strand exchange product was detectable consistently in 3 min, and ~13% of the gDNA was converted to DNA product in 9 min (Figure 6C). When the RecFOR proteins were omitted, the slow spontaneous rate of SSB displacement by RecA protein resulted in the lowest level of DNA strand exchange (2% in 9 min; Figures 6B and 6C); this result is in agreement with the ATP hydrolysis data, which revealed the poorest RecA nucleoprotein complex formation under these conditions. The single omission of either RecF, RecO, or RecR reduced DNA strand exchange at early times (e.g., at 9 min) to only 39%, 17%, or 10% of the complete reaction (Figure 6C), respectively, showing that all three proteins are required for the acceleration of DNA strand exchange. As in the RecA-loading experiments, an ssDNA-dsDNA junction is also required because substitution of the gDNA by ssDNA lowered the efficiency of DNA strand exchange to that observed in the absence of RecFOR proteins (Figure 6B, right-most time course). Last, the yield of DNA heteroduplex product at 90 min was 30%–50% in both the RecFOR reaction and reactions lacking one or all of the RecFOR proteins (but product formation in the slowest reaction was still increasing at 90 min), showing that the RecFOR proteins affect primarily the rate of RecA protein loading onto gDNA and not the final extent of DNA strand exchange. Hence, the RecFOR proteins increase the rate of DNA strand exchange by promoting rapid presynaptic complex formation, and not by affecting the final yield of DNA heteroduplex product. These results not only confirm the conclusions from the ATPase assays but also show that the RecFOR proteins accelerate DNA strand exchange between gDNA and homologous dsDNA by loading RecA protein specifically onto gDNA.

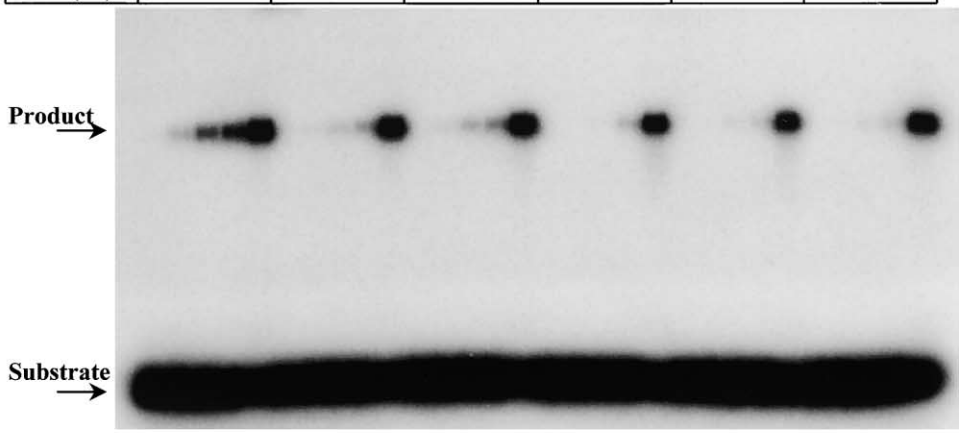
Discussion

Here, we established that the combined functions of the RecF, RecO, and RecR proteins facilitate the loading of

A

B

DNA	gDNA					ssDNA
SSB	+	+	+	+	+	+
RecF	+	−	−	+	+	+
RecO	+	−	+	−	+	+
RecR	+	−	+	+	−	+
RecA	+	+	+	+	+	+
Time (min)	0 3 6 9 90	0 3 6 9 90	0 3 6 9 90	0 3 6 9 90	0 3 6 9 90	0 3 6 9 90



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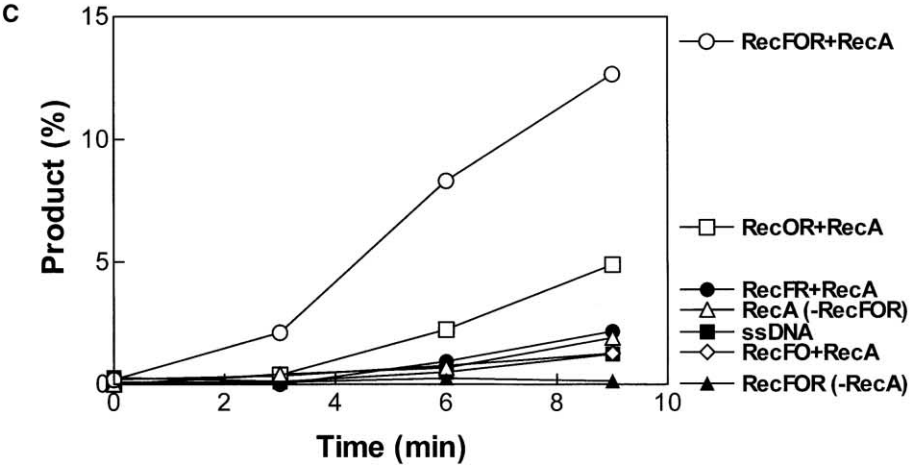

Figure 6. The RecFOR Proteins Facilitate RecA-Promoted DNA Strand Exchange between SSB-Complexed Gapped DNA and Homologous dsDNA

(A) Schematic representation of the DNA strand exchange reaction. The substrates are gapped DNA (gDNA7232) and homologous 35 bp dsDNA (^{32}P-labeled). The ^{32}P-labeled dsDNA has homology to the gDNA 1.8 kb downstream of the ssDNA-dsDNA junction. As a result of DNA strand exchange, the ^{32}P-labeled strand is transferred to the gDNA, resulting in a DNA heteroduplex that can be separated from substrate by gel electrophoresis.

(B) Analysis of the time course of DNA strand exchange. The complete reaction (left five lanes) contained 1.8 μM RecA, 15 nM RecF, 100 nM RecO, 1 μM RecR, and 3 μM SSB proteins, 0.7 nM (molecule) gDNA7232 (5 μM nucleotide), and 1 nM (molecule) ^{32}P-labeled dsDNA. In the lane labeled "ssDNA," M13mp7 circular ssDNA replaced gDNA. Note that for all reactions, except the control reaction without RecA protein, product formation reached 30%–50% at 90 min (B) and was still increasing for the slowest reaction.

(C) Quantification of the time course in (B) from 0 to 9 min.

RecA protein onto SSB-coated gDNA. Omitting any one of the three proteins prevented loading of RecA protein. The reaction requires a base-paired 5′ terminus at the ssDNA-dsDNA junction region, since noncomplementary DNA at this end blocked loading. RecF protein binds stoichiometrically to the gDNA, at the dsDNA-ssDNA junction. Both RecF-RecR and RecR-RecO interactions are necessary, from which we infer that RecR serves to bridge RecF and RecO proteins within the complex.

It is abundantly clear that RecFOR proteins are re-

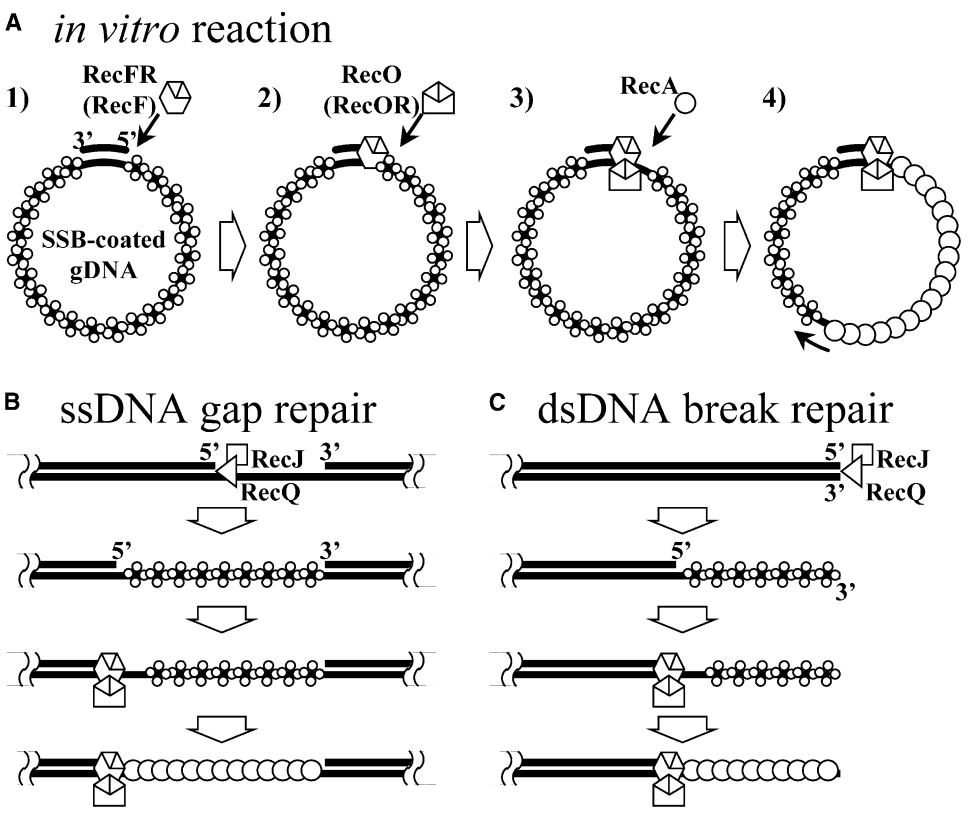

Figure 7. A Model for RecFOR-Facilitated Formation of a RecA Nucleoprotein Filament on Gapped DNA and Tailed DNA

(A) RecFOR-facilitated loading of RecA protein onto gapped DNA. (1) The RecFR complex (or RecF protein) first recognizes and binds to the 5′ end of the dsDNA at the gap. (2) The RecOR complex (or RecO protein) interacts with the RecF(R)-gDNA complex. (3) The RecFOR proteins serve to nucleate RecA protein filament assembly. (4) The RecA filament extends over the ssDNA region by growth in the 5′ to 3′ direction.

(B) Initial steps in the repair of ssDNA gaps by RecFOR proteins. The RecQ and RecJ proteins might process the gapped DNA prior to recognition by the RecFOR proteins (Courcelle and Hanawalt, 1999).

(C) Initial steps in the repair of dsDNA breaks by the RecFOR, RecQ, and RecJ proteins.

quired for the cellular repair of gDNA (Tseng et al., 1994). Other in vivo evidence suggests that RecF, RecO, and RecR proteins are involved in the same step of recombination and that this step can be bypassed by RecA mutants that efficiently displace SSB protein from ssDNA (Volkert and Hartke, 1984; Wang and Smith, 1986; Lavery and Kowalczykowski, 1992; Madiraju et al., 1988, 1992). Therefore, we believe that our finding, that the concerted action of RecF, RecO, and RecR proteins is needed to facilitate the loading of RecA protein onto SSB-coated gDNA, reconstitutes an essential step of the cellular process.

Our results establish that the loading of RecA protein by the RecFOR proteins requires gDNA as a substrate; ssDNA cannot substitute (Figure 1). This observation is particularly significant because, in the absence of RecF protein, the RecOR-mediated reactions that were previously reported do not require gDNA (Figures 1B and 1C; Umezu et al., 1993; Bork et al., 2001); in fact, in those reactions (as we confirmed) RecF protein is inhibitory (Figures 1C and 1D). Therefore, our findings suggest that RecF protein is recognizing a feature of the gDNA, a conclusion that is consistent with a prior report that RecF protein binds preferentially to gDNA (Hegde et al., 1996b). We conclude that the recognition site on the gDNA is the 5′ end of the dsDNA at the gap, because noncomplementary DNA at this end blocked RecF protein function (Figure 2). The interaction between the RecF and RecR proteins that we detect (Figure 4A) may indicate that the RecFR complex, but not RecF protein alone, recognizes and binds to the gDNA. Furthermore, the interaction that we detect between the RecR and RecO proteins suggests that RecO protein binds to this complex via RecR protein, perhaps through the RecR protein that is associated with the RecF protein bound at gap. At present, however, we cannot unambiguously define the pathway of RecFOR assembly at the gap, nor do we know the molecular ratio of RecFOR proteins that assemble at the gap; we know only that RecF protein is essential for gap recognition and that both RecO and RecR proteins are needed in addition.

Assembly of the RecFOR complex at the ssDNA-dsDNA junction facilitates the specific loading of RecA protein onto the SSB-coated ssDNA. We found that RecA loading is species specific, in that neither the T4 phage UvsX nor yeast Rad51 proteins could be loaded by the bacterial RecFOR proteins. In addition, even though the RecOR proteins are capable of mediating an exchange of RecA protein for DNA-bound SSB protein, the complete RecFOR complex acting at DNA gaps is much more effective at loading RecA protein onto ssDNA than is the RecOR complex. Furthermore, be-

cause of the limiting amounts of RecOR protein present in our reactions, only a relatively small amount of RecA protein is being recruited to the ssDNA by the RecOR mechanism; instead, the RecFOR-directed loading of RecA protein onto ssDNA in the gap is the predominant pathway acting in our assays.

Our collective results, therefore, suggest that the reaction is divided into several steps (Figure 7A). The first step is the binding of the RecF protein (or the RecFR complex) onto the gDNA (step 1) by a high-affinity interaction (approximate $K_d < 5$ nM; Figure 3). Then (step 2), the RecOR complex (or the RecO protein) binds to this gDNA-RecF (-RecFR) complex with a K_d of about 30 nM (Figure 4B). This RecFOR complex on the gDNA changes the conformation of gDNA-SSB protein complex and/or displaces some SSB protein so that nucleation of RecA protein can occur in a species-specific manner (step 3). The RecA protein filament then extends over the entire gapped ssDNA region by virtue of its polar (5′ to 3′), cooperative assembly (step 4).

Recognition of 5′ end of the junction on gDNA by the RecFOR proteins is consistent with the expected biological role for these proteins. The RecFOR proteins are needed for gDNA repair as well as DSB repair in a *recBC sbcBC* cell (Figures 7B and 7C). The RecQ helicase and RecJ nuclease can process the break to produce a DNA product with a 3′-ssDNA tail. This tailed duplex has an ssDNA/dsDNA junction with a 5′ end that can also be recognized by the RecFOR system. Subsequently, the RecFOR proteins initiate the nucleation of RecA protein at this junction, followed by RecA protein filament formation on the entire ssDNA region. Finally, this RecA nucleoprotein filament promotes homologous pairing and DNA strand exchange (Figure 6). Thus, the RecFOR proteins catalyze RecA nucleoprotein filament formation, and thereby accelerate DNA strand exchange on substrates complexed with SSB protein. These events are mechanistically similar to those defined for the RecBCD pathway. The RecFORQJ proteins represent the functional analog of the RecBCD helicase/nuclease, and they display all of the activities reported for the RecBCD helicase/nuclease, except for the χ recognition (i.e., helicase, nuclease, and RecA-loading).

Despite the increasingly clear realization that genetic recombination in all organisms shares many common mechanistic features, obvious structural homologs of some key recombination proteins cannot be found in both prokaryotes and eukaryotes. However, recent work reveals that functional analogs of these proteins nevertheless exist (Kantake et al., 2002). For example, the RecO and T4 UvsY proteins are the functional equivalents of eukaryotic Rad52 protein, despite sharing almost no sequence similarity: these proteins both anneal cognate SSB-ssDNA complexes and load their partner DNA strand exchange proteins onto ssDNA (Umezu and Kolodner, 1994; Kantake et al., 2002). Similarly, based on our findings regarding the role of RecF protein in RecA loading, we note that the relationship between Rad51 protein, its paralogs, and Rad52 is also quite similar to that between the RecA, RecFR, and RecO proteins. The Rad51 paralogs of *S. cerevisiae*, the Rad55 and Rad57 proteins, form a stable heterodimer, like the RecFR complex, and overcome the inhibition to Rad51-promoted DNA strand exchange by an ssDNA binding protein, replication protein-A (RPA) (Sung, 1997b). The cytological observation that *rad55* and *rad57* mutant cells do not assemble Rad51 protein foci during meiotic recombination (Gasior et al., 1998) is also consistent with the conclusion that Rad55 and Rad57 proteins facilitate Rad51 loading onto RPA-coated ssDNA. In addition, several Rad51 mutant proteins that suppress the phenotypic defects of *rad55* and *rad57* mutations possess the capacity to efficiently displace RPA from ssDNA without the aid of a recombination mediator protein (Fortin and Symington, 2002); this behavior is identical to that of the mutant RecA proteins (the *srf* alleles) that suppress the defects of *recF* mutations in *E. coli*. Vertebrate Rad51 paralogs also have similar characteristics. Xrcc3 protein, an ortholog of yeast Rad57 protein, is required for Rad51 protein focus formation in Chinese hamster ovary cells (Bishop et al., 1998). Mutation of any of five Rad51 paralogs attenuates Rad51 protein focus formation in chicken DT40 cells (Takata et al., 2001). Thus, although there is no structural homology between the RecFR proteins and the Rad51 paralogs, the Rad51 paralogs are possible functional analogs of the RecFR proteins, and Rad52 protein is the functional analog of RecO protein. The DNA structure that is recognized by RecF protein (Figure 7; the junction of dsDNA and ssDNA, containing a base-paired 5′ end in the DNA duplex) is an intermediate in the double-strand break repair model of DNA recombination (Szostak et al., 1983). Recognizing this structure and loading RecA-like proteins onto this DNA is proposed to be a common step of DNA recombination in all organisms.

Experimental Procedures

Materials

RecA protein (Morimatsu et al., 1995), SSB protein (Dixon and Kowalczykowski, 1991), and yeast Rad51 protein (New et al., 1998) were prepared as described. RecO and RecR proteins were purified by Dr. Noriko Kantake as described (Kantake et al., 2002). UvsX protein was generously provided by Dr. Scott W. Morrical (University of Vermont). M13mp7 ssDNA was prepared as described (Messing, 1983). ATP, β-nicontinamide adenine dinucleotide reduced form (NADH), phosphoenolpyruvate (PEP), pyruvate kinase, and lactate dehydrogenase were purchased from Sigma.

RecF protein was prepared as described (Hegde et al., 1996a) with minor modification. A Superdex-200 gel filtration column (Pharmacia) was used at the last step of purification. The purity of the RecF preparation was confirmed by SDS-polyacrylamide gel electrophoresis, using Coomassie staining, to be 98%.

Preparation of Gapped DNA Substrates

All oligonucleotides, am-55 (33-mer), KAT-41 (35-mer), KAT-42 (35-mer), KAT-43 (35-mer), am-55-3′T (43-mer), and am-55-5′T (43-mer), were purchased from OPERON and purified by polyacrylamide gel electrophoresis. The sequences of am-55, KAT-31, KAT-32, and KAT-33 are complementary to the M13mp18 genome viral ssDNA sequences of 226–256, 1005–1039, 2511–2545, and 4726–4760, respectively. am-55-3′T or am-55-5′T contains 10 thymidines at the 3′- or 5′ end of am-55. The gDNA7200 substrate was prepared by incubating a solution containing 0.069 μM (molecule concentration; 500 μM nucleotides) M13 mp7 ssDNA and 0.35 μM (molecule concentration) am-55 in 10 mM Tris-HCl, 1 mM EDTA, 50 mM NaCl (pH 8.0) at 70°C followed by cooling to 25°C over a period of 1 hr. To prepare the gDNA7200-3′T or gDNA7200-5′T, am-55-3′T or am-55-5′T was used instead of am-55. The gDNA3600 and gDNA1800 substrates were prepared using the same procedure, except that two oligonucleotides (am-55 and KAT-32) or four oligonucleotides (am-55, KAT-31, KAT-32, and KAT-33) were used, respectively.

Since elimination of the free excess oligonucleotides did not affect the activity of RecFOR proteins (data not shown), these substrates were directly used without further purification.

ATPase Assay

The DNA was first incubated with SSB protein in 90 μl of buffer (20 mM Tris-acetate [pH 7.5], 10 mM magnesium acetate, 1 mM DTT, 5% glycerol, 100 μg/ml BSA, 1 mM ATP, 1.5 mM PEP, 0.75 mM NADH, 20 units/ml pyruvate kinase, and 20 units/ml lactate dehydrogenase) at 30°C for 5 min. RecF, RecO, and RecR proteins were incubated at 30°C for 5 min in 10 μl of the same buffer solution and were then added to the SSB-ssDNA complex. The sample was incubated for 10 min further at 30°C, and then the reaction was started by adding RecA protein. Unless otherwise indicated, the standard reaction contained 5 μM (nucleotide) ssDNA (or gDNA), 3 μM SSB protein, 0.015 μM RecF protein, 0.1 μM RecO protein, 1 μM RecR protein, and 3.6 μM RecA protein in the final mixture (total volume of 100 μl). The kinetics of ATP hydrolysis were followed by measuring the absorption of NADH at 380 nm with an HP-8453 spectrophotometer; the absorption at 450 nm was used for background correction. The rate of ATP hydrolysis was calculated from the absorption change over a 2 min interval.

DNA Strand Exchange

Gapped DNA (gDNA7232) was prepared by annealing KAT-32 to M13mp7 ssDNA. The dsDNA was prepared by annealing KAT-33, which was ^{32}P-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase, to its complementary 35-mer oligonucleotide, KAT-38.

DNA strand exchange was performed at 30°C. The gDNA was first incubated with SSB protein in 90 μl of buffer (20 mM Tris-acetate [pH 7.5], 10 mM magnesium acetate, 1 mM DTT, 5% glycerol, 100 μg/ml BSA, 1 mM ATP, 1.5 mM PEP, and 20 U/ml pyruvate kinase) for 5 min. RecF, RecO, and RecR proteins were incubated for 5 min in 10 μl of the same buffer solution and were then added to the SSB-gDNA complex. The sample was incubated for 5 min further, and then the ^{32}P-labeled dsDNA was added. After an additional incubation for 5 min, adding RecA protein started the reaction. Unless otherwise indicated, the standard reaction contained 5 μM (nucleotide) gDNA (0.7 nM molecule), 1 nM (molecule) ^{32}P-dsDNA, 3 μM SSB protein, 0.015 μM RecF protein, 0.1 μM RecO protein, 1 μM RecR protein, and 1.8 μM RecA protein in the final mixture (total volume of 100 μl). Aliquots (24 μl) were taken at the indicated time, mixed with 5 μl of stop solution (5% SDS and 250 mM EDTA), and chilled on ice. After the reaction, samples were treated with Proteinase-K by adding 2 μl of 1 mg/ml Proteinase-K in buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, and 0.05% bromophenol blue), followed by incubating for 30 min at 37°C. The samples were loaded onto a 1% agarose gel with TAE-buffer (40 mM Tris-acetate [pH 8.0] and 1 mM EDTA) containing 50 μg/ml of ethidium bromide. After electrophoresis, the gel was dried and analyzed by autoradiography using a Storm 820 PhosphorImager with ImageQuaNT software.

Acknowledgments

We thank Mark Dillingham, Naofumi Handa, Noriko Kantake, Maria Spies, and Tomohiko Sugiyama for valuable discussions on this work, and Andrei Alexeev, Ichiro Amitani, Cynthia Haseltine, Jim New, Zeynep Ozsoy, Edgar Valencia-Morales, and Yun Wu for comments on the manuscript. We are especially grateful to Drs. Noriko Kantake and Scott W. Morrical for providing RecOR proteins and UvsX protein, respectively. This work was supported by NIH grant GM-62653.

Received: November 26, 2002
Revised: March 27, 2003
Accepted: May 1, 2003
Published: May 22, 2003

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