Effect of RecF protein on reactions catalyzed by RecA protein

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ABSTRACT

RecF protein is one of at least three single strand DNA (ssDNA) binding proteins which act in recombination and repair in *Escherichia coli*. In this paper we show that our RecF protein preparation complexes with ssDNA so as to retard its electrophoretic movement in an agarose gel. The apparent stoichiometry of RecF-ssDNA-binding measured in this way is one RecF molecule for every 15 nucleotides and the binding appears to be cooperative. Interaction of the other two ssDNA-binding proteins, RecA and Ssb proteins, has been studied extensively; so in this paper we begin the study of the interaction of RecF and RecA proteins. We found that the RecF protein preparation inhibits the activity of RecA protein in the formation of joint molecules whether added before or after addition of RecA protein to ssDNA. It, therefore, differs from Ssb protein which stimulates joint molecule formation when added to ssDNA after RecA protein. We found that our RecF protein preparation inhibits two steps prior to joint molecule formation: RecA protein binding to ssDNA and coaggregate formation between ssDNA-RecA complexes and dsDNA. We found that it required a much higher ratio of RecF to RecA protein than normally occurs *in vivo* to inhibit joint molecule formation. The insight that these data give to the normal functioning of RecF protein is discussed.

INTRODUCTION

The gene product encoded by *recF* plays an important role in several cellular processes as inferred from the UV sensitivity, mitomycin C sensitivity, recombination deficiency and other phenotypes associated with *recF* mutations in different genetic backgrounds (1). A method that has been used in several laboratories for understanding RecF function at a physiological level is to isolate and characterize extragenic suppressor mutations. The general conclusion from all these studies is that RecF protein facilitates or modulates RecA protein activities (2, 3, 4). Biochemical characterization of the gene product encoded by one such suppressor mutation, *recA803*, led to a hypothesis that RecF protein helps RecA protein in overcoming any limitation of recombination and repair caused by secondary structure in ssDNA or Ssb protein (5).

Understanding the function of *recF* at a biochemical level has been limited by the availability of a purified protein. As a first step to attain that understanding, we purified and characterized RecF protein with respect to its DNA binding properties. Our studies revealed that the protein is a single-stranded DNA binding protein (Madiraju and Clark, in preparation). While a description of those studies was in preparation a report describing purification of RecF protein and preliminary characterization of its ssDNA binding properties appeared (6).

In homologous genetic recombination the ability of RecA protein to bind to ssDNA plays a crucial role in carrying out subsequent reactions namely synapsis and strand exchange reactions (7). Since *in vitro* RecF protein has been shown to bind ssDNA (Madiraju and Clark, in preparation, 6), we hypothesized that RecF protein's ability to bind ssDNA will have profound effects on RecA protein catalyzed reactions, thus influencing DNA recombination and repair reaction processes *in vivo*. In order to obtain further insights into the possible roles of RecF protein in genetic recombination, we have investigated the consequences of RecF protein binding to ssDNA by examining its affect on RecA protein catalyzed joint molecule reaction, a reaction presumed to represent a recombination reaction occurring *in vivo*.

MATERIALS AND METHODS

DNA substrates and proteins

PstI cut Phi X174 linear dsDNA was prepared as described (5). Circular M13mp7 was used to prepare linear single-stranded DNA. M13mp7 has a 29 base pair palindrome creating perfect restriction sites for *Bam*HI, *Pst*I, *Sal*I, *Acc*II; *Hinc*II and *Eco*RI enzymes (8). We restricted the M13mp7 circular ssDNA in the stem region with *Bam*HI followed by phenol precipitation. 5' ends were then labelled with ATP-gamma-³²P using polynucleotide kinase essentially as described by Sambrook et al (9) DNA concentrations were expressed as micromoles of nucleotide residues per liter. RecA protein was purified essentially as described (5). RecF protein used in the present study was purified from a *recA* deletion strain JC15875 containing the *recF* expressing plasmid pSJS140 (10). Detailed purification methods will be presented elsewhere. Briefly, a clear cell lysate from RecF protein overproducing cells was prepared and treated with ammonium sulfate to 70% saturation. The protein pellet obtained

after centrifugation was dialysed and chromatographed on two successive columns of Phosphocellulose P-11 (Whatman) and PBE-94 (Pharmacia) columns in R buffer (20 mM Tris-HCl pH 7.5; 1 mM dithiothreitol (DTT); 0.1 mM EDTA; and 10% glycerol) containing 100 mM NaCl. Peak fractions containing RecF protein were pooled and brought to 70% ammonium sulfate saturation. The pellet obtained after centrifugation was resuspended in R buffer containing 1 M NaCl. Proteins were fractionated on a ACA-54 ultrogel (3000 – 70,000 cut off IBF) sizing column and peak fractions containing the monomeric protein (40,000 KDa) were pooled, dialysed directly in R buffer containing 50% glycerol and subsequently used in all assays. This protein is more than 92% pure as judged on SDS polyacrylamide gels and also sequencing the first 20 aminoacids from N-terminus of the purified RecF protein preparation. RecF protein preparations showed no detectable single-stranded exo, endo nuclease and double-stranded exonuclease activities (Madiraju and Clark, in preparation).

Buffers used

Unless otherwise mentioned, R buffer containing 20 mM Tris-HCl, pH 7.5; 1 mM DTT (dithiothreitol); 0.1 mM EDTA and 10% glycerol was used. Purified protein was dissolved in a storage buffer, i.e. R buffer containing 50% glycerol.

Joint molecules formation reaction

A nitrocellulose filter assay for detecting the joint molecules (11) was performed essentially as described (5). We prepared reaction cocktails on ice containing 20 mM Tris-HCl, pH 7.5; 10 mM MgCl₂; 0.1 mM EDTA and 1 mM DTT; 1 mM ATP; RecA protein (1.0 μ M final concentration) and 3 μ M ssDNA. Samples were pre-warmed to 37°C for 2 minutes and the reactions were initiated by the addition of linear dsDNA (6.0 μ M final concentration). Incubations were continued for 10 minutes and at the end of 10 minutes, samples in duplicate were processed to detect joint molecules (5). RecF and Ssb (single-stranded DNA binding) proteins when present were added where indicated. In all the experiments reported here, RecF protein stocks were diluted in 1 $\times$ D-loops buffer (i.e. 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA) and were added immediately to the reaction cocktail (containing ATP and ssDNA) to obtain reproducible results. Similar results were also obtained when RecF protein stocks were either directly added or were diluted in storage buffer and added to the reaction cocktail. The final glycerol concentrations was adjusted to 0.5% when such dilutions were carried out. By contrast, reproducible results were not obtained when RecF protein stocks were diluted in 1 $\times$ D-loops buffer and incubated for various times before adding to the cocktail.

ATP hydrolysis assay

We have used a spectrophotometric assay (12) that measures the hydrolysis of ATP as described (13). In this assay ADP that is generated due to the hydrolysis of ATP by RecA protein is converted to ATP, in a regenerating system consisting of pyruvate kinase, lactate dehydrogenase and NADH (14, 15). Reaction mixtures in a 500 microliter volume contained 20 mM Tris-HCl, pH 7.5; 10 mM MgCl₂; 0.1 mM DTT; 0.1 mM EDTA; 1 mM ATP; 1 mM phosphoenolpyruvate; 2 units each of pyruvate kinase and lactate dehydrogenase; 3.0 μ M circular ssDNA; 1.0 μ M RecA protein and indicated amounts of RecF protein.

Gel retardation assay

This assay was carried out essentially as described (16). RecF protein was incubated in 20 microliters of standard buffer used for joint molecule formation reaction (see above) containing no RecA protein and no dsDNA. Radiolabelled linear ssDNA was added to a final concentration of 0.1 μ M. RecF protein concentration was as described. RecF protein stocks were diluted in 1 $\times$ D-loops buffer and used immediately. Reactions were incubated at 37°C for 10 minutes and at the end of incubation period 3 microliters of 0.5 M EDTA was added to stop the reaction. Samples were mixed with the loading buffer and loaded on to 0.7% agarose gels, electrophoresed, dried and autoradiographed (9). We have also noted variation in the total DNA retarded when we used RecF protein that was incubated for several minutes on ice after dilution. For example, incubating the diluted RecF protein in buffer for 30 minutes on ice before adding to the reaction cocktail caused no detectable retardation at 1 RecF to 5 ssDNA residues (data not shown) as compared to 100% retardation when used immediately (data not shown. See Table 2).

Coaggregation assay

We have used the method of Tsang et al. for determining the coaggregates of protein-ssDNA and dsDNA (17) with the following modifications. In our experiments we used cold circular ssDNA and tritium labelled phi X174 dsDNA. We incubated RecA or RecF proteins with ssDNA (either M13mp8 or phiX174) in buffer containing 10 mM MgCl₂ for 10 minutes followed by the addition of tritium labelled *Pst*I cut linear dsDNA. Reaction samples were incubated for 10 more minutes and the tubes were spun in a Fisher microcentrifuge for 4 minutes. Radioactivity associated with each fraction was determined by counting in a liquid scintillation counter and the DNA present was examined on agarose gels.

RESULTS

RecF protein reduces electrophoretic mobility of ssDNA

While testing various purification methods, we found that protein with the electrophoretic mobility of RecF protein bound to ssDNA cellulose columns tightly, requiring as much as 150 mM NaCl for elution. We confirmed the identity of the protein by amino acid sequencing (data not shown). Furthermore we confirmed the ssDNA binding ability of the purified RecF protein by measuring protein-induced mobility shift of ssDNA in agarose gels, also known as gel retardation assay. We carried out this assay with different RecF protein concentrations and at a fixed concentration of linear M13ssDNA, labelled at the 5' end. The results of such an experiment are shown in Fig. 1. All the DNA was found to be retarded at amounts greater than or equal to one molecule of RecF protein for every 15 nucleotides of ssDNA (see Fig. 1). As the amount of RecF protein to DNA decreased, the amount of DNA retarded also decreased proportionately. Densitometric scanning of the gels revealed that about 68, 63, 36 and 25 percent DNA was retarded at 1:20; 1:30, 1:40; and 1:60 ratios of RecF protein to DNA ratio respectively. A rough estimate based on the densitometric scan data indicated that each increment of RecF protein retarded the amount of DNA that could be bound at an average of one RecF protein monomer to 15 nucleotide residues (data not shown). We have calculated the percent ssDNA not bound by RecF, i.e. free DNA, under these

Figure 1. Mobility shift assay for analyzing RecF protein ssDNA complexes : We have incubated 5' end labeled linearized M13mp7 ssDNA with RecF protein at indicated concentrations for 10 min. At the end of 10 min a gel loading buffer was added and the samples were loaded on to a 0.7% agarose gel, electrophoresed and autoradiographed as described (9).

conditions and showed the data as a line graph (see Fig. 2A). We noted no detectable retardation of linear dsDNA with short single strand ends at any concentration of RecF protein (data not shown). More details of the DNA binding characteristics will be reported elsewhere (Madiraju and Clark, in preparation).

RecF protein interferes with joint molecule formation catalyzed by RecA protein and the extent of interference depends on the RecF protein concentration

Several DNA substrates have been used for studying the reactions catalyzed by RecA protein (11). We chose to start studying the interaction of RecF and RecA proteins by looking at formation of joint molecule intermediates using circular single stranded DNA and linear double stranded DNA. We preincubated ssDNA with RecF and RecA proteins together in 10 mM MgCl₂ and initiated the reaction by adding linear dsDNA. When we carried out this reaction at 0.5 μ M and 0.2 μ M RecF protein concentrations we noticed almost 90% interference in joint molecule formation (data not shown). At these concentrations the ratios of RecF:RecA:ssDNA were 1:2:6 and 1:5:15 respectively. To determine at what concentration of RecF protein there is no interference, we determined the joint molecule forming ability of RecA protein at different RecF protein concentrations (Fig. 2 B). The results show almost 90% interference at concentrations above 0.15 μ M RecF protein, confirming the preliminary results. Below this concentration down to 0.02 μ M RecF protein, the degree of interference is directly proportional to the RecF protein concentration. 50% interference was noted by interpolation at 0.066 μ M. Below a concentration of 0.02 μ M RecF protein, interference was not observed.

Interference in joint molecule formation by RecF protein does not depend on ssDNA termini of linear dsDNA

A possibility exists that RecF may bind to the ssDNA overhangs of restriction nuclease cut dsDNA and that this binding interferes with the formation of joint molecules. To test this we examined the formation of joint molecules between covalent closed circular dsDNA and circular ssDNA. With these DNA substrates only

Figure 2. (A): Percent ssDNA not retarded by RecF protein: We have determined the amount of free DNA available at different RecF protein concentrations by scanning the autoradiogram of Fig. 1. This was represented as a line graph where RecF protein concentration was shown on X-axis and the amount of free DNA on Y-axis. (B): Joint molecule formation ability of RecA protein in presence of varying concentrations of RecF. Reaction mixtures on ice containing 20 mM Tris, pH 7.5; 0.1 mM EDTA; 1.0 mM DTT, 10mM MgCl₂; 1mM ATP; 3 μ M ssDNA, 1.0 μ M RecA protein and indicated amounts of RecF protein were prepared. Samples were prewarmed to 37°C for 2 min before the addition of linear dsDNA to 6.0 μ M final concentration. At the end of 10 min incubation period, samples were removed and processed to detect joint molecules. (C): Comparative plots showing joint molecule interference (panel B), amount of free DNA (panel A) and the final rates of ATP hydrolysis at different RecF protein concentrations. We have superimposed the panel A and panel B figures. We have also determined the final rates of ATP hydrolysed at 6 min from the data shown in Fig. 3. (open circles) and then plotted the values on the graph showing joint molecule interference (dark diamonds). Also we plotted the percent ssDNA not retarded by RecF protein (filled circles).

paranemic joint molecules are made by RecA protein (18). Under these conditions we noted that upto 65% of the total input dsDNA was retained on to the nitrocellulose filters by RecA protein in the absence of RecF protein as compared to 4% retained by RecA protein in the presence of RecF protein (data not shown). Therefore, RecF protein's interference in joint molecular reaction does not depend on the nature of DNA termini.

Table 1. Order of addition of RecA and RecF proteins does not affect interference by RecF protein with the joint molecule formation ability of RecA

Reaction conditions*	Relative % Joint molecules formed**
1. RecA alone	100
2. RecA and RecF added together	8
3. RecF added 2 min. after RecA	7
4. RecF added 2 min. before RecA	8
5. RecF alone	0

*Reaction samples were prepared in Tris-HCl pH 7.5; 0.1mM EDTA; 0.1 mM DTT; 10mM MgCl₂; 3.0 μ M ssDNA and 1.0 μ M RecA. RecF protein was added where indicated to a final concentration of 0.2 μ M. Samples were incubated from 2 min at 37°C on a water bath before adding the linear dsDNA to a final concentration of 6.0 μ M. Reaction samples in duplicate were removed at the end of 10 min for determining the total joint molecules formed.

**Results obtained in the absence of RecF were normalized to 100% and all others were compared to this number. RecA protein under these conditions made up to 38% joint molecules.

Order of the addition of RecF protein does not influence the interference

In the above reactions, we added both RecA and RecF proteins together to the ssDNA. In a two protein component system containing RecA and Ssb proteins, the order in which they are added to ssDNA has been shown to affect dramatically the joint molecule formation ability of RecA protein, i.e. Ssb protein when added before RecA protein addition inhibits and when added after or along with RecA protein stimulates joint molecule formation (11). To find out whether the order of addition of RecF protein alters the interference, we added RecF protein to ssDNA 2 minutes before, 2 minutes after or at the same time as RecA protein (Table 1). In all three cases we observed about 90% interference with the joint molecule reaction. Thus RecF protein apparently does not act like Ssb protein and order of addition does not alter the interference. Heat-treated (100°C for 15 minutes) RecF protein did not show any interference confirming that the effects are due to a protein and not to a heat stable small molecule. We also observed that RecF protein alone was incapable of catalyzing joint molecule formation (Table 1).

Increasing RecA protein concentration does not relieve the interference caused by RecF protein

If RecF protein is acting as a competitive inhibitor for ssDNA, then adding more RecA protein should overcome the interference. We tested the effect of increasing RecA protein at 0.066 and 0.12 μ M RecF protein concentrations. These concentrations were expected to produce 50% and 80% interference respectively when RecA protein was 1 μ M (see Fig. 2B). Our results indicated that concentrations up to 3 micromolar RecA protein did not change the amount of interference due to RecF protein suggesting that the ratio between RecF protein to RecA protein may not be important and indicating that other factors, such as the ratio between RecF protein to DNA, may be important. Consequently, we varied the ratio between RecF protein and ssDNA. In previous reactions we had used 1 RecA: 3 ssDNA : 6 dsDNA at 0.066 and 0.12 μ M RecF protein. We now doubled the concentration of RecA and DNA, i.e. 2 μ M RecA: 6 μ M ssDNA: 12 μ M dsDNA and used three RecF protein concentrations, 0.066; 0.12 and 0.2 μ M. If the RecF protein to DNA ratio is not important, then we should get 50% interference at 0.066 μ M RecF protein and approximately 80% at 0.12 mM RecF and 90% at 0.2 μ M

Figure 3. ATP hydrolysis ability of RecA protein in presence of RecF protein. Reaction mixtures on ice containing 20 mM Tris-HCl, pH 7.5; 10 mM MgCl₂; 0.1 mM EDTA; 0.1 mM DTT; 3.0 μ M circular ssDNA and 1 mM ATP were prepared. Samples were prewarmed to 37°C before the addition of 1.0 μ M RecA protein. RecF protein concentrations were 0.0 (control); 0.05; 0.1; 0.2 and 0.3 μ M. RecF protein when included was added along with RecA protein.

RecF protein (see Fig. 2B). Our results show that the interference is less than expected, i.e. 30, 50 and 80 percent respectively. This result suggests that the high level interference depends on the relative RecF to DNA ratio. We did not vary either ssDNA or dsDNA alone in these experiments because joint molecule formation is most efficient at the ratio of ssDNA to dsDNA used.

RecF protein blocks net synthesis of joint molecules

In an attempt to determine whether the interference by RecF protein with the yield of joint molecules is due to its ability to prevent the formation process or to destroy preformed joint molecules, we prepared presynaptic complexes of RecA protein and ssDNA by incubating them for 2 minutes at 37°C in the presence of ATP. Linear dsDNA was added and then at various times RecF protein was added and the incubations were continued. In the absence of RecF protein, at 10 minutes after the dsDNA addition, 41 percent of the total dsDNA was converted to joint molecules. This value was normalized to 100% and the joint molecules formed in the presence and absence of RecF protein were determined (data not shown). Our results indicated that at any given time RecF protein prevented any net increase in formation of joint molecules. From this, therefore, we cannot decide whether RecF protein interferes with the formation of joint molecules or destroys joint molecules at the rate they are forming.

RecF protein interferes with the ability of RecA protein to coaggregate ssDNA and dsDNA

The formation of the coaggregates of RecA-ssDNA-dsDNA constitutes an important step in joint molecule formation (18). Since RecF protein interferes with the joint molecule forming ability of RecA protein, it might prevent formation of these coaggregates. To test this we added RecF and RecA proteins together to a mixture of ss circular and ds linear DNAs. Our results indicated that when RecA protein was present alone, almost all of the radioactive dsDNA was found as easily

Table 2. RecF protein exerts minimal interference effects when ssDNA is not available for interaction

Condition*	% joint molecules formed	
	- RecF	+ RecF
1. RecA alone	38	4
2. RecA + SSB	100	90*

*Joint molecules reaction was carried out in standard buffer containing 20 mM Tris-HCl pH 7.5; 0.1 mM DTT; 0.1 mM EDTA; 10 mM MgCl₂; 1 mM ATP, 3 μ M ssDNA and 1 mM RecA. Samples were preincubated for 2 minutes before adding linear dsDNA for determining the joint molecules formed. Ssb protein when present was added after RecA; RecF protein was added two min after Ssb.

sedimentable coaggregates, whether the dsDNA and ssDNA were homologous or not (data not shown). Addition of RecF protein prevented coaggregate formation completely (data not shown). By itself RecF protein had little if any ability to coaggregate ss and dsDNAs.

RecF protein interferes with the ssDNA dependent ATPase activity of RecA protein

A prior step in the formation of coaggregates is the formation of a complex between ssDNA and RecA protein (11, 18). As a measure of the amount of such complex formed, we used the ability of RecA protein to hydrolyze ATP when bound to ssDNA. We measured ATP hydrolysis at a ratio of 1 RecA protein molecule to 3 nucleotides and at different RecF protein concentrations (Fig.3). The results indicate that the degree of interference varies with the RecF protein concentration. Slow and continuous increase in the rates of ATP hydrolysis at different RecF protein concentrations suggests that RecF protein may bind ssDNA more rapidly than RecA protein and may be displaced slowly by RecA protein. Alternatively, with increasing time RecA protein may change from a poor ATP-hydrolyzing form (e.g. scattered molecules separated by RecF protein molecules) to a more efficient hydrolyzing form (e.g. continuous chains of RecA protein molecules). Our results also show that the steady state complexes of RecA-ssDNA-ATP are formed in about 4 min. Presently, we are carrying out detailed biochemical experiments to test whether the amount of interference by RecF protein on ssDNA dependent ATPase activity and joint molecules reactions changes when RecF is added to the steady-state complexes of RecA-ssDNA-ATP.

We also carried out the ATPase assay under the standard joint molecules assay conditions, i.e. by adding linear dsDNA 2 minutes after the addition of RecF protein and RecA protein. No stimulation or further reduction in ATPase rates was observed after an additional 10 minutes incubation (data not shown).

RecF protein does not interfere with formation of joint molecules when Ssb protein is added prior to RecF protein

Our experiments measuring the interference of the formation of joint molecules by RecF protein were carried out under suboptimal *in vitro* conditions, in which secondary structures are present in the ssDNA substrate. To remove secondary structures and optimize conditions for efficiently forming presynaptic complexes i.e. RecA-ssDNA nucleoprotein complexes, SSB protein is added after RecA protein to the reaction mixture. To test whether or not RecF protein can interfere under such conditions, we performed the experiment described in Table 2. We found that there was only minimal interference, if any.

DISCUSSION

Purified RecF protein binds to ssDNA (6) (this study and Madiraju and Clark, in preparation). In this article we show that RecF protein can decrease the electrophoretic mobility of ssDNA in a concentration dependent fashion. As RecF protein concentration increases, the fraction of ssDNA retarded increases. Dividing the differential increase in RecF protein molecules by the differential amount to DNA retarded averages one RecF molecule for every 15 nucleotides. This also corresponds to the minimum amount of RecF protein required to retard all the ssDNA. One explanation of this result would be the cooperative interaction of RecF protein molecules each binding to 15 nucleotides. The binding ratio, however, must be considered an underestimate because we are assuming that 100% of the RecF protein binds DNA. At present we do not know the fraction of active molecules in the preparation of RecF protein used.

Cooperativity of RecF protein in binding to ssDNA is suggested by the maximal retardation of a fraction of DNA and the normal mobility of the remaining fraction. Another possible explanation is the formation of a protein-protein aggregate with a limited capacity to entrap added DNA. The inability of RecF protein to coaggregate an appreciable fraction of ssDNA and dsDNA as we noticed does not support this possibility, however.

We found that RecF protein interferes with the catalysis of three reactions by RecA protein: (1) ssDNA-dependent ATPase, (2) ssDNA-dsDNA coaggregation, and (3) joint molecule formation. Interference with two of these was measured at enough RecF protein to ssDNA ratios to compare the effectiveness of inhibition with the fraction of ssDNA retarded electrophoretically. The comparative plots (as shown in Fig.2C) show that interference with the ATPase is less efficient than with joint molecules formation. If we assume that the amount of ATPase is proportional to the amount of RecA protein bound to ssDNA, then it would appear that RecF protein does not displace RecA protein until RecF protein exceeds a concentration of 1 RecF:60 nucleotides (Fig.2C). However, at 1 RecF:60 nucleotides, joint molecule formation has been reduced by 40%. Thus RecF may interfere with joint molecule formation at a step after RecA protein binding to ssDNA, i.e. after presynaptic complex formation. At first we thought this latter step might be coaggregate formation (17). A recent paper has, however, shown that coaggregate formation is not required for the formation of joint molecules (19). As a result, we do not know at what step RecF protein interferes with joint molecule formation.

An alternative way of interpreting the differential interference is differential structural requirements of RecA-ssDNA complexes for joint molecule formation and ATPase activity. In other words, a small disruption of the structure of *recA*-ssDNA complexes by RecF protein may inactivate them for joint molecule formation while not altering their ATPase activity to the same extent.

A third alternative is suggested by the close apposition of the curves illustrating the effect of RecF protein on joint molecule formation and its ability to retard ssDNA in agarose gel electrophoresis (Fig.2C). This suggests that the fraction of ssDNA combined with RecF protein is inactive in joint molecule formation. To explain the differential interference with ATPase activity, there are two possibilities: First, RecF-ssDNA complexes may combine with RecA protein and hence support ATPase activity. Second, the presence of RecF protein or RecF-ssDNA complexes may stimulate the ATPase activity of RecA-ssDNA complexes so that the net effect appears to be a higher RecF protein concentration for interference with ATP hydrolysis.

One question which arises is why RecF protein should interfere with the activities of RecA protein *in vitro* when *in vivo* evidence indicates that it should stimulate those activities (5). One possibility is that the contaminating proteins present in our protein preparations may be contributing to the interference. To test this we are seeking to remove the contaminating proteins to see if the amount of interference changes. We are also considering three other possibilities: (1) perhaps we used non physiological RecF: RecA ratios, (2) perhaps proteins required for stimulatory function are absent and (3) perhaps proteins inhibiting the interference by RecF protein are absent.

To assess the physiological ratio of RecF and RecA proteins we used polyclonal antibodies raised against purified RecF protein to determine the *in vivo* levels of RecF. We found less than 190 molecules of RecF (Madiraju and Clark, unpublished information). under the conditions where wild type cells would have about 10,000 molecules of RecA (20). Thus the physiological ratio of RecF to RecA protein is less than 1:50. At low RecF concentration (i.e. RecF to RecA close to 1:50) we see minimal interference effects by RecF protein (Fig.2B). The interference effects noticed at high RecF to RecA protein ratios tempt us to predict that conditions that alter the *in vivo* equilibrium between RecF and RecA proteins will have profound effects on the RecA protein catalyzed reactions. Assuming that the joint molecule reaction represents an hypothetical recombination reaction occurring *in vivo* (18), we can then predict that cells overproducing RecF protein *in vivo* will have decreased ability to carry out recombination and recombinational repair of damaged DNA. Recently, Moreau (21) reported a related finding with cells overproducing SSB protein, another single-stranded DNA binding protein involved in DNA recombination, replication and repair. Currently, experiments are underway to test these predictions.

Second RecF protein may require other proteins to fulfill its *in vivo* role of stimulating RecA protein activities. Several gene products are involved in the RecF pathway of recombination: namely RecF, RecJ, RecO, RecR, RecN, RecQ and Ruv proteins (1, 5). Recent work carried out in our laboratory indicate that *recA803* partially suppresses the recombination and repair defects due to *recO* and *recR* mutations like it suppresses *recF* mutations (Madiraju, Clark and Templin, unpublished results). R. Lloyd and his colleagues (22) also argue that *recF*, *recO* and *recR* belong to the same epistasis group. These findings suggest that *in vivo* RecF works in concert with RecO, RecR proteins. Absence of those proteins in our reactions may contribute to the non-physiological interference we have observed. Third, we found that SSB protein inhibits interference by RecF protein (Table 2). SSB protein *in vivo* may do likewise. To assess this possibility we need to do more experiments on the interactions of RecF and SSB proteins and both proteins with RecA protein.

Assuming that RecF protein's binding to ssDNA represents at least one property important for its *in vivo* activity, we can suggest several hypothetical roles for RecF protein in recombination. A detailed description of these roles have been reported by one of us recently (23). For example, by binding to ssDNA ends of dsDNA, such as gaps or double stranded breaks, RecF protein might organize a functional protein complex or facilitate entry of RecA protein to the dsDNA. This implies RecF is a nucleation protein (23). Alternatively, RecF protein could prevent loss of ssDNA, a key intermediate in recombination, from nuclease attacks. Another possibility is that RecF protein could be a processivity subunit for a complex

helicase that might remove potential secondary structures or SSB protein from ssDNA.

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