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An Interaction between the *Escherichia coli* RecF and RecR Proteins Dependent on ATP and Double-stranded DNA*

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The DNA binding and ATPase activities of RecF protein are modulated by RecR protein. Stoichiometric amounts of RecF protein bind to double-stranded (ds) DNA (about 1 RecF monomer/4–6 base pairs) in the presence of adenosine 5′-*O*-(3-thio)triphosphate (ATPγS), forming a homogeneous protein coating on the DNA. Little or no cooperativity is evident in the binding process. In the presence of ATP, RecF binding to dsDNA is much weaker, and no RecF protein coating forms. Instead, small numbers of RecF protomers are interspersed randomly along the DNA. RecR protein does not bind appreciably to the dsDNA under these same conditions. However, a protein coating, similar to that which was observed with RecF protein alone in the presence of ATPγS, was produced when both RecF and RecR proteins were incubated with dsDNA in the presence of ATP. An interaction between RecF and RecR enables both proteins to bind tightly to the dsDNA in an approximately 1:1 molar ratio. We also report a weak ATP hydrolytic activity of RecF which is stimulated by RecR.

The products of the *recF*, *recO*, and *recR* genes are believed to act at a similar and early stage of recombinational DNA repair and homologous recombination in *Escherichia coli* (1–5). These three genes show remarkably similar mutant phenotypes. The frequency of recombination between plasmids is reduced 100-fold by a mutation in any one of these genes in a wild-type background (6–8). In excision repair deficient cells (*uvrA*), mutations in *recF*, *recO*, or *recR* produce a major deficiency in the repair of daughter strand gaps (9). In addition, the SOS response to DNA damage is significantly delayed in cells harboring a mutation in any of the three (10, 11). How the RecF, RecO, and RecR proteins are able to act on such a wide variety of DNA substrates is not known. Biochemical characterization of the individual proteins is beginning to offer some clues.

The RecF protein has been shown to bind ssDNA[1] (12, 13) and, in the presence of ATP or ATPγS, dsDNA (14). The RecO protein also binds to both ssDNA and dsDNA, and promotes renaturation of complementary ssDNA molecules in the absence of ATP (15). *E. coli* RecR does not bind to ssDNA (16), and no information regarding its dsDNA binding properties has been published, although the *Bacillus subtilis* RecR has been shown to bind both ssDNA and dsDNA (17). The majority of the biochemical studies involving the combination of these proteins have focused on their interaction with the RecA protein in the presence of single-stranded DNA. The RecO and RecR proteins in combination have been shown to help the RecA protein overcome the inhibitory effects of the single-stranded DNA binding protein on joint molecule formation *in vitro* (4, 16). Surprisingly, the RecF protein inhibits most of the activities of the RecA protein *in vitro*, including ssDNA binding, joint molecule formation (13), and LexA cleavage.[2] Although the primary structure of each of the three proteins displays a nucleotide-binding fold, ATP hydrolysis has not been reported for any of them.

Since these three proteins are thought to act at the same step of recombination and have been proposed to function as a complex (3), they may manifest a physical interaction with each other under the right conditions. Recently, the RecO and RecR proteins were shown to interact in the absence of any DNA or nucleotide cofactor (16). In this study we show that the RecF and RecR proteins interact in the presence of ATP and dsDNA. This interaction enables both to bind tightly to the DNA. We also provide evidence for a weak ATP hydrolytic activity of RecF protein.

MATERIALS AND METHODS

Enzymes and Reagents—E. coli RecF and RecR proteins were purified as described below. The concentration of RecF and RecR proteins were determined by the method of Bradford (18) with bovine serum albumin as the protein standard. RecA protein was purified as described elsewhere (19) and its concentration was determined by absorbance at 280 nm, using an extinction coefficient of $\epsilon_{280} = 0.59\ A_{280}\ mg^{-1}$ ml (20). Restriction endonucleases and Vent DNA polymerase were purchased from New England BioLabs. Bovine serum albumin and ATP were from Sigma. Oligonucleotides were synthesized by the University of Wisconsin Biochemistry Department Synthesis Facility. Plasmids pET21a and pET21d were purchased from Novagen. The Sequenase version 2.0 sequencing kit was from U. S. Biochemical Corp. Isopropyl-1-thio-β-galactopyranoside (IPTG) was from Bachem. Phosphocellulose P11 was from Whatman Ltd. PBE94, phenyl-Sepharose CL-4B, and Sepharose 4B resins were from Pharmacia Biotech Inc. Ultragel AcA54 gel filtration resin was from Sepracor. Cibacron blue 3GA-agarose type 100 resin was from Sigma. [α-^{32}P]ATP (3000 Ci/mmol) was from Amersham Corp. Mahcery-Nagel polyethyleneimine cellulose thin layer chromatography (polyethyleneimine/UV_{254}) sheets were purchased from Brinkmann.

*DNA—*ϕX174 RF II DNA was purchased from New England BioLabs. Bacteriophage M13mp8.1037 is derived from M13mp8 (21), having a 1037-bp insertion (*Eco*RV-*Eco*RV fragment from the *E. coli gal*T gene) in the *Sma*I site (22). Supercoiled circular duplex DNA from bacteriophage M13mp8.1037 was prepared using a method described previously (23–25). Complete digestion of supercoiled M13mp8.1037 by

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[1] The abbreviations used are: ssDNA, single stranded DNA; dsDNA, double-stranded DNA; bp, base pair(s); FII DNA, nicked and circular form of dsDNA; ATPγS, adenosine 5′-*O*-(3-thio)triphosphate; PAGE, polyacrylamide gel electrophoresis; MES, 2-(*N*-morpholino)ethanesulfonic acid; IPTG, isopropyl-1-thio-β-galactopyranoside.

[2] B. L. Webb, unpublished results.

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*Msc*I resulted in linear duplex DNA. The concentration of DNA stock solutions was determined by absorbance at 260 nm, using 50 mg ml^{-1} A_{260}^{-1} as the conversion factor. DNA concentrations are expressed in terms of total nucleotides.

Polymerase Chain Reaction Cloning of the E. coli recF and recR Genes—The *recF* and *recR* genes were individually isolated from the *E. coli* genome (26) (strain MG1655, a gift from George Weinstock) by polymerase chain reaction using a Perkin-Elmer DNA thermal cycler. This was accomplished by designing DNA primers which were complementary to the 5′ DNA sequence and to the 3′ DNA sequence of each gene. For the *recF* gene, the 5′ DNA primer corresponded to bases 6–21 of the published DNA sequence (27), with the sequence 5′-CCTCATGAG added to the 5′ end to provide a *Bsp*HI restriction site. This primer, designed to facilitate cloning, changed the second codon of the gene from TCC to AGC, though both encode for serine. The 3′ DNA primer corresponded to bases 1057–1074, with the sequence 5′-CCCAAGCTT added to the 5′ end to provide a *Hin*dIII restriction site. For the *recR* gene, the 5′ DNA primer corresponded to bases 584–598 of the published sequence (28), with the sequence 5′-CGTGATCCATATGCAG added to the 5′ end to provide a *Nde*I site. This primer was designed to change the second codon of *recR* from CAA to the more preferred codon for glutamine in *E. coli*, CAG (29). The 3′ DNA primer corresponded to bases 1164–1183, with the sequence 5′-CCCAAGC added to the 5′ end to create a *Hin*dIII restriction site. These primers were used to amplify DNA fragments of the appropriate size by the polymerase chain reaction. The *recF* gene fragment was digested with *Bsp*HI and *Hin*dIII and then ligated to the cloning vector pET21d (Novagen). This plasmid containing the *recF* gene was designated pBLW20. The *recR* gene fragment was digested with *Nde*I and *Hin*dIII and subsequently ligated to pET21a (Novagen). This plasmid containing the *recR* gene was designated pBLW22. The integrity of the entire *recF* and *recR* genes in these constructs was verified by direct sequencing (Sequenase kit and protocol, U. S. Biochemical Corp.). For the purpose of overexpressing RecF protein, pBLW20 was transformed into the *E. coli* strain K38/pGP1-2 (30). For overexpressing RecR protein, pBLW22 was transformed into the *E. coli* strain BL21(DE3) (31, 32).

Purification of E. coli RecF Protein—A 30-liter culture of *E. coli* strain, K38/pGP1-2, with the plasmid pBLW20 was grown with aeration in a New Brunswick MPPF 30 liter fermentor at 30 °C in Luria broth (supplemented with 100 μg/ml ampicillin and 40 μg/ml kanamycin) to an OD_{650} = 0.5. Expression of RecF protein was induced by adding IPTG to a final concentration of 0.5 mM and by raising the temperature of the medium to 42 °C for 30 min. The culture was then incubated at 25 °C for 5 h, after which the cells were collected by centrifugation. Outgrowth of the cells at 25 °C rather than 37 °C greatly increased the amount of soluble RecF protein. The cell pellet (480 g) was resuspended in lysis buffer (50 mM Tris-HCl, 80% cation (pH 7.5), 10% sucrose) at 1.65 ml/g of wet cells, frozen in liquid nitrogen, and stored at −70 °C.

RecF protein was purified as previously described (12, 13), with the following modifications. The fractions from the phosphocellulose column containing RecF protein were pooled and treated with ammonium sulfate to 70% saturation. The precipitate was harvested by centrifugation at 15,000 rpm for 30 min in a Beckman JA21 rotor. The pellet was resuspended in a volume of buffer A (20 mM Tris-HCl (pH 7.5 at 4 °C), 0.1 mM EDTA, 10% (w/v) glycerol, 10 mM 2-mercaptoethanol) such that the conductivity was equal to that of buffer A + 100 mM NaCl. This solution was then loaded onto a PBE94 column (35-ml bed volume, 1.0 cm × 44 cm) that had been previously equilibrated in buffer A + 100 mM NaCl. The column was then washed with two bed volumes of buffer A + 100 mM NaCl and developed with a 160-ml linear gradient of buffer A with from 100 to 600 mM NaCl. Peak fractions (around 200 mM NaCl) were pooled and precipitated with ammonium sulfate to 70% saturation. After centrifugation, the pellet was resuspended in buffer A + 1 M NaCl. This solution was loaded onto a Ultragel AcA54 gel filtration column (125-ml bed volume, 1.5 cm × 71 cm) equilibrated in buffer A + 1 M NaCl. The high salt concentration was required to minimize interaction between RecF protein and the column resin. The pooled peak fractions were then dialyzed against buffer A + 60% glycerol [w/v] + 100 mM NaCl, and stored at −70 °C. The final yield of RecF protein from 100 g cells was 3.6 mg. The RecF proteins was at least 95% pure as estimated by SDS-PAGE (Fig. 1) and contained no detectable endo- or exonuclease activities.

Purification of E. coli RecR Protein—A 30-liter culture of *E. coli* strain, BL21(DE3), with the plasmid pBLW22 was grown with aeration in a New Brunswick MPPF 30-liter fermentor at 37 °C in Luria broth (supplemented with 100 μg/ml ampicillin) to an OD_{650} = 1.5. RecR protein expression was induced by adding IPTG to a final concentration of 0.4 mM. The culture was then incubated at 37 °C for 9 h. The cells were then collected by centrifugation (200 × *g*), resuspended in lysis buffer at 2 ml/g of wet cells, frozen in liquid nitrogen, and stored at −70 °C.

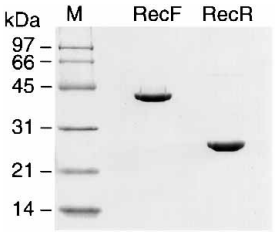


FIG. 1. **SDS-PAGE of purified RecF and RecR proteins.** The molecular masses of the RecF and RecR proteins are 40.5 and 22 kDa, respectively. RecR migrates at 26 kDa (28). *Lane 1*, molecular mass markers; *lane 2*, RecF protein (3 μg); *lane 3*, RecR protein (3 μg).

RecR protein was purified as described elsewhere (4). The final yield of RecR protein from 26 g of cells was 7.6 mg. The RecR protein was estimated by SDS-PAGE (Fig. 1) to be at least 95% pure and was free of detectable endo- or exonuclease activities.

Standard Reaction Conditions—All reactions were carried out in a standard reaction buffer containing 20 mM Tris-HCl (80% cation, pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM dithiothreitol. DNA and nucleotide cofactors were added as indicated. The pH at 37 °C after addition of all reaction components was 7.01.

Electron Microscopy—Proteins were incubated with 3 μM DNA (FII or linear dsDNA as indicated) at 37 °C in the standard reaction buffer for 10 min. Samples were prepared directly from reaction mixtures, without cross-linking, by the following adsorption procedure. (*a*) A carbon film was evaporated onto a set of freshly cleaved mica disks at an angle of 90 °C. Carbon-coated disks were used after aging for at least 2 weeks and were usable for at least 1 year. The carbon film was made as thin as possible while retaining the capacity to resist breakage during subsequent steps. The carbon film was floated off the mica by sliding the disk through a clean air-water interface at an angle of 45 °C, and pieces of the film were picked up from below on 400 mesh copper electron microscope grids. After drying, the grids were stored in a desiccator. (*b*) Immediately before use, the carbon-filmed grids were briefly dipped sequentially into three separate beakers containing $CHCl_3$ and dried under a heat lamp. Carbon films were then "activated" by floating the grids on a 50-μl drop of 0.002% Alcian blue in 0.03% acetic acid, for 5–10 min followed by a wash on a 1-ml drop of water for 5–10 min. The excess water was removed, and grids were dried under a heat lamp. The above activation and washing steps were not necessary for visualization of the DNA-protein complexes discussed in this report, but were used to increase the yield of any unreacted DNA adsorbed to the carbon film and thus give a more realistic view of the contents of a reaction mixture. (*c*) The grids were floated on a 10-μl drop of the reaction mixture (immediately after the reaction incubation) for 30 s followed by floating on three 1-ml drops of washing solution containing 15% glycerol, 20 mM Tris (pH 7.5), 0.1 mM EDTA, and 10 mM $MgCl_2$ for 1 s, 5 min, and 10 min, respectively. (*d*) The grids were then briefly touched to a 50-μl drop of staining solution containing 10% glycerol and 5% uranyl acetate, and then floated on a second drop of the same staining solution for 40 s. The grids were then washed by touching briefly to four drops of 10% glycerol in water, and the remaining liquid was withdrawn with a capillary pipette. The grids were dried under a heat lamp for 2 min. Finally, the sample was rotary shadowed with platinum in a vacuum evaporator.

The uranyl acetate solution mentioned above was made up every 3 months, initially filtered through a 0.22-μm Millipore membrane and stored in a dark bottle at room temperature. Before each use, the solution was spun for 1 min in a microcentrifuge, and only the top part of the supernatant was used. The Alcian blue solution was made up each day by dilution of a stock solution containing 0.2% Alcian blue in 3% acetic acid.

Photography and measurements of the protein-DNA complexes were carried out as described previously (33). Copies of the electron micrographs discussed in this publication are available for viewing or downloading using a World-Wide Web Client at the URL: http://phage.bocklabs.wisc.edu/.

Conditions for RecA Binding to dsDNA—RecA (2 μM) was incubated with ϕX174 RF II DNA (6 μM) and 3 mM ATP in 25 mM MES-NaOH (56% cation, pH 6.27), 10 mM magnesium acetate, 2% glycerol (v/v) with an ATP-regenerating system (5 units ml^{-1} creatine phosphokinase, 6

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mM phosphocreatine), 10 min at 37 °C. After this incubation period, ATPγS was added to a final concentration of 0.5 mM, and the reaction was incubated an additional 10 min. To neutralize the pH of this reaction mixture prior to spreading, it was diluted with an equal volume of a solution containing the same components described above except the buffer was 25 mM Tris acetate (40% cation, pH 8.4) and the RecA and DNA were not present.

Restriction Endonuclease Protection Assay—Nicked, circular φX174 dsDNA (FII; 10 μM) was preincubated with either RecF protein (2 μM), RecR protein (2 μM), or their corresponding storage buffers (as a control), 10 min at 37 °C in a 60-μl reaction mixture containing the standard reaction buffer. Nucleotide cofactors were included as indicated. A 15-μl aliquot was then removed (for a zero time point with respect to digestion by the restriction endonuclease). To the remaining reaction mixture was added NEBuffer no. 4 and *Bsp*HI (New England Biolabs) to final concentrations of 1× and 0.1 units/μl, respectively (a total addition of 6 μl). Aliquots (25 μl) of the restriction digestion reactions were removed at the indicated time points and the reactions were stopped by the addition of 5 μl of gel loading buffer (30% glycerol, 0.03% bromphenol blue, 30 mM EDTA, 5% SDS). Samples were then electrophoresed in a 1.0% agarose gel.

Agarose Gel Retardation Assay—An agarose gel assay patterned after a published procedure (14) was used to detect complexes between linear dsDNA and RecF and RecR proteins. Proteins as indicated were incubated in 35-μl reaction mixtures containing 10 μM linear dsDNA in the standard reaction buffer at 37 °C for 10 min. At the end of the incubation period 5 μl of a gel loading buffer (25 mM Tris-HCl (80% cation, pH 7.5), 50% glycerol, and 0.02% bromphenol blue) were added. Samples were then loaded onto an 0.8% agarose gel and electrophoresed at 2.5 V/cm with recirculation of the running buffer (20 mM Tris-HCl (80% cation, pH 7.5), 4 mM sodium acetate, and 0.1 mM EDTA). The DNA was then detected by ethidium bromide staining.

Gel Filtration of RecF-RecR-DNA Complexes—A Sepharose 4B gel filtration column (3.85-ml bed volume, 0.7 cm × 10 cm) was used to isolate RecF-RecR-DNA complexes. Proteins and linear dsDNA at the indicated concentrations were incubated in 60-μl reaction mixtures in the standard reaction buffer at 37 °C for 10 min. This reaction mixture was loaded onto a Sepharose 4B column that had been previously equilibrated with buffer A + 100 mM NaCl + 1 mM ATP and chromatographed, 2 ml/h at room temperature. Fractions (115 μl) were collected and analyzed for DNA and protein content. A portion of each fraction was run on a 0.8% agarose gel to determine the elution volume of the DNA, which was found be in the column void volume (2.7 ml). A portion of each fraction was also run on a 15% polyacrylamide minigel to visualize the elution profile of the RecF and RecR proteins. RecF protein and RecR protein bound to the DNA were found in the void volume, while that which was unbound was found to elute at 5.78 ml and 4.62 ml, respectively. After each experiment the column was washed with 2 M NaCl to wash off any protein which had adsorbed to the column resin.

Quantitation of the RecF-RecR-dsDNA Complex—The concentration of DNA in the void volume of individual experiments was determined by running a sample of the peak fraction alongside a DNA titration of known concentration on the same 0.8% agarose gel to generate a standard curve. The photographic negative of the ethidium bromide-stained gel was scanned using a Molecular Dynamics Personal Densitometer SI and the DNA bands quantitated using ImageQuant NT software. The amount of RecF protein and RecR protein bound to the DNA was determined by running a sample of the same fraction on a 15% polyacrylamide minigel, including a RecF protein titration and a RecR protein titration to generate standard curves. After silver staining the gel, the protein bands were quantitated in the same fashion as the DNA.

Thin Layer Chromatography Assay for ATP Hydrolysis Measurements—The polyethyleneimine-cellulose plates were first developed in water overnight, a step recommended by the manufacturer to clean the plates prior to use. The plates were then dried and stored at 4 °C. Reaction mixtures (50 μl) containing the standard reaction buffer, [α-^{32}P]ATP, various concentrations of cold ATP, and DNA as indicated, were preincubated at 37 °C for 5 min. The reaction was started by the addition of RecF protein. RecR protein was added at this time as well, as indicated. At various time points, aliquots (1 μl) of the reaction were spotted to prewashed TLC plates to quench the hydrolysis reaction. The TLC plates were developed in 1 M formic acid, 0.5 M LiCl. The plates were dried and exposed to storage phosphor screens (Molecular Dynamics). The exposed phosphor screens were scanned with a Molecular Dynamics PhosphorImager (model 425E). The radioactivity in the ATP and ADP spots were quantified using ImageQuant NT software, and the percent ATP hydrolyzed was calculated and plotted *versus* time. The percent ATP hydrolyzed per min was calculated by a linear least squares fit. The rate of ATP hydrolysis was calculated using the expression:

$$\text{Rate of ATP hydrolysis} = ((\text{percent ATP hydrolyzed/min}) \times [\text{ATP}]_0)/100$$

where $[\text{ATP}]_0$ is the concentration of ATP at time 0 (before the addition of RecF protein).

RESULTS

Experimental Design and Summary—An interaction between the RecF and RecR proteins was inferred from the effects of RecR on RecF activities *in vitro* as described below, and from the detection of complexes containing both proteins. Binding of RecF protein to dsDNA was characterized by electron microscopy, endonuclease protection, gel retardation, and gel filtration. A standard assay for ATP hydrolysis was adapted to detect a low level ATPase activity of RecF protein.

RecF Binds Noncooperatively to and Coats dsDNA in the Presence of ATPγS—RecF has been shown to retard linear dsDNA on an agarose gel in the presence of ATP and its nonhydrolyzable analog, ATPγS (14). Electron microscopy was used to visualize the RecF-dsDNA complex formed in the presence of ATPγS. Unbound nicked circular dsDNA is shown in Fig. 2*A* for reference. Addition of RecF protein, at concentrations equivalent to 1 monomer/1.25 base pairs of DNA resulted in a complete coating of the DNA with RecF protein (Fig. 2, *B* and *C*). These RecF-DNA complexes were sufficiently stable to survive the spreading procedure without cross-linking. DNA topology did not affect RecF binding, as similar results were seen with supercoiled and linear double-stranded DNA (data not shown). Further experiments showed that a full coating of the dsDNA was obtained at RecF concentrations corresponding to 1 RecF monomer/5 bp and greater. As described in more detail below, this coating of the DNA is not observed when ATP replaces ATPγS.

We also measured the contour length of RecF-bound DNA to determine if binding resulted in a measurable extension of the DNA. Also shown in Fig. 2 is a comparison of RecF-coated dsDNA and the same dsDNA substrate bound by RecA protein in the presence of ATPγS. RecA is known to form a right-handed helical protein filament on linear dsDNA under these conditions (34). We found that RecA lengthened the dsDNA by 62% relative to protein-free DNA under these spreading conditions (Fig. 2, *D versus A*), somewhat more than the 53% extension reported previously (35, 36). RecF (Fig. 2, *B* and *C*) had a more modest effect, increasing the contour length of dsDNA by 9% in the presence of ATPγS.

RecF complexes formed on dsDNA at concentrations of RecF insufficient to coat the DNA suggested that the binding was largely noncooperative. Two intermediate points in the titration, at RecF to base pair ratios of 1:20 and 1:7, along with protein-free and fully coated DNA, are shown in Fig. 3, with increasing protein concentration from left to right. By counting the number of RecF dots per DNA molecule on a representative number of DNA molecules within each sample, we were able to demonstrate that the average number of dots bound per DNA does increase with increasing RecF concentration (data not shown). Also evident is that the dots accumulated in an apparently random fashion over the entire length of the DNA molecule, rather than as a single tract of protein, as would be expected if the binding were as cooperative as that observed with RecA protein (36). These results are consistent with titrations using gel retardation (data not shown) in which increasing RecF concentrations kept the entire DNA band relatively compact while decreasing its mobility in incremental steps.

RecR Protein Facilitates RecF Protein Binding to dsDNA in the Presence of ATP—Using the digestion of linear dsDNA by

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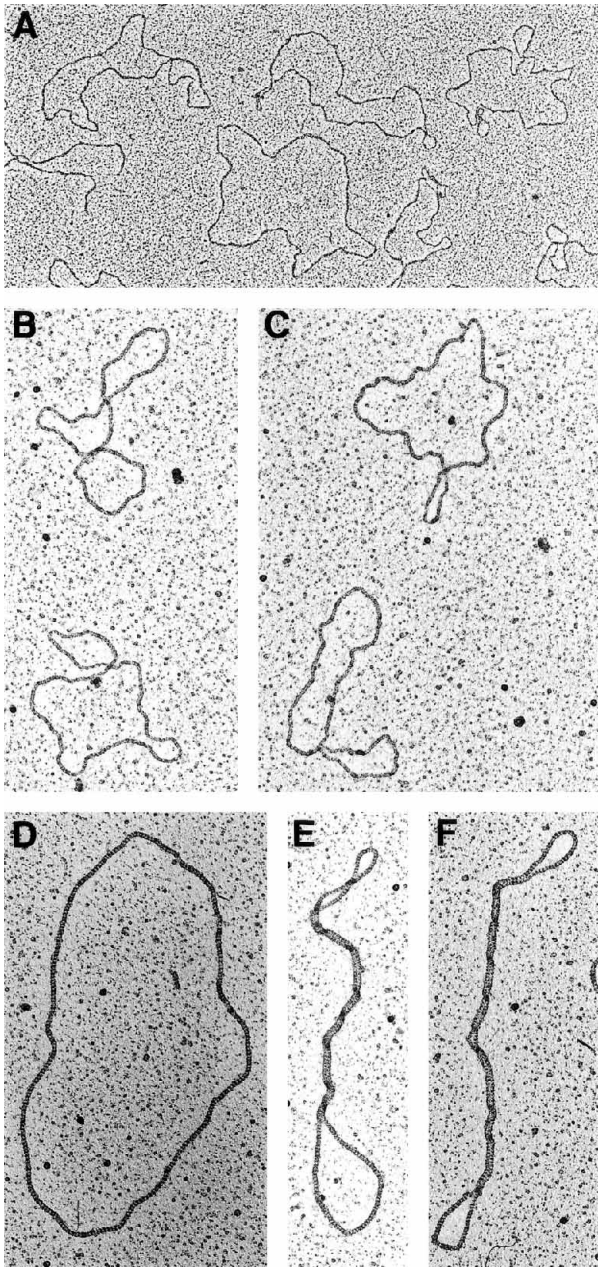


FIG. 2. **Visualization of RecF and RecA proteins bound to FII DNA by electron microscopy.** RecA protein (2 μM) was bound to ϕX174 FII DNA (3 μM) as described under "Materials and Methods." RecF protein (2.4 μM) was incubated for 10 min at 37 °C in a 30-μl reaction mixture containing 3 μM ϕX174 FII DNA and 1 mM ATPγS in standard reaction buffer. Both samples were spread for electron microscopy without cross-linking as described under "Materials and Methods." *A*, FII DNA with no protein added; *B* and *C*, FII DNA coated with RecF protein; *D–F*, FII DNA coated with RecA protein. The molecules were chosen to be representative of the respective samples. Careful determinations of contour lengths revealed that RecF protein binding produced a 9% extension in the DNA, compared to a 62% extension produced by RecA protein under these conditions.

the restriction endonuclease *Bsp*HI as a measure of protein binding, we found that RecF protein (at 1 monomer/5 bp) completely protected the DNA from digestion in the presence of ATPγS (Fig. 4). A partial protection was observed when ATP replaced ATPγS. RecR protein alone (at 1 monomer/5 bp) afforded no protection to the DNA. However, including the RecR protein in the reaction with RecF protein led to complete protection of the DNA in the presence of ATP (Fig. 4). The partial protection of the DNA afforded by RecF protein alone and ATP reflected an incomplete but apparently stable interaction. The pattern of bands shown in Fig. 4 was stable for at least 40 min of *Bsp*HI digestion. No protection was afforded by the RecF and RecR proteins together in the absence of ATP (data not shown).

Complementary results were obtained with gel retardation assays (Fig. 5). RecF protein binding to dsDNA was again much more complete when ATPγS was used as cofactor, as indicated by the greater retardation of band migration. RecR protein alone had little effect on band migration in the presence of ATP. RecF protein caused a slight shift in DNA band mobility under these conditions, but the shift was less than that produced with ATPγS. However, when the two proteins were added to the reaction together in the presence of ATP, they produced a substantial retardation in the band migration. In many experiments, the retardation was comparable to that observed with RecF in the presence of ATPγS. The results indicate that the RecR protein facilitates the binding of RecF protein to DNA in the presence of ATP.

The complexes formed with RecF and RecR proteins on linear dsDNA in the presence of ATP were examined by electron microscopy (Fig. 6). As stated earlier, when RecF alone was incubated with dsDNA in the presence of ATP, very little RecF was observed to be bound to the DNA, even when the RecF concentration was increased to give 1 RecF monomer/2 bp (Fig. 6*A*). A reaction mixture of RecR alone (1 monomer/2 bp) incubated with dsDNA and ATP and spread similarly is shown in Fig. 6*B*. We believe that the observed large black spots represent RecR protein rather than a spreading artifact. No other protein stock solutions (RecA, RecF) produced comparable spots, the number of spots on the grid was directly dependent on the amount of the RecR protein stock solution spread, and the addition of proteinase K eliminated the spots. The spots are large enough to contain 2–6 RecR monomers, allowing for uncertainties imposed by the shadowing of the complexes. In some cases, the spots show a certain degree of fine structure. Similar spots were observed in electron microscopic observations of the *B. subtilis* RecR protein (17).

When the RecF and RecR proteins were incubated together with dsDNA and ATP and the reaction mixture spread, the DNA was seen to be coated with protein (Fig. 6*C*). The order of addition of the proteins did not seem important, as a similar result was obtained if the DNA was preincubated with RecF 10 min prior to RecR addition (Fig. 6*D*) or visa versa. Also, consistent with the gel retardation results, the DNA appeared essentially unbound when ATP was omitted from the reaction containing both proteins (data not shown). When ATP was omitted, the overall concentration of DNA on the grid was consistently lower, an effect described further below.

The protein-DNA structure formed by RecF, dsDNA, and ATPγS (Fig. 2, *B* and *C*) appears very similar to that formed by RecF, RecR, dsDNA, and ATP (Fig. 6*C*). The large spots characteristic of RecR protein are not evident in Fig. 6*C*, and there is no feature of the protein coat on the DNA to indicate the presence or absence of RecR within it. However, the absence of RecR protein in the background implies its presence in the protein coat.

RecR and RecF Proteins Form a Joint Complex on dsDNA in the Presence of ATP—To determine if RecR is a component of the protein coat formed on dsDNA in the presence of RecF, RecR, and ATP, we purified preformed complexes by chromatography on a gel filtration column that excluded the linear dsDNA and any protein bound to it but included unbound proteins. The column fractions were then analyzed both by agarose and SDS-polyacrylamide gel electrophoresis to determine the elution profiles of the DNA and proteins, respectively. When either RecF (1 monomer/3.4 bp) or RecR (1:3.4) alone was

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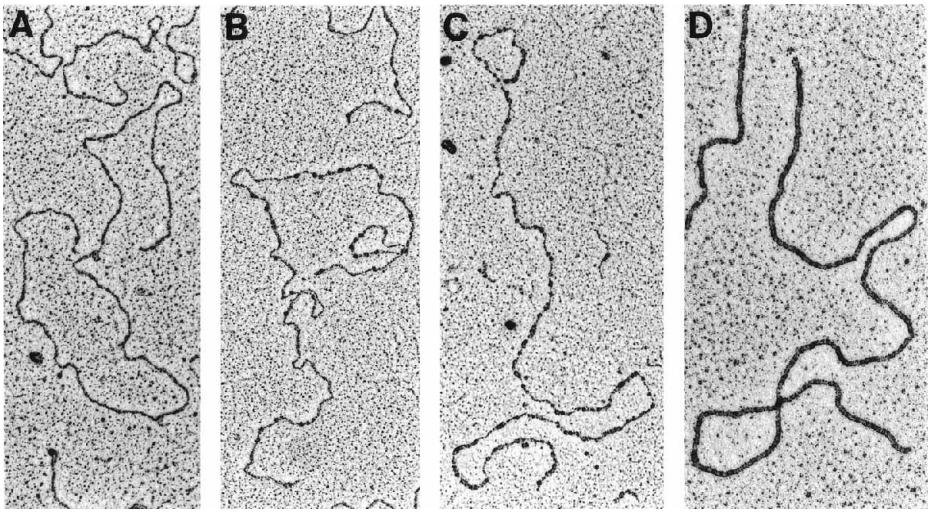


FIG. 3. **RecF protein titration on linear dsDNA in the presence of ATPγS.** Linear M13 mp8.1037 dsDNA (3 μM) was incubated with RecF protein at protein to DNA bp ratios of *A*, 0; *B*, 1:20; *C*, 1:7; and *D*, 1:2 in the presence of ATPγS as described in Fig. 2.

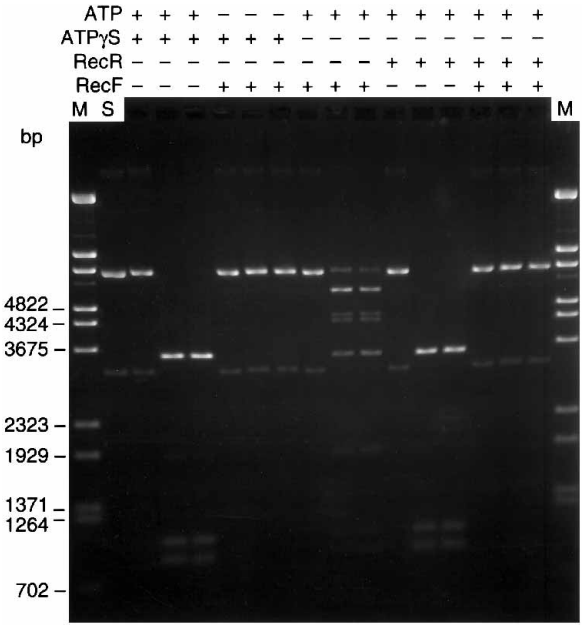


FIG. 4. **Protection of dsDNA from restriction endonuclease digestion by RecF and RecR proteins.** φX174 FII DNA (5386 bp) has three *Bsp*HI sites within it, yielding fragments of 3503, 1017, and 866 bp upon complete digestion. Reactions were carried out with 10 μM φX174 FII DNA with RecF (2 μM) and/or RecR (2 μM) proteins as described under "Materials and Methods." Reactions also contained 1 mM ATP or ATPγS as indicated. The φX174 FII DNA preparation contained approximately 10% supercoiled DNA molecules. The marker lanes (*M*) contain λ DNA digested with *Bst*EII. The position of φX174 FII DNA alone is shown in the second lane (*S*). The next 15 lanes are divided into five reactions (three lanes each) in which the DNA was incubated with the components indicated at the top for 10 min prior to *Bsp*HI addition. In each case, the three lanes show the status of the DNA at 0, 5, and 10 min after addition of *Bsp*HI. In the third time course, containing RecF protein and ATP, the observed DNA fragments were produced by incomplete cleavage of the DNA: a single cut produces linear dsDNA (5386 bp), while pairs of fragments (3503 and 1883, 4369 and 1017, or 4560 and 860 bp) are produced by the three possible combinations of double cuts.

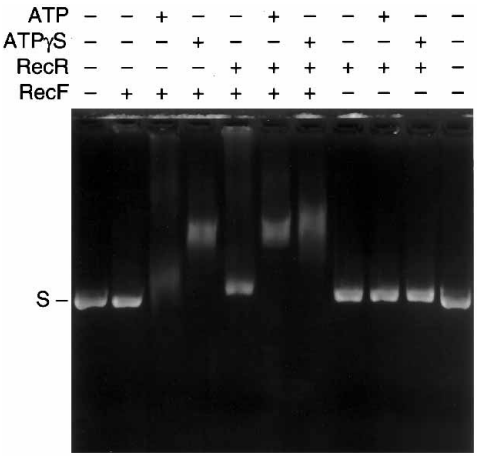


FIG. 5. **Agarose gel retardation assay to detect RecF and RecR binding to linear dsDNA.** Reactions were carried out as described under "Materials and Methods" with linear M13mp8.1037 dsDNA (10 μM) and with RecF protein (0.5 μM) and/or RecR protein (2 μM). ATP and ATPγS at 1 mM were included as indicated. The first and last lanes show the linear DNA substrate without added proteins. The remaining lanes reflect 10-min reactions with the components indicated at the top.

incubated with dsDNA and ATP and then chromatographed, neither protein could be detected in the void volume (2700 μl) with the DNA (Fig. 7, *A* and *B*). In the experiment with RecF alone, the elution volume of RecF was 5775 μl. In the experiment with RecR alone, RecR eluted at 4620 μl. This finding that RecR (22 kDa) elutes prior to RecF (40.5 kDa) is consistent with the report that RecR exists as a dimer in solution (16). Interestingly, unless the column was equilibrated and run with buffer containing 1 mM ATP, the RecF protein was retained on the column, presumably by nonspecific interaction with the column matrix, and did not elute (data not shown). The requirement for ATP in the elution buffer could be alleviated by using ATPγS in the reaction mixture with RecF and dsDNA, instead of ATP. Under these conditions, RecF co-eluted with the DNA in the void volume (data not shown), again demonstrating the increased binding of RecF protein to dsDNA when ATPγS replaces ATP.

When both RecF and RecR were included in the reaction with dsDNA and ATP, each at protein to nucleotide ratios of 1:3.4, a substantial fraction of the total concentration of both proteins was found associated with the DNA in the void volume. The remaining, unbound RecF and RecR eluted later at volumes corresponding to the individual proteins (Fig. 7*C*). A similar result was obtained when ATPγS was used in the reaction (not shown). This provides evidence that RecR is retained in the RecF-DNA complex in the presence of ATP and ATPγS.

We note that, when ATP was omitted from the reaction mixture, some RecF and RecR was found to elute in the void volume with the DNA (the amount was generally substantially reduced and variable relative to the amounts found associated with DNA in the presence of ATP; data not shown). The most plausible explanation for this finding is that, in the absence of

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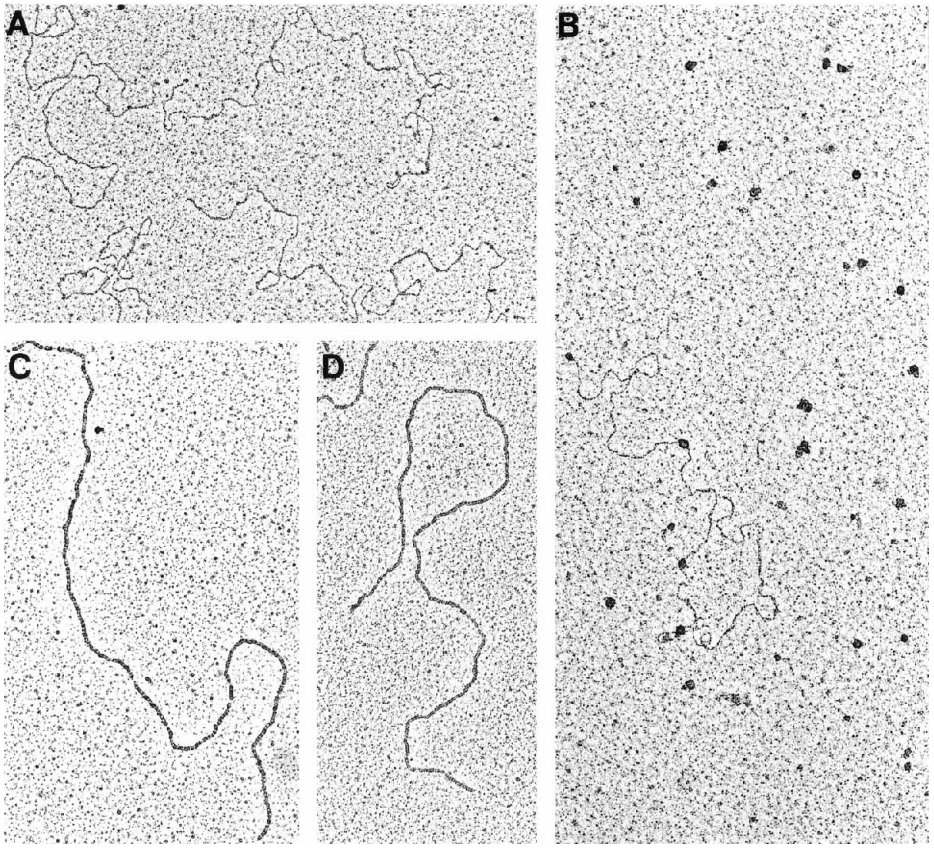


FIG. 6. **Visualization of RecF-RecR-dsDNA complexes by electron microscopy.** Linear M13 mp8.1037 dsDNA (3 μM) was incubated with the indicated proteins at 37 °C for 10 min in a 40-μl reaction mixture containing the standard reaction buffer and 1 mM ATP. Samples were spread directly for electron microscopy as described under "Materials and Methods." *A*, 1.5 μM RecF protein; *B*, 1.5 μM RecR protein; *C*, RecF and RecR proteins (both at 1.5 μM). In *D*, the DNA was incubated 10 min at 37 °C with 1.5 μM RecR protein, and then 1.5 μM RecF was added followed by an additional 10 min of incubation prior to spreading. In this and other experiments it was determined that the order of protein addition had no effect on the result.

ATP, RecF and RecR form large nonspecific aggregates with dsDNA. This is supported by the observation of DNA retained in gel wells (Fig. 5, *lane 5*) and by the reduction in concentration of free DNA molecules seen on electron microscopy grids under these conditions. As described above, the RecF and RecR proteins together do not protect the DNA from digestion by the endonuclease *Bsp*HI unless ATP is present.

To determine if RecF and RecR interact in the absence of dsDNA, both proteins were incubated under identical conditions except that DNA was omitted from the reaction. Fig. 7*D* shows that both proteins eluted at their normal elution volume, indicating that any interaction between the RecF and RecR proteins in the presence of ATP is DNA dependent.

The Bound Complexes Contain Approximately Equal Concentrations of RecF and RecR Proteins—To determine the molar ratios of RecF and RecR within the complex formed with dsDNA, void volume fractions from the gel filtration experiments were analyzed for protein and DNA content as described under "Materials and Methods." The reactions analyzed were those described above in which RecF protein and/or RecR protein were incubated at 1 monomer/3.4 bp of dsDNA with ATP or ATPγS. When RecF protein was incubated with DNA and ATPγS, there was 1 monomer found per 4–6 bp of DNA in the eluted complex in three experiments. In the complexes with RecF, RecR, DNA, and ATP, the amount of RecF appeared to decrease somewhat, with 1 monomer/6–9 bp of DNA in most experiments. RecR protein was also evident in these complexes, at a RecF to RecR ratio of approximately 1:1 (range = 1:0.7 to 1:1.4).

RecF Protein Exhibits a Weak ATPase Activity That Is Stimulated by RecR Protein—Thin layer chromatography was used to analyze the ATP hydrolytic activity of RecF. We detected a weak ATP hydrolytic activity in the absence or presence of linear dsDNA, with a K_m and k_{cat} of approximately 73 μM and 0.21 min^{-1}, respectively (Fig. 8). The observed ATPase activity was stimulated 2.5-fold by RecR protein, which itself shows no detectable hydrolytic activity under these conditions. The measured K_m and k_{cat} for RecF-mediated ATP hydrolysis in the presence of dsDNA and RecR protein was approximately 83 μM and 0.52 min^{-1}, respectively. The ATPase was detected in two successive preparations of RecF protein, with comparable activities in both. When the purified RecF protein was subjected to gel filtration, the ATPase activity co-eluted with it (data not shown).

DISCUSSION

The principal findings of this study are the detection of an ATP- and dsDNA-dependent interaction of the RecF and RecR proteins and a weak ATP hydrolytic activity of RecF protein. We have also further characterized the dsDNA binding properties of RecF, showing it to bind noncooperatively to and completely coat dsDNA in the presence of ATPγS, resulting in a modest extension of the DNA.

Previously it was shown that the RecF and RecR proteins do not physically interact in the absence of DNA (16). The genetic studies suggesting that these proteins act together *in vivo* prompted us to look for conditions in which they would show an interaction. The interaction we detected is highly dependent on DNA and ATP. The RecR protein greatly facilitates RecF protein binding to dsDNA in the presence of ATP. The absence of detectable DNA binding with RecR alone indicates that the effect is not merely additive with respect to the binding observed with RecF or RecR alone. Both proteins are present in the resulting complex. In gel filtration experiments, the RecF and RecR proteins elute together with the DNA in a complex containing approximately equal numbers of both protomers.

The electron microscopy experiments further characterize the binding of RecF protein to dsDNA. RecF binds noncooperatively to dsDNA in the presence of ATPγS, forming a homogeneous protein coating on the DNA that exhibits saturation (in

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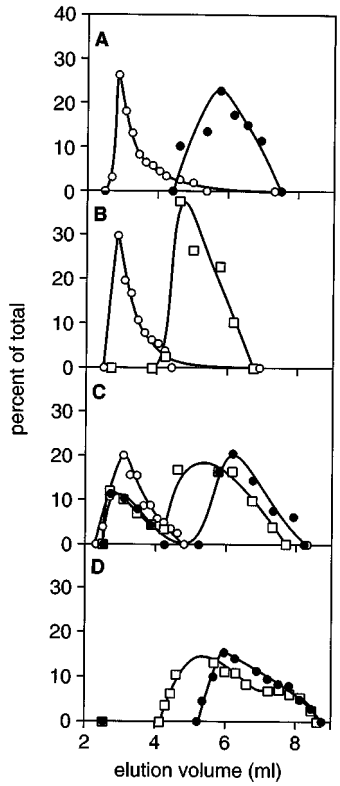


FIG. 7. **RecF-RecR-dsDNA complexes detected by gel filtration.** RecF protein (11.7 μM) and/or RecR protein (11.7 μM) were incubated at 37 °C for 10 min with 40 μM linear M13 mp8.1037 dsDNA in a 60 μl reaction mixture containing the standard reaction buffer and 1 mM ATP. The reaction mixture was then loaded onto a Sepharose 4B column (0.7 cm × 10 cm) that was equilibrated with buffer A + 100 mM NaCl + 1 mM ATP at room temperature. The column was developed and the results quantitated as described under "Materials and Methods." *A*, RecF protein; *B*, RecR protein; *C*, RecF and RecR protein. In *D*, the DNA was omitted from the reaction. Data point symbols are (○) DNA, (●) RecF protein, and (□) RecR protein.

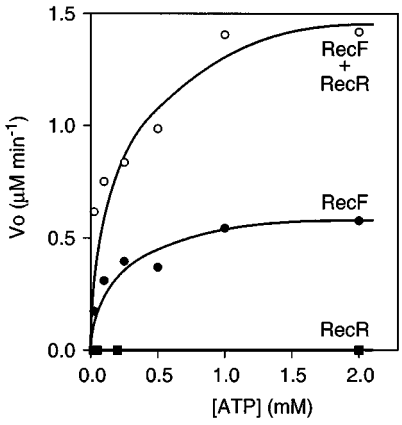


FIG. 8. **Stimulation of RecF protein mediated ATP hydrolysis in the presence of linear dsDNA by RecR protein.** Reactions were carried out as described under "Materials and Methods." Reactions contained 10 μM linear M13mp8.1037 dsDNA, 2.5 μM RecF protein and/or 2.5 μM RecR protein, and the indicated concentration of ATP. Symbols are (■) RecR, (●) RecF, and (○) RecR and RecF.

gel filtration experiments) at approximately 1 RecF monomer/5 bp. Although the binding of RecF appears to extend the DNA slightly (about 9%), there are no regular structural features comparable to the striations in a RecA filament that are evident at the resolution limit of these samples. RecF protein binding to dsDNA in the presence of ATP is much weaker, and as a result no such RecF coating of the DNA occurs. However, the addition of RecR to a reaction mixture of RecF, dsDNA, and ATP leads to both RecF and RecR becoming stably complexed to the dsDNA. Both proteins together produce a fairly uniform protein coat on dsDNA in the presence of ATP. We do not know if RecR protein is bound directly to the DNA or indirectly via protein-protein interactions with RecF. The latter seems more likely since RecR alone does not bind to the dsDNA under these conditions.

In some gel mobility shift experiments and in the electron micrographs, the complexes formed with RecR and RecF proteins on dsDNA in the presence of ATP exhibit an apparent similarity to those formed with RecF protein alone in the presence of ATPγS. We draw no structural conclusions from these observations. The experiments have a relatively low resolution with respect to molecular structure, and may not reflect alterations in the complexes which must logically accompany the incorporation of RecR protein. The gel filtration experiments suggest that somewhat less RecF protein is bound to the DNA in the presence of RecR protein than is present with saturating amounts of RecF protein alone and ATPγS. Some replacement of RecF protein with RecR protein may lead to only modest changes in the total mass of the two kinds of complexes, providing perhaps a partial explanation for their apparent similarity.

A search for an ATPase activity in RecF was motivated by the existence of a nucleotide binding fold in the primary structure of the RecF protein, similar to that present in other known ATPases (37), and by the observed differences in the dsDNA binding properties of RecF when ATP replaced ATPγS. The ATP hydrolytic activity of RecF protein in the presence of dsDNA is sufficiently weak that it is difficult to eliminate the possibility that it is associated with a contaminating protein. The activity has been detected in two different RecF preparations, and it co-elutes with the RecF protein in gel filtration experiments. The fact that the ATPase activity is stimulated 2.5-fold by RecR provides additional evidence that it is intrinsic to RecF protein. RecR protein alone shows no detectable ATPase activity.

We are also addressing the possibility that at least some of the observed ATP hydrolysis reflects a latent activity of RecR that is stimulated by RecF. We have recently constructed, expressed, and purified a RecF mutant protein (RecF K36R) which alters the putative ATP binding site. The mutation is equivalent to that in the *recF4101* mutant described by Sandler *et al.* (37). A comparable mutation in the conserved ATP binding site of the RecA protein (RecA K72R) creates an altered protein that binds, but does not hydrolyze ATP (38). The RecF K36R mutant protein binds to dsDNA in the presence of ATP (B. L. Webb, unpublished results). However, RecF K36R does not hydrolyze ATP, and no ATP hydrolytic activity of RecR protein is observed in the presence of the mutant protein,[2] providing perhaps the best evidence that all of the observed ATPase activity is intrinsic to RecF.

The molecular role of the ATPase activity is not clear. The activity may become more robust upon addition of components not yet tested in the system, or the weak hydrolytic activity may reflect a timing or regulatory function (39). Indeed, the molecular functions of the RecF and RecR proteins are not known. Given the recently demonstrated joint activity of the RecO and RecR proteins in modulating the binding of RecA protein to ssDNA (16), one might postulate RecF and RecR as an alternative functional pairing. Our results showing a dsDNA-dependent interaction between RecF and RecR may signal a role in plasmid recombination. We do not yet know if RecO protein can interact with the RecF-R complexes, nor have we tested the effects of the RecF and RecR proteins on RecA reactions *in vitro*.

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