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RecFOR Proteins Target RecA Protein to a DNA Gap with Either DNA or RNA at the 5′ Terminus

*IMPLICATION FOR REPAIR OF STALLED REPLICATION FORKS**[S]

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Background: Mediator proteins regulate assembly of RecA onto single-stranded DNA complexed with SSB protein.
Results: RecFOR targets RecA to the 5′-end of DNA gaps, regardless whether terminated by DNA or RNA.
Conclusion: RecFOR loads RecA onto RNA-DNA substrates that mimic unfinished lagging strand replication gaps.
Significance: This work provides biochemical evidence for recognition by RecF of gaps created when DNA replication halts.

The repair of single-stranded gaps in duplex DNA by homologous recombination requires the proteins of the RecF pathway. The assembly of RecA protein onto gapped DNA (gDNA) that is complexed with the single-stranded DNA-binding protein is accelerated by the RecF, RecO, and RecR (RecFOR) proteins. Here, we show the RecFOR proteins specifically target RecA protein to gDNA even in the presence of a thousand-fold excess of single-stranded DNA (ssDNA). The binding constant of RecF protein, in the presence of the RecOR proteins, to the junction of ssDNA and dsDNA within a gap is 1–2 nM, suggesting that a few RecF molecules in the cell are sufficient to recognize gDNA. We also found that the nucleation of a RecA filament on gDNA in the presence of the RecFOR proteins occurs at a faster rate than filament elongation, resulting in a RecA nucleoprotein filament on ssDNA for 1000–2000 nucleotides downstream (5′ → 3′) of the junction with duplex DNA. Thus, RecA loading by RecFOR is localized to a region close to a junction. RecFOR proteins also recognize RNA at the 5′-end of an RNA-DNA junction within an ssDNA gap, which is compatible with their role in the repair of lagging strand gaps at stalled replication forks.

DNA lesions caused by exogenous and endogenous sources are lethal if left unrepaired (1). Just one DNA break or replication-blocking lesion is highly toxic to the organism: such damage can block cell division and, in the case of multicellular Eukarya, may lead to cancer (2). In *Escherichia coli*, several pathways are responsible for the repair of such DNA lesions (3). These pathways include nucleotide or base excision repair, homologous recombinational repair, and lesion bypass. The expression of some of the proteins involved in these repair pathways is controlled by the LexA repressor and RecA protein in a DNA damage-inducible system termed the SOS response (4).

Single-stranded DNA (ssDNA)[3] gaps in double-stranded DNA (dsDNA) are one type of damaged DNA. Such gapped DNA (gDNA) can be produced when DNA synthesis stops at a DNA lesion during DNA replication (1). The RecF pathway of recombination repairs gDNA and requires the functions of at least RecA, RecF, RecG, RecJ, RecN, RecO, RecQ, RecR, RuvA, RuvB, RuvC, and SSB proteins (5). RecA protein plays a central role in recombination: it promotes the homology search and associated DNA strand exchange (6–9). The RecF, RecO, and RecR proteins mediate the loading of RecA protein onto ssDNA, functioning as the RecOR or RecFOR complexes; these proteins are often called "mediator" proteins. Mutation of *recF*, *recO*, or *recR* results in a delay of SOS induction (10–13). However, this delay and the loss of DNA repair are suppressed by specific mutations in the *recA* gene, called *srf* (suppressors of recF mutation) mutations (14, 15). The RecA803 protein is the product of one *srf* suppressor; it binds efficiently to ssDNA that is complexed with SSB protein, whereas the wild-type RecA protein does not (16). These and other *in vivo* and *in vitro* observations strongly suggested that the RecFOR proteins were needed for assembly of the RecA filament on SSB-coated ssDNA.

SSB protein is required for efficient homologous recombination *in vivo*; the frequency of homologous recombination is reduced in *ssb* mutant strains (17–19). However, SSB protein can either stimulate or inhibit the DNA strand exchange activity of RecA protein *in vitro* (20, 21). When RecA protein is incubated with ssDNA prior to SSB protein or when substoichiometric amounts of SSB protein relative to ssDNA are used, SSB protein stimulates DNA strand exchange by removing secondary structure in ssDNA. When stoichiometric amounts of SSB protein are incubated with ssDNA prior to RecA protein filament assembly, SSB protein inhibits DNA strand exchange by kinetically impeding access of RecA to the ssDNA. Because SSB protein binds ssDNA faster than RecA protein (22, 23), in the cell, SSB protein binds ssDNA prior to RecA protein, preventing the assembly of RecA filament and recombinational repair (24). The RecFOR proteins as well as the *srf* mutations in

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[3] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; gDNA, gapped DNA; SSB, single-stranded DNA-binding.

RecA protein overcome this inhibitory effect by SSB protein as described above.

In vitro analyses of RecA, RecFOR, and SSB proteins revealed the mechanism of RecA loading onto ssDNA or gDNA. It was first reported that RecA protein assembles onto SSB-coated ssDNA more quickly in the presence of RecOR proteins compared with their absence (25). Subsequently, the specific binding of RecFOR proteins to gDNA was reported (26). The RecFR complex was found to bind to the 3′-end of a ssDNA-dsDNA junction within gDNA to stop the growth of RecA protein beyond the junction (27). Finally, it was then discovered that RecFOR proteins recognize the 5′-end of a dsDNA-ssDNA junction flanking an ssDNA gap and nucleate formation of the RecA filament onto SSB-covered ssDNA (28). Elongation of the RecA nucleoprotein filament in 5′ to 3′ direction results in displacement of SSB protein and the subsequent pairing with homologous dsDNA (28).

The genetic evidence suggests that the RecFOR proteins have the ability to target RecA protein onto gDNA in the presence of competing ssDNA, and also onto gDNA that forms when lagging strand synthesis is impeded: such gDNA will have an RNA primer at its 5′ terminus. In this study, we examine the substrate specificity of RecFOR protein-mediated loading of RecA protein onto SSB-ssDNA complexes.

EXPERIMENTAL PROCEDURES

Materials—RecA (29), SSB (30), RecF (28), and RecOR proteins (31) were purified as described. M13mp7 ssDNA (32) and ϕX174 ssDNA (33) were prepared as described. ATP, β-nicotinamide adenine dinucleotide reduced form (NADH), phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase were purchased from Sigma.

Preparation of DNA Substrates—Oligodeoxynucleotides KAT-32, KAT-33, KAT-38, KAT-71, KAT-72, KAT-73, KAT-74, and KAT-76 (all 35-mers), as well as ϕX174–117F and ϕX174–166R (both 50-mers), were purchased from Operon and purified by PAGE. The sequences of KAT-32 and KAT-33 were complementary to the M13mp18 viral genome sequences 2511–2545 and 4726–4760, respectively. The sequences of KAT-71, KAT-72, KAT-73, KAT-74, and ϕX-166R were complementary to the ϕX174 viral genome sequences 5368–0016, 1392–1426, 2704–2738, 4084–4118, and 117–166, respectively. KAT-38, KAT-76, and ϕX-117F were complementary to KAT-33, KAT-71, and ϕX-166R, respectively. The DNA sequences of these oligodeoxynucleotides are shown in supplemental Table 1. The RNA-containing oligonucleotides (35-mer) were the same sequence as KAT-71 except that KAT71RD contained 10 ribonucleotides at the 5′-end (thymine was replaced by uracil), and KAT71RNA was all RNA (35 ribonucleotides). These RNA-containing synthetic oligonucleotides were purchased from Sigma.

M13 gDNA and ϕX174 gDNA were prepared by annealing KAT-32 to M13mp7 ssDNA and KAT-74 to ϕX174 ssDNA, respectively, as described (28). These gDNA were used as the M13- and ϕX174-gDNAs, unless otherwise indicated. The gDNA-100, -4100, -1500, and -2800 were made by annealing KAT-71, KAT-72, KAT-73, and KAT-74 to ϕX174 ssDNA, respectively. RNA-containing gDNAs were prepared by annealing the RNA-containing oligonucleotides to ϕX174 ssDNA. The ^{32}P-M13 dsDNA and ^{32}P-ϕX174 dsDNA (both 35 bp) were prepared by annealing 5′-^{32}P-labeled KAT-33 to cold KAT-38 and 5′-^{32}P-labeled KAT-71 to cold KAT-76, respectively. The 50-bp ^{32}P-ϕX174 dsDNA was prepared by annealing 5′-^{32}P-labeled ϕX-117F to cold ϕX-166R.

DNA Strand Exchange Reactions—Reactions were performed at 30 °C. Complexes of gDNA and/or ssDNA and SSB protein in 90 μl of buffer (20 mM Tris acetate (pH 7.5), 10 mM magnesium acetate, 5% glycerol, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 1 mM ATP, 1.5 mM phosphoenolpyruvate, and 20 units/ml pyruvate kinase) were formed by incubating for 5 min. RecFOR proteins were incubated for 5 min in 10 μl of the same buffer solution and were then added to the SSB-DNA complex. The sample was further incubated for 10 min. During this incubation, 5 min after adding RecFOR proteins, ^{32}P-labeled dsDNA was added. Finally, adding RecA protein to the mixture started the reaction. Unless otherwise indicated, the standard reaction contained 5 μM (nucleotide) gDNA/ssDNA (0.7 nM molecule for M13-based DNA; 0.9 nM molecule for ϕX174-based DNA), 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; all protein concentrations are given in terms of monomer concentrations). When M13 gDNA/ssDNA and ϕX174 gDNA/ssDNA were used simultaneously, the concentrations of these DNAs were both 5 μM so that the sample contained a total of 10 μM gDNA/ssDNA. Aliquots (24 μl) were taken at the indicated time, mixed with 5 μl of stop solution (5% SDS and 250 mM EDTA), and kept on ice. After the reaction, all of the aliquots were treated by addition of 2 μl of 1 mg/ml proteinase K in buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.05% bromphenol blue), followed by incubation for 30 min at 37 °C. The samples were loaded onto a 1% agarose gel in TAE (40 mM Tris acetate (pH 8.0) and 1 mM EDTA) containing 0.5 μg/ml ethidium bromide. After electrophoresis, the gel was dried and analyzed by autoradiography using a Storm 820 PhosphorImager with ImageQuaNT software (GE Healthcare).

ATPase Assays—ATP hydrolysis was measured using reactions that couple ADP formation to NADH oxidation as described previously (28). The concentration of NADH was measured by its absorption at 380 nm every 20 s. The ATP hydrolysis rate was determined from the change in absorption (*i.e.* ATP hydrolysis) over a 2-min interval. Unless otherwise indicated, the standard reaction contained 5 μM (nucleotide) gDNA (0.7 nM molecule), 3 μM SSB protein, 0.1 μM RecO protein, 1 μM RecR protein, and 1.8 μM RecA protein in the final reactions (total volume of 100 μl). The concentration of RecF protein is indicated in the figure legends.

RESULTS

RecA Protein Is Specifically Loaded onto Gapped DNA in the Presence of the RecFOR Proteins—To determine the role of the RecFOR proteins in RecA protein-mediated DNA strand exchange, we developed the substrates shown in Fig. 1*A*. One substrate is gapped DNA made by annealing an oligonucleotide to either ϕX174 or M13 ssDNA. The second substrate is duplex DNA (35 bp) that is homologous to the ssDNA region of the

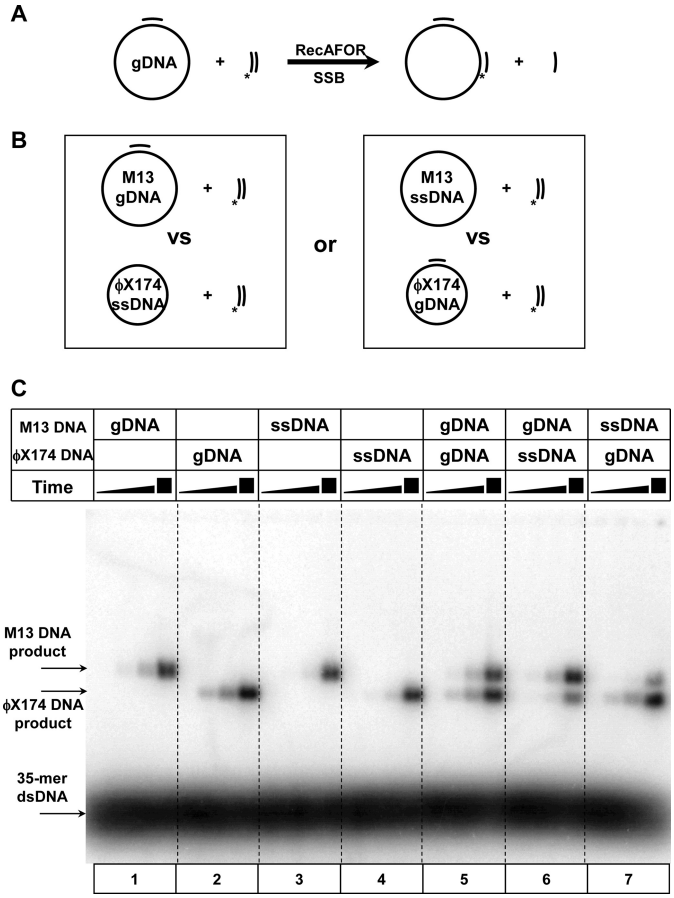

FIGURE 1. **RecAFOR-mediated DNA strand exchange occurs specifically with gapped DNA.** *A,* schematic representation of RecAFOR-mediated DNA strand exchange reaction between gDNA and homologous dsDNA. The gapped DNA contains a 35-bp dsDNA region and 7.2 kb (M13-based DNA) or 5.4 kb (ϕX174-based DNA) of ssDNA. DNA strand exchange with ^{32}P-labeled (shown as an *asterisk* at the 5′-end of one strand) dsDNA (35 bp) homologous to ssDNA within the gDNA (either M13 or ϕX174) results in transfer of the labeled strand to the gDNA. *B,* schematic representation of competition between gDNA and ssDNA for the loading of RecA protein by RecFOR proteins. M13 gDNA and ϕX174 ssDNA (*left side*) or M13 ssDNA and ϕX174 gDNA were simultaneously used in DNA strand exchange reactions to determine onto which DNA RecA is preferentially loaded by RecFOR protein. Because of the differences in sizes, the different products of DNA strand exchange can be distinguished by agarose gel electrophoresis. *C,* DNA strand exchange between the gDNA or ssDNA and homologous dsDNA. The ssDNA-containing DNA substrates indicated were first incubated with SSB, and then RecFOR proteins were added; subsequently, labeled dsDNA was added. Reactions were started by adding RecA protein and analyzed by agarose gel electrophoresis and autoradiography at 0, 5, 10, and 90 min. *Arrows* indicate the positions of M13 and ϕX174 DNA products and 35-mer dsDNA.

gDNA substrate. The strand of dsDNA that is complementary to the ssDNA region is ^{32}P-labeled so that DNA strand exchange results in transfer of the labeled strand to the larger gDNA. Because the dsDNA is short (35 bp), the branch migration step of DNA strand exchange is very fast. Therefore, the extent of DNA strand exchange reflects the amount of RecA protein on the gDNA. The gDNA is preincubated with SSB protein to prevent rapid assembly of RecA protein onto the gDNA. The RecFOR protein complex can accelerate the assembly of RecA protein onto the gDNA by binding to the ssDNA-dsDNA junction in the gDNA at the 5′-end (28). In the absence of a junction (*i.e.* with ordinary circular ssDNA), the RecFOR-facilitated loading of RecA protein does not occur efficiently (28).

To determine how efficiently RecFOR targets RecA onto gDNA *versus* ssDNA, when both the gDNA and ssDNA are present simultaneously, substrates based on either M13 ssDNA or ϕX174 ssDNA were used (Fig. 1*B*). The sizes of these DNA species are different so that each can discriminated using agarose gel electrophoresis. Therefore, one can determine whether the RecA nucleoprotein filament formation occurred on the ssDNA or the gDNA.

Fig. 1*C* shows the results of DNA strand exchange with various combinations of gDNA and ssDNA in the presence of RecA, RecFOR, and SSB proteins. Aliquots at 0, 5, and 10 min were analyzed by gel electrophoresis to determine the initial kinetics, and a 90-min time point determined the yield at a late stage of the reaction (28). In experimental sets 1–4, the assembly of RecA onto ssDNA and gDNA was examined. As reported previously (28), RecA protein was loaded by RecFOR proteins onto either M13 gDNA or ϕX174 gDNA (Fig. 1*C*, *experimental sets 1* and *2*) faster than onto ssDNA (*sets 3* and *4*). The products of DNA strand exchange appeared at 5 min with the gDNA (Fig. 1*C*, *experimental* sets *1* and *2*), but only a negligible amount of product was found at early times with ssDNA (*sets 3* and *4*). When M13 gDNA and ϕX174 gDNA were both present simultaneously in a competition experiment, RecA protein was loaded equivalently onto either gDNA (Fig. 1*C*, *experimental set 5*). When the competition was between gDNA and ssDNA (Fig. 1*C*, *experimental sets 6* and *7*), RecA protein was more efficiently loaded onto the gDNA than onto ssDNA; RecA protein was targeted to M13 gDNA in experimental set 6 and to ϕX174 gDNA in set 7. These results demonstrate that when gDNA and ssDNA are present in a competitive experiment, RecFOR directs RecA protein onto gDNA in kinetic preference to ssDNA.

RecF, RecO, and RecR Proteins Are All Required for the Targeting of RecA Protein to Gapped DNA—To examine whether all the RecFOR proteins are required for this targeting of RecA protein to gDNA, each of the RecFOR proteins was omitted individually (Fig. 2*A*). When all of the RecFOR proteins were present, RecA protein was specifically loaded onto the gDNA (Fig. 2*A*, *experimental sets 2* and *3*), but when any one of the RecFOR proteins was absent, the specific loading of RecA protein onto gDNA was not observed (Fig. 2*A*, *experimental sets 4–6*). Instead, RecA was distributed equally between each DNA, and the gDNA substrate behaved as though it was a simple ssDNA substrate, as seen in Fig. 2*A*, *experimental set 1*. Thus, RecF, RecO, and RecR proteins are required to load RecA protein specifically onto gDNA when competing ssDNA is present.

Previously, RecF protein was shown to interact with the ssDNA-dsDNA junction, but RecO and RecR proteins were not (28). To determine the optimal amount of RecF protein required for targeting, the concentration of RecF protein was varied in the presence of both gDNA and ssDNA (Fig. 2*B*). A limiting concentration of RecA protein was used in these experiments to restrict RecA assembly onto the kinetically preferred substrate. In the absence of RecF protein, the final extents of DNA strand exchange were 19% for M13 ssDNA and 24% for ϕX174 gDNA (Fig. 2*B*). Note that the ϕX174 ssDNA is a slightly

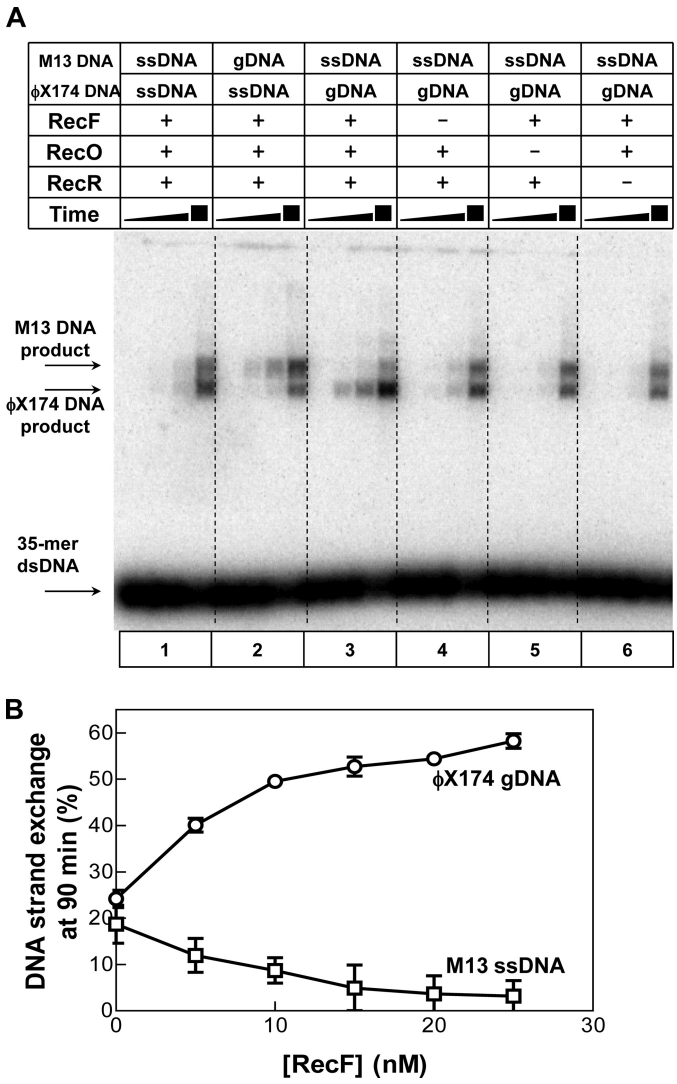

FIGURE 2. **RecF, RecO, and RecR proteins are required for targeting of RecA protein to gapped DNA in preference to ssDNA.** *A,* competitive DNA strand exchange reactions. The ssDNA-containing substrates were preincubated with SSB protein and RecF, RecO, and RecR proteins as indicated for 10 min. Both homologous ^{32}P-labeled dsDNA pairing partner substrates were added, and the reaction was started by adding RecA protein (0.9 μM). Aliquots were taken at 0, 5, 10, or 90 min and analyzed by agarose gel electrophoresis and autoradiography. *Arrows* indicate the positions of M13 and ϕX174 DNA products and 35-mer dsDNA. *B,* dependence of DNA targeting preference on the concentration of the RecF protein. M13 ssDNA and ϕX174 gDNA were used with standard assay conditions. The yields of DNA strand exchange products for M13 ssDNA (*squares*) and ϕX174-gDNA (*circles*) at 90 min after adding RecA protein (0.9 μM) are shown. The experiments were done twice, and *error bars* show standard errors, unless smaller than the symbol.

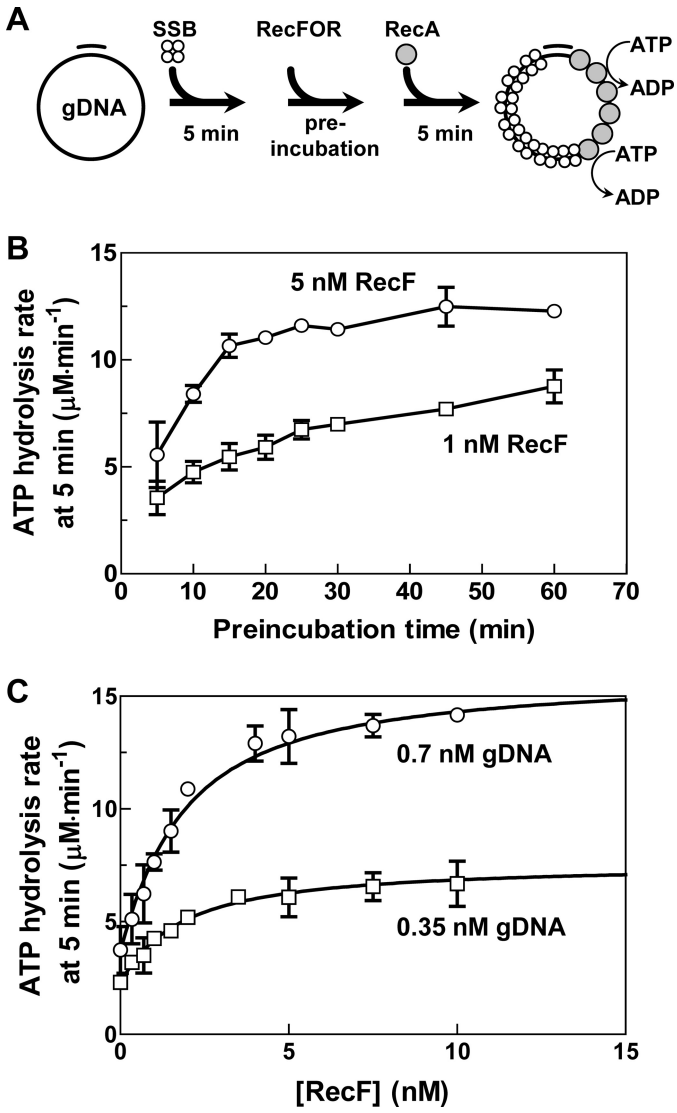

FIGURE 3. **RecFOR complex assembles slowly on gapped DNA, but binds tightly.** *A,* illustration of RecA-loading onto gDNA by the RecFOR proteins as monitored by the DNA-dependent ATPase activity of RecA protein. ATP hydrolysis by RecF protein is negligible. *B,* time dependence of RecFOR preincubation with gDNA, prior to addition of RecA protein. M13 gDNA that was first coated by SSB proteins was mixed with RecFOR proteins and incubated for the indicated times. The reaction was started by adding RecA protein, and ATP hydrolysis was measured. *Graph* shows ATPase activity at 5 min after RecA protein addition. The concentrations of RecF protein were 5 nM (*circles*) and 1 nM (*squares*). The experiments were done twice, and *error bars* show standard errors, unless smaller than the symbol. *C,* ATP hydrolysis as a function of RecF protein concentration. SSB-complexed gDNA was mixed with the indicated concentration of RecF protein, with RecOR proteins at standard assay concentrations. After incubation for 60 min, the reaction was started by adding RecA protein, and its ATPase activity was followed. *Graph* shows the ATPase activities at 5 min after RecA protein addition. The concentrations (in molecules) of gDNA were 0.7 nM (*circles*) and 0.35 nM (*squares*). The experiments were done twice, and *error bars* show standard errors, unless smaller than the symbol. The *curves* are least squares fits of the data to a hyperbola with dissociation constants of 1.5 (±0.4) and 1.6 (±0.3) nM for 0.7 nM and 0.35 nM gDNA, respectively.

better substrate than M13 ssDNA (Fig. 2*A*, *experimental set 1*), probably due to the lower amount of secondary structure in ϕX174 ssDNA than in M13 ssDNA. When the concentration of RecF protein was increased, the final extent of DNA strand exchange with the ϕX174 gDNA increased and reached a plateau at around 15 nM; at the same time, DNA strand exchange with the M13 ssDNA decreased to nearly background levels because most of the RecA protein was loaded onto ϕX174 gDNA (Fig. 2*B*). The optimal concentration of RecF protein (15 nM) for RecA loading onto gDNA was the same as in our previous study (28).

Binding of RecF Protein to Gapped DNA in the Presence of RecOR Proteins—We examined the length of time that is required to make a productive gDNA-RecFOR complex. For these experiments, we used the M13 gDNA and measured the appearance of the ATPase activity from RecA protein (Fig. 3*A*). Because the ATPase activity of the RecA protein is DNA-de-

pendent, the observed ATP hydrolysis rate is proportional to the amount of RecA-DNA complex formed; the ATPase activity of RecF protein is negligible due to its low concentration and low k_{cat} value (34). Fig. 3*B* shows the efficiency of RecA loading onto M13 gDNA, followed by using the ATPase activity of RecA protein, as a function of the preincubation time of the RecFOR complex with gDNA. Two different RecF concentrations (1 and 5 nM) were used; the concentrations of RecO and RecR were in excess, at 100 and 1000 nM, respectively (28). In the presence of 5 nM RecF protein, at least 20–30 min was required for maximum loading efficiency (Fig. 3*B*, *circles*); for 1 nM RecF protein, the RecA-loading efficiency increased continuously for 60 min (Fig. 3*B*, *squares*).

In the presence of 5 nM RecF protein, the RecA-loading efficiency was saturated by 60 min of preincubation, so a 60-min preincubation time was used to examine the effect of RecF concentration on RecA loading (Fig. 3*C*). Two different M13 gDNA concentrations (0.35 and 0.7 nM molecule) were used for the RecF titrations. For both concentrations of gDNA, the binding is hyperbolic and could be fit to yield an apparent dissociation constant, K_d, of 1.6 (±0.3) and 1.5 (±0.4) nM, respectively (Fig. 3*C*).

Gapped DNA Is Recognized by RecFOR Proteins in the Presence of a 1000-Fold Excess of ssDNA—To test the ability of RecFOR complex to recognize gDNA in a mixture of ssDNA and gDNA, we varied the molar ratio of ssDNA and gDNA. The RecFOR-facilitated loading of RecA onto gDNA was measured by DNA strand exchange (Fig. 4*A*). In this experiment, ϕX174 gDNA and M13 ssDNA were used. The concentration of M13 ssDNA was fixed at 5000 nM (nucleotides) and the ϕX174 gDNA was varied from 5 to 500 nM. The concentration of RecA protein was also varied. In the presence of 500 nM ϕX174 gDNA, DNA strand exchange efficiently occurred both in the presence of excess (2000 nM) and limiting (200 nM) amounts of RecA protein (Fig. 4*A*, *experimental set 1*). When the concentration of ϕX174 gDNA was reduced to 50 nM, DNA strand exchange decreased (Fig. 4*A*, *experimental set 2*). Fig. 4*B* shows the percentage of gDNA converted to DNA strand exchange product; in the presence of 2000 nM RecA protein, nearly 100% of the gDNA was converted to product when its concentration was 500 nM. When the concentration of gDNA was reduced to 50 or 5 nM, the reaction efficiencies per gDNA were only slightly reduced (Fig. 4*B*). In the presence of 200 nM RecA protein and with as little as 5 nM gDNA, 75–80% of the gDNA was converted to DNA strand exchange product (Fig. 4*B*). Note that this DNA strand exchange is occurring with a substoichiometric amount of RecA protein relative to the ssDNA present; the 200 nM RecA protein cannot completely cover the 5000 nM competitor ssDNA. Thus, the RecFOR proteins can target RecA protein onto gDNA even in the presence of a 1000-fold excess of ssDNA.

RecA Protein Is Loaded onto ssDNA Adjacent to the ssDNA-dsDNA Junction and Grows Outward—We also investigated the ability of RecFOR protein to function at limiting concentrations of RecA protein to determine the distance over which RecA protein can assemble into a filament capable of DNA strand exchange. To investigate the relationship between RecA concentration and the length of RecA filament assembly on

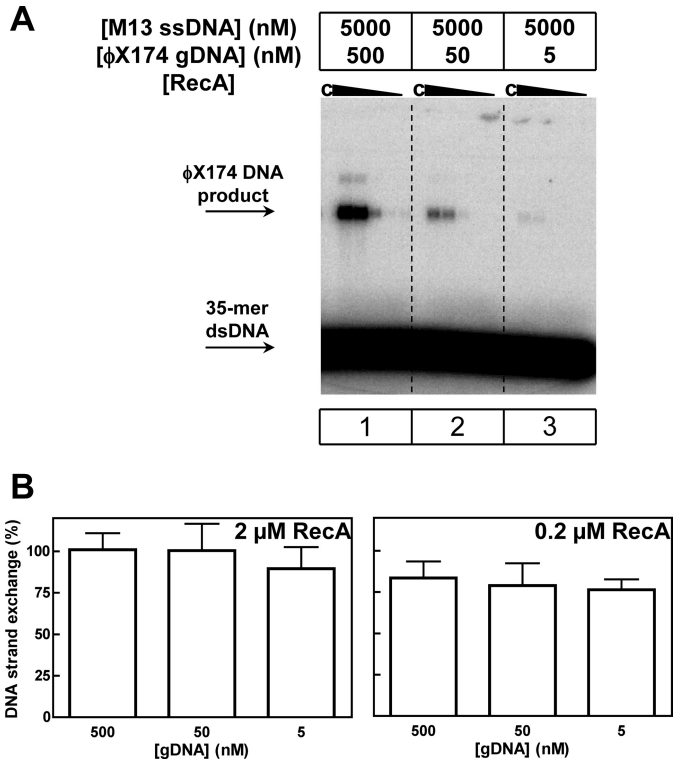

FIGURE 4. **RecFOR proteins load RecA protein onto gapped DNA in the presence of a large excess single-stranded DNA.** *A*, DNA strand exchange was measured using M13 ssDNA (5000 nM) and ϕX174 gDNA (500, 50, or 5 nM). SSB protein and RecFOR proteins were incubated for 10 min with the DNA. ^{32}P-Labeled ϕX dsDNA was then added, and the reaction was started by adding RecA protein. After 90 min, the sample was taken for analysis by agarose gel electrophoresis and autoradiography. The RecA concentrations were 2, 0.2, 0.02, 0.002, or 0 μM in each set (from *left to right*); "*c*" represents the control sample taken at 0 min in the presence of 2 μM RecA protein. *Arrows* indicate the positions of ϕX174 DNA product and 35-mer dsDNA. The *faint bands* at the *top* of gel correspond to a small amount of contaminant in ϕX174 DNA substrate with a higher molecular weight. *B, graphs* showing the yield of DNA strand exchange product, normalized for the amount of gDNA present, were determined from agarose gel electrophoresis and autoradiography for the two RecA protein concentrations, 2 and 0.2 μM. The *graphs* show the results from 2 or 3 independent experiments, and *error bars* show standard error.

gDNA, we constructed the gDNA substrates named gDNA-100, -1500, -2800, and -4100, in which the dsDNA-ssDNA junctions were located 0.1, 1.5, 2.8, and 4.1 kilobases, respectively, upstream of the position where DNA strand exchange occurred (Fig. 5*A*). In the presence of 250 nM RecA protein, DNA strand exchange with gDNA-100 occurred efficiently, whereas the reactions with gDNA-1500, -2800, and -4100 were negligible (Fig. 5*B*). For gDNA-1500, more than 900 nM RecA protein was required for the same DNA strand exchange efficiency seen with gDNA-100. For gDNA-2800 and -4100, even higher concentrations of RecA protein were required, consistent with the idea that RecA nucleation occurred at the RecFOR bound to the junction, and then the filament grew over the increasing distances toward the homologous DNA pairing site.

For the gDNA-100 substrate, 50 nM RecA protein is theoretically required to bind between the junction and the region of DNA strand exchange; for gDNA-1500, -2800, and -4100, the amount of RecA protein required for this intervening region is 470, 900, and 1300 nM, respectively. Fig. 5*B* (*triangles*) shows that the yield of DNA strand exchange product for gDNA-100 started to increase at ~50 nM. For gDNA-1500, the increase in

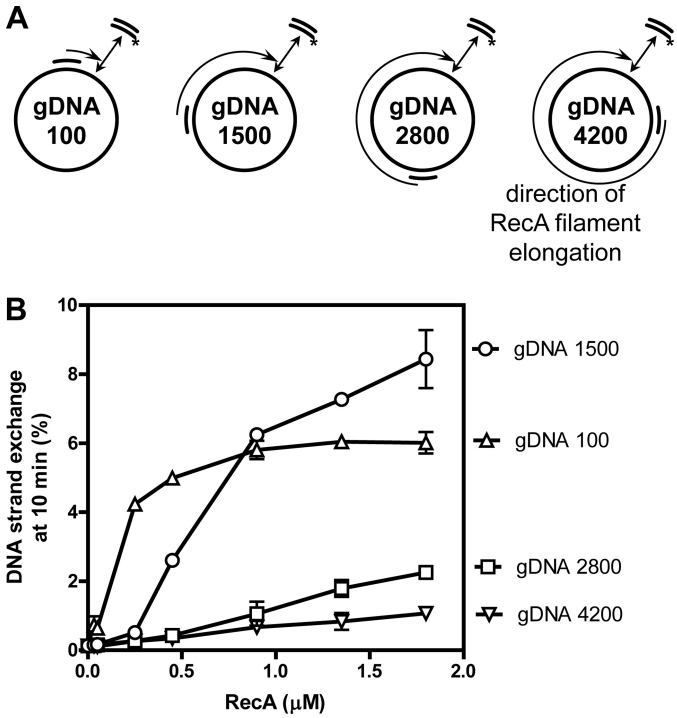

FIGURE 5. **From an ssDNA-dsDNA junction, RecA filaments nucleated by the RecFOR proteins grow for ~1500 nucleotides.** *A,* gDNA substrates used in these experiments are shown. The positions for DNA strand exchange are 100, 1500, 2800, and 4100 bp downstream of ssDNA-dsDNA junction in gDNA-100, -1500, -2800, and -4100, respectively. These are gDNA derived from ϕX174. *B,* DNA strand exchange with SSB-coated gDNA in *A* was performed using standard assay conditions. Product formation at 10 min was determined from agarose gel electrophoresis and autoradiography, and was quantified and plotted for two independent experiments; *error bars* show standard error.

reaction efficiency started at ~500 nM (Fig. 5*B*, *circles*). These results show that, in the presence of limited amounts of RecA protein, only the region downstream of the ssDNA-dsDNA junction (downstream of the RecA filament elongation direction, which is in a net 5′ → 3′ direction) becomes occupied by RecA protein and that the functional length of RecA-ssDNA filament is increased by using higher concentrations of RecA protein. For gDNA-2800 and -4100 (Fig. 5*B*, *squares* and *inverted triangles*), for which 900 and 1300 nM RecA protein are needed to saturate the region between the junction and the DNA strand exchange locus, respectively, product yields are significantly lower than those for gDNA-100 and -1500, suggesting that the rate of RecA nucleoprotein filament growth is too slow to support efficient DNA strand exchange at a distance of 2800 nucleotides.

RecFOR Proteins Can Also Recognize the 5′-End of RNA-DNA Hybrids to Load RecA Protein onto the ssDNA in the Gap—Okazaki fragments contain RNA at their 5′-end. Such 5′-RNA remains as a hybrid RNA/DNA-ssDNA junction when lagging strand synthesis is stalled. To determine whether the 5′-RNA at such a junction can be recognized by RecFOR protein and direct the loading of RecA protein onto the ssDNA within the gapped region, we used gDNA containing RNA. The RNA-containing gDNA has 10 ribonucleotides of RNA-DNA hybrid at the 5′-end of the double-stranded region.

The abilities of gDNA-100 and an otherwise identical RNA-containing gDNA (except for 10 ribonucleotides at the 5′-end)

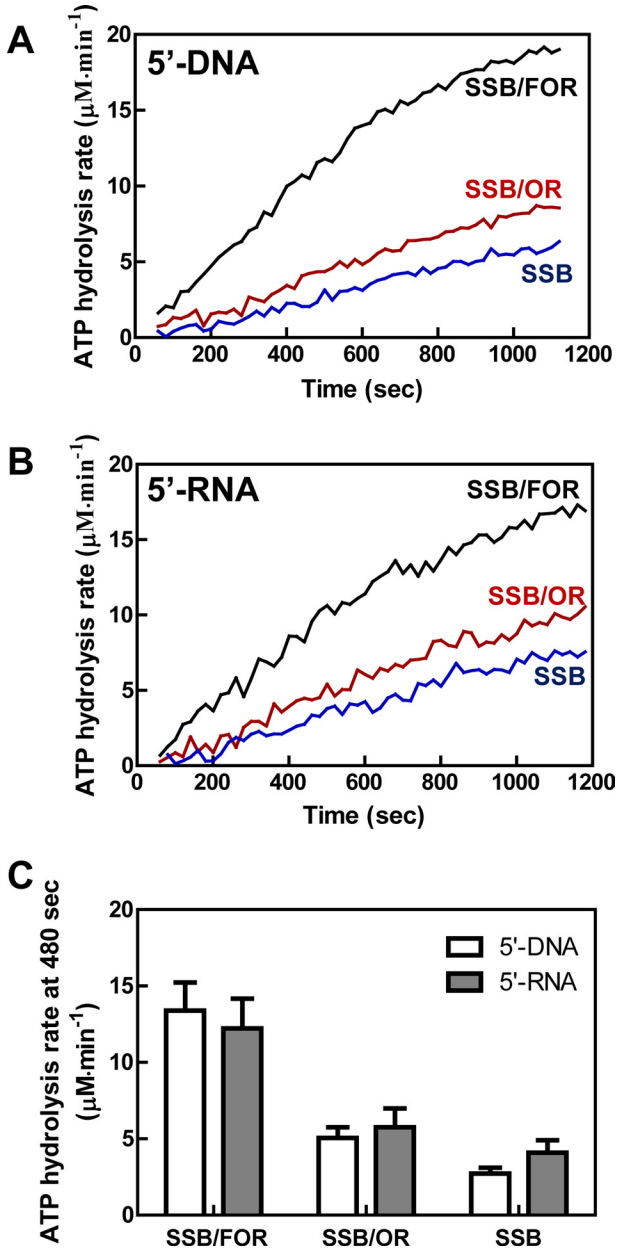

FIGURE 6. **RecFOR proteins can load RecA protein onto gapped DNA containing RNA at 5′-end.** *A,* kinetics of ATP hydrolysis were followed. ATPase activity was measured as in Fig. 3. The concentration of RecF protein was 15 nM. The DNA substrate was gDNA-100. The reaction was done in the presence of SSB and RecFOR proteins (*black*), SSB and RecOR proteins (*red*), or SSB protein (*blue*). The ATP hydrolysis rate is the average rate over a 2-min interval. *B,* gDNA-100 substrate containing 10-nucleotides of RNA at the 5′-end was used; conditions were the same as for the experiment done in *A*. *C, bar graphs* showing ATPase activity for each set of reactions. The ATP hydrolysis rate at 480 s was determined for two independent experiments, and *error bars* show standard error.

to promote RecFOR-mediated loading of RecA were first compared using the ATPase assay (Fig. 6). The kinetics of the increase in ATP hydrolysis rate were similar for the two substrates (Fig. 6, *A* and *B*), with behavior similar to that observed previously for gapped substrates (28). Supplemental Fig. 1 confirms that the RNA was not artifactually degraded during these assays. These results show that the RecFOR proteins recognized 5′-RNA that mimicked the RNA primer in an Okazaki fragment and that this interaction supported the loading of

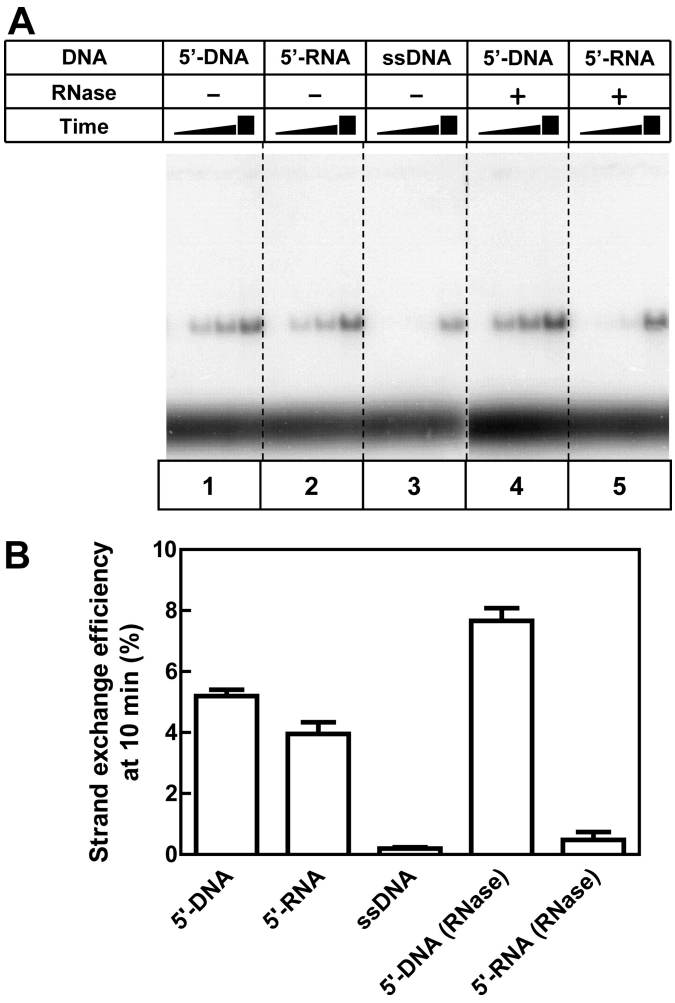

FIGURE 7. **RNA at the 5′-end of gapped DNA supports RecFOR-mediated RecA loading for DNA strand exchange.** *A,* DNA strand exchange between gDNA and 35-mer ^{32}P-dsDNA. The substrates are gDNA-100 (indicated as 5′-DNA), gDNA-100 with 35-mer RNA (5′-RNA), and ϕX174 ssDNA. Concentration of RecA protein is 200 nM. Samples were taken at 0, 5, 10, and 90 min after adding RecA protein and analyzed by electrophoresis and autoradiography. In the right-most two experimental sets, 5′-DNA gDNA or 5′-RNA gDNA stock (500 μM nucleotides, 10 μl) was treated with 0.2 μg of RNase A for 72 h before using. *B,* DNA strand exchange for each condition is shown. *Error bars* show standard error from two independent experiments.

RecA protein onto the adjacent ssDNA region that corresponds to the lagging strand of unfinished DNA replication.

The ability of RecFOR proteins to load RecA protein onto RNA-containing gDNA to produce filaments that were functional for DNA strand exchange was also tested (Fig. 7). The gDNA used in this experiment contained a 35-mer ribonucleotide without any deoxyribonucleotides to eliminate the possibility that degradation of the RNA in a chimeric oligonucleotide might produce an oligonucleotide with DNA at the 5′-end. Fig. 7 shows that DNA strand exchange occurred efficiently for both the conventional DNA- and RNA-containing gDNA (Fig. 7, *A*, *experimental sets 1* and *2*, and *B*). As expected, the control reaction with ssDNA was low, with only negligible amount of product at early times (Fig. 7). Treatment of the RNA-containing gDNA with RNase A reduced DNA strand exchange to about the same extent as for the ssDNA control (Fig. 7, *experimental A, set 5,* and *B*), but the same treatment of the conventional DNA-containing gDNA with RNase A did not affect the yield (Fig. 7, *A, experimental set 4,* and *B*). Supporting the ATPase assays, these results demonstrate that the RecA protein loaded onto ssDNA, which is adjacent to 5′-RNA, is also capable of promoting DNA strand exchange with homologous dsDNA.

DISCUSSION

In this study, we demonstrated that RecFOR proteins efficiently target RecA protein specifically onto gDNA in the presence of competitor ssDNA. The targeting to gDNA occurs even in the presence of a 1000-fold excess of ssDNA. The dissociation constant, K_d, for RecF protein binding to gDNA in the presence of RecOR proteins was determined to be ~1–2 nM, a value that is similar to the K_d value of the RecBCD-dsDNA interaction (35). If an *E. coli* cell contained only 10 molecules of RecF protein, its concentration would be about ~10 nM (based on the volume of an *E. coli* cell of $\sim 1 \times 10^{-15}$ liter). Therefore, RecF protein can bind even just one gDNA site in the cell.

The RecFOR proteins loaded RecA proteins onto gDNA when the concentration of RecA protein was as low as 200 nM; at such a limiting concentration of RecA (1 RecA molecule per 25 nucleotides DNA), only a part of gDNA was covered by RecA protein. In the presence of higher concentrations of RecA protein, an active nucleoprotein filament could be initiated at the junction that extended along ssDNA for 1.5–2.8 kb of the gap. We also showed that the RecFOR proteins could recognize an RNA-DNA hybrid junction and load RecA protein onto the ssDNA adjacent to the 5′-end of the RNA. This substrate mimics the end of an Okazaki fragment formed as a part of DNA replication, and our finding is consistent with the *in vivo* loading of RecA protein onto an ssDNA gap created when a replisome is unable to complete synthesis of the lagging strand.

Based on Fig. 3*C*, we can roughly calculate the stoichiometry between gDNA and RecF protein: more than 2.8 nM RecF protein was required for 0.7 nM gDNA to reach the saturation of activity, yielding a ratio of ~4 RecF proteins per gDNA. Because the interaction between RecF protein and gDNA is not stoichiometric under our conditions, this ratio is an overestimate. In addition, our RecF protein stock may not be 100% active, also contributing to an overestimated value. NMR and gel filtration analysis of *Thermus thermophilus* RecF and RecR protein suggested formation of a 4:2 (RecR/RecF) heterohexamer (36), a stoichiometry confirmed for the proteins from *Deinococcus radiodurans* (37). Therefore, in conjunction with our results, one or two protomers of such a heterohexameric complex would be sufficient to promote the loading of RecA protein onto gDNA. It is still unknown how much RecO protein is part of the RecA-loading RecFOR complex. Our previous study suggested a weaker interaction between RecFR and RecO protein than between RecFR and gDNA (28). Because RecO protein interacts with both ssDNA (38, 39) and SSB-covered ssDNA (31, 39), the amount of RecO protein required for RecA loading in this study does not reflect the stoichiometric ratio for formation of a complex between RecFR and RecO proteins. Our preliminary results indicate that when a lower concentration of ssDNA is present, a nearly equimolar concentration of RecO relative to RecF protein is sufficient for RecA loading (data not

shown). The need for an excess amount of RecR is unclear: it may be the consequence of a low affinity RecR for RecO in the presence of SSB (39) or a low specific activity of our protein stock. The stoichiometry of the RecFOR-SSB complex that is assembled on the DNA junction and is active in RecA loading is undefined, partly due to the complexity of competing binding equilibria. In this regard, it is known that RecO interacts with both SSB and RecR, but the interaction with SSB is stronger and 1:1:1 complexes can form when proteins are present at micromolar concentrations; however, optimal stimulation of RecA loading by RecOR does not require such a stoichiometric complex (39). How RecF might affect these complicated multiple equilibria is presently unknown.

The RecFOR proteins recognize the 5′-end of a dsDNA-ssDNA junction to target RecA protein onto the ssDNA in the adjacent gap (28). The assembly of a RecA filament on ssDNA requires two steps as follows: nucleation and elongation of filament. Recognition of the 5′-end of the gap, instead of the 3′-end, is consistent with the net direction of filament growth by RecA protein on ssDNA, 5′ to 3′ (40). Our collective results show that the RecFOR proteins provide a site for the nucleation of the RecA filament with net growth occurring in the 5′ to 3′ direction. In the presence of substoichiometric amounts of RecA protein, binding of RecA protein onto gDNA in the presence of the RecFOR proteins is restricted to the region that is just downstream of the nucleation site on the gDNA (Fig. 5*B*, *e.g.* 250 nM RecA protein). When higher concentrations of RecA are used, growth proceeds further into the downstream region. Our results show that in the presence of RecFOR, the nucleation step is no longer limiting for nucleoprotein filament formation. If the elongation step was faster than nucleation, then each nucleated RecA filament would rapidly grow to the most distal downstream regions of the gDNA substrates, resulting in the similar efficiencies of DNA strand exchange (Fig. 5). Therefore, the kinetics of RecA binding (Fig. 6*A*, *black line*) reflect the speed of replacement of SSB protein by RecA protein. The increase in the ATP hydrolysis rate is ~1.5 μM/min^2 (supplemental Fig. 2); this value corresponds to the binding of ~60–80 RecA molecules/min on the SSB-ssDNA complex in the gap.

Fig. 8 shows a simplified model for RecFOR-facilitated formation of a RecA filament on gDNA in the context of a stalled replication fork. The replication fork migrates with a speed of 1000 bases/s, whereas the Okazaki fragments are generated every second (Fig. 8, *A* and *B*). Even though RecFOR proteins can recognize the 5′-end of RNA in Okazaki fragments (Figs. 6 and 7), recognition does not occur sufficiently quickly relative to a rapidly moving replication fork because of the many minutes required for the binding of RecFOR proteins to the junction *vis à vis* the ~1-s lifetime of a lagging strand gap (Fig. 3*B*). However, once an ssDNA gap persists due to stalling of a polymerase (Fig. 8*B*), the RecFOR complex can bind (Fig. 8*C*) and rapidly promote nucleation of the RecA filament (Fig. 8*D*), with uncatalyzed elongation of the RecA filament now being a relatively slower step (Fig. 8*E*). If not repaired or otherwise replicated within this time, the ssDNA region of the gap is covered by RecA proteins within 10–15 min. Finally, DNA strand exchange with the sister copy permits recombinational bypass of the lesion. In the case of a lesion on the leading strand template (Fig. 8*B*, *left*), the replication fork can be re-started downstream *de novo* before repair of the DNA lesion (41). Restart in this case requires priming by the DnaG primase, so that the gap in the leading strand also contains RNA at 5′-end (Fig. 8*B*), which would be recognized by RecFOR. The RecOR proteins can also mediate RecA loading downstream of the junction, in a DNA structure-independent manner (data not shown) (39). Thus, through RecFOR-dependent loading of the RecA protein initiated at the ssDNA/dsDNA junction, the RecA filament formed on the ssDNA gap can promote recombinational repair via steps, including DNA strand exchange with the intact daughter duplex, branch migration, and resolution or dissolution of Holliday junctions (42, 43).

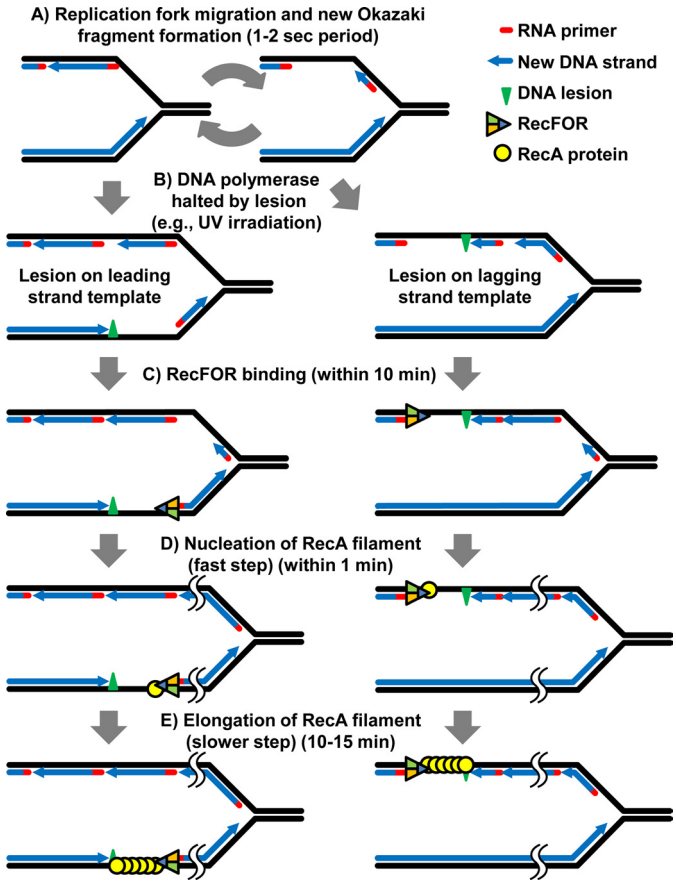

FIGURE 8. **Model for the nucleation of RecA protein filaments by RecFOR protein on gapped DNA.** In the absence of a DNA lesion, Okazaki fragments are generated every second or 2 s (*A*). UV irradiation (*B*) or other DNA damage can create a lesion that stalls the DNA polymerase to create an ssDNA gap within the replicating DNA on either the leading strand (*left*) or lagging strand (*right*) template. The ssDNA/dsDNA junction is recognized by RecFOR proteins (*C*) to commence loading of RecA protein into the ssDNA gap (*D* and *E*) and initiate its ultimate repair via recombination with the intact daughter dsDNA. See text for details.

The loading of eukaryotic Rad51 onto gDNA by the *Ustilago maydis* BRCA2 homologue (Brh2) was reported using DNA strand exchange assays similar to those we described (28); DNA strand exchange was found to be higher at positions near the ssDNA-dsDNA junction (44). The structure recognized by Brh2 is the same as that recognized by RecFOR, the 5′-end of an ssDNA-dsDNA junction (44). The direction of *U. maydis* Rad51 filament elongation is also the same as that of the RecA filament, 5′ to 3′ (44). This observation suggests that the mech-

anism for recombinational repair of gDNA is conserved between bacteria and this unicellular eukaryote. However, human BRCA2 recognizes junctions with either 5′- or 3′-ends, as well as ssDNA without junctions (45). Thus, whereas the RecFOR and Brh2 proteins recognize only one of ssDNA-dsDNA junctions in a gap, the human BRCA2 recognize both ends of a gap and can target RAD51 proteins onto any form of ssDNA. The recognition of ssDNA by BRCA2 is thus unique and suggests an evolutionary separation of function for the mammalian recombinational DNA repair proteins (45). Thus, a functional path of evolutionary progression from RecFOR to the various eukaryotic analogues can be envisioned.

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