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Purification and characterization of the RecF protein from *Bacillus subtilis* 168

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ABSTRACT

Genetic evidence suggests that the *Bacillus subtilis recF* gene product is involved in DNA repair and recombination. The RecF protein was overproduced and purified. NH_2-terminal protein sequence analysis of RecF was consistent with the deduced amino acid sequence of the *recF* gene. The RecF protein (predicted molecular mass 42.3 kDa) bound single- and double-stranded DNA in a filter binding and in a gel retarding assay. The RecF–ssDNA or –dsDNA complex formation proceeds in the absence of nucleotide cofactors. RecF–ssDNA interaction is markedly stimulated by divalent cations. The apparent equilibrium constants of the RecF–DNA complexes are ~110–130 nM for both ssDNA and dsDNA. The binding reaction shows no cooperativity. The RecF protein does not physically interact with the RecR protein. Under our experimental conditions an ATPase activity was not associated with the purified RecF protein or with the RecF and RecR proteins.

INTRODUCTION

In *Bacillus subtilis*, postreplication repair and transformational recombination occur primarily by activities classified within the α epistatic group (counterpart of *Escherichia coli* RecF pathway), whereas conjugational recombination in wild-type *E.coli* occurs mainly through the RecBCD pathway (counterpart of *B.subtilis* functions classified within the β epistatic group) (1–5). Genetic analysis in both *E.coli* and *B.subtilis* shows that recombination via these functions comprised within the RecF or α group is dependent, at least, on the RecA, RecF, RecR, RecL (genetic counterpart of *E.coli* RecO protein) and single-stranded DNA-binding (SSB) proteins (1–5). Furthermore, in both *E.coli* and *B.subtilis*, the *recF*, *recR* and *recO*(*recL*) strains have a similar phenotype and share indirect suppressors, therefore, it was assumed that the RecF, RecR and RecO(RecL) functions act at a similar stage (1–5).

The biochemical activities of the *E.coli* and *B.subtilis* products classified within the α epistatic group or required for the RecF pathway are currently being characterized. Unless otherwise stated, the indicated genes and products are of *B.subtilis* origin. The *E.coli* RecR protein (*Eco*RecR), which shows 44% identity to the RecR protein, binds neither single-stranded DNA (ssDNA) (6) nor double-stranded DNA (dsDNA) (7). The RecR protein, however, binds both ssDNA and dsDNA (8–10). In the presence of ATP and divalent cations (Mg^{2+} and Zn^{2+}), the affinity of the RecR protein for ssDNA is ~3-fold lower than for dsDNA (8,10). A RecR homomultimer is frequently located at the intersection of two duplex DNA strands in an interwound DNA molecule generating DNA loops of variable length (9).

The *Eco*RecO protein, which binds ssDNA and dsDNA, renatures homologous ssDNA, and forms D-loops (6,11). Direct interactions between *Eco*RecO and *Eco*RecR, *Eco*RecF and *Eco*SSB have been demonstrated biochemically and immunologically (6,12). The *Eco*RecO–*Eco*RecR complex promotes the binding of *Eco*RecA to ssDNA and facilitates homologous pairing by *Eco*RecA (6,11). At present, a *B.subtilis recL* gene (phenotypic counterpart of *EcorecO*) has not been identified.

The *Eco*RecF protein, which shows only a 26% identity to the RecF protein, is unable to complement a *B.subtilis* strain bearing a *recF* null allele (data not shown). The *Eco*RecF protein exhibits a weak ATPase activity and possesses ATP-independent ssDNA binding and ATP-dependent dsDNA binding activities (7,13,14). The addition of *Eco*RecF to an assay for *Eco*RecA-promoted DNA strand exchange blocks the reaction (11). To investigate the biochemical properties of the RecF protein we have overproduced and highly purified the protein. We show that the RecF protein binds to ssDNA or dsDNA with a similar apparent dissociation constant (K_{app}), in the order of 110–130 nM, in the absence of any nucleotide cofactor. The reaction did not show cooperativity.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli strains BL21(DE3) (15) and JM109 (16) were used. *Bacillus subtilis* strain YB886 and its isogenic derivatives BG129 (*recF*15) (17) and BG376 (*recF*36R) were used. Phage M13 mp18 (16) and plasmids pUC18 (16), pBT95 (17), pHP13 (18) and pLysS (15) have been previously described. Plasmid pCB72 was constructed as follows: the 2.0 kb *Hin*dIII–*Sal*I DNA fragment containing the *recF* gene from plasmid pBT95 was cloned into *Hin*dIII–*Sal*I-cleaved pHP13.

Enzymes and reagents

The *Eco*RecA protein was from Gibco-BRL and the *Eco*SSB protein was from Pharmacia. The protease inhibitor PMSF was

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from Boehringer Mannheim, and BIGCHAP (*N,N*-bis-3-D-gluconamidopropyl-cholamide) and IPTG (isopropylthiogalactoside) were from Calbiochem. S-Sepharose and Protein A–Sepharose were from Pharmacia.

The rNTPs, dNTP and ATP[γS] were purchased from Boehringer Mannheim. The nucleotides were dissolved as concentrated stock solutions at pH 7.0 and their concentration was determined spectrophotometrically.

[^{32}P]dNTPs, [^{32}P]NTPs and [^{35}S]methionine were from Amersham Corp. Ultrapure acrylamide was from Serva. The low molecular weight (LMW) protein marker was obtained from Gibco-BRL.

DNA manipulations

Covalently closed circular plasmid DNA was purified by using the sodium dodecyl sulphate (SDS) lysis method (19). End-labeling of ssDNA and dsDNA was performed as described by Sambrook *et al*. (19). Oligonucleotides were synthesized on an Applied Biosystem 380B DNA synthesizer and purified through non-denaturing polyacrylamide gel electrophoresis (ndPAGE) by standard procedures.

The concentration of DNA was determined using molar extinction coefficients of 8780 and 6500 M^{-1} cm^{-1} at 260 nm for ssDNA and dsDNA, and the amount of DNA is expressed as mol of nucleotides (ssDNA) or base pairs (dsDNA).

Synthetic oligonucleotides with a 50% (50 nt) or a 33% (60 nt) of dC + dG content in their ssDNA were synthesized. A 50 nt (5′-AGAGGATCCCCGGGTACCGAGCTCGAATTCCATTAG-TACCAGTATCGACA-3′) and a 60 nt (5′-CTCCTATTATGCTC-AACTTAAATGACCTACTCTATAAAGCTATAGTACTGCTA-TCTAATC-3′) long oligonucleotides were used.

The ssDNA was 5′-end-labeled with γ-^{32}P and the dsDNA was 3′-end-labeled with α-^{32}P as described by Sambrook *et al*. (19).

Protein manipulations

The RecR protein was purified as previously described (8). RecF was purified as follows: a culture (3 l) of *E.coli* BL21(DE3) strain containing pBT95 and pLysS was grown in L medium and induced as described by Alonso and Stiege (17). The cells were harvested by centrifugation at 4°C and mixed with a similar cell lysate containing RecF protein labeled with [^{35}S]methionine as previously described (17). The cell paste (10 g wet weight) was resuspended in 50 ml buffer A (50 mM Tris–HCl pH 7.0, 0.5 mM EDTA, 0.2 mM PMSF, 5% glycerol) containing 500 mM NaCl. The cells were lysed by sonication (15 × 15 s pulses of 100 W using an M.S.E. sonicator). The overexpressed RecF protein was readily sedimented by low speed centrifugation (Fig. 1, lanes 3 and 4). The pellet was washed in buffer A and resuspended in buffer B (50 mM Na_2HPO_4/NaH_2PO_4 pH 7.0, 0.5 mM EDTA, 0.2 mM PMSF, 10% glycerol) containing 50 mM NaCl and 2 M deionized urea. The pellet was collected and resuspended in 50 ml of buffer B containing 50 mM NaCl and 7 M urea. Diluted H_3PO_4 was added to the supernatant to bring the solution to pH 5.0. The supernatant (Fig. 1, lane 5) was loaded onto an SP–Sepharose column equilibrated with buffer C (50 mM Na_2HPO_4/NaH_2PO_4 pH 5.0, 0.5 mM EDTA, 0.2 mM PMSF, 10% glycerol) containing 50 mM NaCl and 7 M urea. The column was washed with buffer C containing 75 mM NaCl and 7 M urea and eluted by a step gradient from 75 to 250 mM NaCl, 7 M urea. The fractions corresponding to the radioactive material, which coincides with the pure RecF protein, were pooled (Fig. 1, lane 6). The pooled fractions were concentrated in a second SP–Sepharose as described above (Fig. 1, lane 7). The refolding conditions were chosen to minimize formation of aggregates. Urea was slowly removed by dialysing against equal volumes of buffer D [50 mM Tris–HCl pH 7.0, 1.5 M potassium glutamate (KGlu), 4% BIGCHAP, 5% glycerol]. Samples were stored at –20°C (Fig. 1, lane 8). The RecF protein concentration was determined by using the molar extinction coefficient of 29 300 $M^{-1}cm^{-1}$ at 280 nm and is expressed as mol of protein protomers.

Rabbit polyclonal antibodies against RecF and RecR proteins were obtained by the use of conventional techniques (19).

Filter binding assay

The formation of RecF–DNA complexes was measured by using alkali-treated filters (Millipore, type HAWP 0.45 μm) as described by Alonso *et al.* (8). The standard reaction (25 μl) was carried out in a solution of 4 ng of ^{32}P-labeled 60 nt ssDNA (480 nM) or 8 ng of ^{32}P-end-labeled pUC18 dsDNA (480 nM) and the indicated amount of the RecF protein in buffer E (50 mM Tris–HCl pH 7.0, 200 mM KGlu, 4 mM $ZnSO_4$, 0.16% BIGCHAP) and incubated for 15 min at 37°C. The binding reactions were performed in buffer E, unless stated otherwise.

Ice-cold buffer E (1 ml) was added to the reaction mixture to stop it. The reaction was then filtered trough KOH-treated filters. Filters were dried and the amount of radioactivity bound to the filter was determined by scintillation counting. The DNA retained on the filter was corrected for the retention of radiolabeled DNA in the absence of RecF protein. The specific activity of the labeled DNA was measured as TCA precipitable material. All reactions were performed in duplicate.

Quantitative equilibrium binding measurements were also performed by using the filter binding assay. Protein RecF–DNA complexes were formed at increasing concentrations of protein RecF to establish the protein–DNA equilibrium. The apparent equilibrium binding constant was determined by the method of Riggs *et al.* (20). Dissociation measurement was initiated by addition of a 50-fold molar excess of the unlabeled DNA. Aliquots were taken at the indicated times, chilled on ice and measured as indicated above.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as previously described (9,19), except that a low ionic strength buffer (7 mM Tris–HCl pH 7.9, 3 mM sodium acetate, 0.3 mM EDTA) was used (19). The standard reaction (25 μl) was carried out in a solution of 4 ng of a ^{32}P-labeled 50 nt ssDNA (480 nM) or 8 ng of ^{32}P-end-labeled 50 bp *Eco*RI–*Hin*dIII pUC18 dsDNA (480 nM) and increasing concentrations of RecF protein in buffer F (50 mM Tris–HCl pH 7.0, 4 mM $MgCl_2$, 4 mM $ZnSO_4$, 0.16% BIGCHAP) containing 50 mM NaCl, and incubated for 15 min at 37°C. Samples were transferred to ice and 3 μl of a solution containing 30% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol were added. The protein–DNA complexes formed were resolved on an 8% ndPAGE (80:1 acrylamide/bis), ran at 3 mA at 4°C and dried prior to autoradiography.

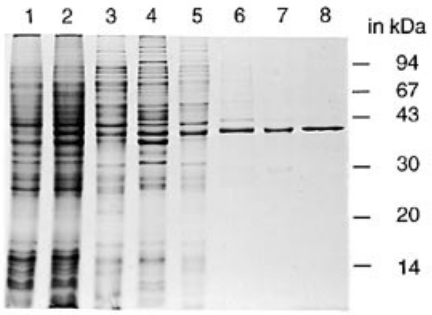

Figure 1. SDS–polyacrylamide gel electrophoresis of RecF protein purification. Coomassie blue-stained 12.5% SDS–PAGE. Lane 1, cell lysate (non-induced cells); lane 2, cell lysate (induced cells); lanes 3 and 4, supernatant and pellet of the lysis, respectively; lane 5, supernatant of 7 M urea; lane 6, elution from SP-Sepharose at 125 mM NaCl in buffer C containing 7 M urea; lane 7, elution from the concentrating SP-Sepharose column; lane 8, renatured RecF protein. The molecular mass standards (in kDa) are indicated.

Protein affinity chromatography

The protein–protein interactions were assayed by affinity chromatography. The RecF, RecR or BSA proteins (6 µM) were covalently cross-linked to the Affi-Gel-10 (1 ml) resin as recommended by the manufacturer (BioRad). The RecR, *Eco*RecA or *Eco*SSB protein (1 µM) was loaded onto an affinity column that has been equilibrated with binding buffer F containing 50 mM NaCl. Bound fractions were eluted with 5 vol of binding buffer containing 1 M NaCl and 5 vol of the same buffer containing 1% SDS. Fractions of 100 µl were collected and analyzed by SDS–PAGE.

Antibodies against RecF were coupled to a Protein A–Sepharose column as recommended by the supplier (Pharmacia). RecF (1 µM) and RecR (1 µM) proteins were incubated together or separated at 30°C for 15 min in binding buffer (50 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$, 1 mM $ZnSO_4$, 2 mM ATP) containing 100 mM NaCl and then loaded onto the AntiRecF–Protein A–Sepharose column (50 µl column) equilibrated with the same buffer. The columns were then washed with 5 column vol of binding buffer containing 100 mM NaCl, 1 M NaCl and 6 M urea. Fractions were analyzed by SDS–PAGE.

Other methods

The N-terminal amino acid sequence of the RecF protein was determined by Helga Gaenze (Max-Planck-Institut für molekulare Genetik, Berlin, Germany) with an automated Edman degradation in a pulsed-liquid phase sequencer (model 476, Applied Biosystems).

The ATPase activity of RecF was measured as described by Ayora *et al.* (21).

RESULTS

Purification of RecF protein

The pBT95-encoded RecF protein (17) was specifically labeled with [^{35}S]methionine with the help of an *in vivo* expression system (15). The RecF polypeptide, under the expression conditions described in Materials and Methods, accounts for ~2% of total protein mass (Fig. 1, lanes 1 and 2). The purification of the RecF polypeptide was monitored by following radioactively labeled RecF protein (42 kDa). A major fraction of the overproduced 42 kDa polypetide (predicted molecular mass 42 304) was insoluble. The RecF aggregates could, however, be dissolved in the presence of 7 M urea (Fig 1, lane 5). This property was exploited in our purification scheme to release unwanted proteins. Figure 1 shows the progressive purification of the 42 kDa RecF polypeptide. After the last purification step, the (42 kDa) RecF polypeptide is >98% pure, as judged by SDS–PAGE (Fig. 1, lane 8).

Two putative initiator codons were predicted for the RecF protein. The initiator codon could be either a UUG or an internal AUG codon, 40 codons downstream of the UUG (17 and references therein). The N-terminus of the purified protein was sequenced by automatic Edman degradation. The N-terminal sequence of the first 15 residues of the purified 42 kDa polypeptide was determined to be MYIQNLELTSYRNYD. The N-terminal amino acid sequence was identical to the sequence predicted from the nucleotide sequence of the *recF* gene starting with the UUG codon and confirmed that the 42 kDa purified protein was encoded by the *recF* gene (17).

We have verified that the *recF* gene used for overexpressing the RecF protein, from plasmid pBT95, encodes for a wild-type product by subcloning the DNA segment containing the *recF* gene into a *B.subtilis* replicon (generating plasmid pCB72) and confirming that pCB72-borne *recF* gene product fully restored the phenotypes of the *recF*15 strain (data not shown).

Characterization of RecF activities

The ability of RecF protein to act as an ATP-dependent or ATP-independent nuclease (dsDNA or ssDNAexo- and/or endonuclease), DNA helicase, and to bind to dsDNA or ssDNA were assayed (see below). Binding to ssDNA and dsDNA were the only activities observed. The ability of RecF protein to bind to DNA was assayed by filter binding. The RecF protein (180 nM) is able to bind a linear ^{32}P-labeled ssDNA (60 nt) (480 nM) (dG + dC content 33%) or linearized ^{32}P-labeled dsDNA (pUC18, 2686 bp) (480 nM) (dG + dC content 50%) to nitrocellulose membrane filter. The protein–DNA complex formation is not enhanced by the presence of 2 mM ATP (Table 1). The same results were observed when a 50 nt (dG + dC content 50%) ssDNA or a 166 bp (dG + dC content 32%) dsDNA was used in the binding reaction in the presence of the RecF protein (data not shown). Since no homology (>3 nt) was detected between the different substrates and the RecF protein binds to these substrates with a similar efficiency, it is likely that the RecF protein forms a complex with DNA in a sequence-independent manner.

As shown in Table 1, the binding of RecF protein (180 nM) to the 60 nt ssDNA or 2686 bp dsDNA is independent of nucleotide cofactors. When 1–2 mM GTP was added to the reaction mixture, RecF–ssDNA complex formation was about half as efficient as in the absence of the nucleotide cofactor (Table 1). Furthermore, the addition of 1–2 mM UTP or ATPγS has an inhibitory effect in both RecF–ssDNA and RecF–dsDNA complex formation.

The binding of the RecF protein to ssDNA (480 nM), and to a lesser extent to dsDNA (480 nM), is enhanced by the addition of Mg^{2+} and Zn^{2+}. When the RecF protein is present in limiting amounts (110 nM), the rate of RecF–ssDNA complex formation is increased by the addition of Mg^{2+} up to 4 mM and Zn^{2+} up to 8 mM. The same values are obtained for the RecF–dsDNA complex (Table 1).

Table 1. Reaction requirements for RecF–DNA binding activity

	Experimental condition	% DNA retained on filter	
		ssDNA	dsDNA
a	Complete	100	100
	– RecF	3.4	2.6
	+ 2 mM ATP	73	70
	+ 2 mM dATP	84	96
	+ 2 mM GTP	58	89
	+ 2 mM dGTP	82	85
	+ 2 mM UTP	62	63
	+ 2 mM dTTP	107	99
	+ 2 mM CTP	108	94
	+ 2 mM dCTP	88	95
	+ 2 mM ATPγS	45	55
b	– Zn^{2+}	100	100
	+ 1 mM Zn^{2+}	192	115
	+ 4 mM Zn^{2+}	200	155
	– Mg^{2+}	100	100
	+ 1 mM Mg^{2+}	132	105
	+ 4 mM Mg^{2+}	182	126

The binding reactions were performed at 37°C for 15 min in a 25 µl volume with 4 ng of ^{32}P-labeled 60 nt (480 nM) ssDNA or 8 ng of ^{32}P-labeled 2686 bp dsDNA (480 nM), and a given concentration of RecF protein: 180 nM (**a**) and 110 nM (**b**) in buffer F, with the omission or additions indicated. The DNA retained on filter and the quantitation of binding products were as described in Figure 2. Under the conditions used in the complete reaction, 66 ± 3% of the ssDNA and 70 ± 4% of the dsDNA [in (a)] and 40 ± 2% of the ssDNA and 45 ± 2% of the dsDNA [in (b)] were converted to protein–DNA complexes, and this amount of binding is indicated as 100%.

DNA-binding activity of the RecF protein

The rate of RecF–ssDNA and RecF–dsDNA complex formation was determined as a function of RecF protein concentration (Fig. 2). The K_{app}, which in this case is equal to half-maximal protein concentration, is 110 and 120 nM at pH 7.0 and 37°C for ssDNA and dsDNA, respectively. At the protein concentration midpoint about one RecF protomer binds to ~2–3 nt of ssDNA or 2 bp of dsDNA in a non-cooperative manner.

To determine the affinity of RecF protein for ssDNA and dsDNA further, binding reactions were performed in the presence of various DNA concentrations while the RecF protein level was kept constant (112 nM). As revealed in Figure 3, the data were plotted as the total amount of RecF divided by the concentration of bound substrate versus the inverse of the total substrate concentration (13). From the analysis of this data we confirm that the reaction did not show cooperativity (Fig. 3).

The slope of the line equals the K_d constant for RecF binding to the DNA substrate, provided that each RecF monomer has one functional DNA binding site (13). The K_d for RecF protein binding to the 60 nt ssDNA and 2686 bp dsDNA substrate, which were calculated from the slope of the line, were 108 and 128 nM, respectively. These values are very similar to those obtained by measuring the K_{app} (see above).

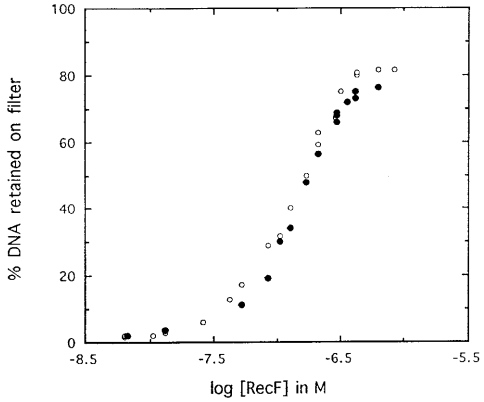

Figure 2. Binding of DNA to membrane filters in the presence of RecF protein. Four ng of ^{32}P 60 nt (480 nM) ssDNA (closed circles) or 8 ng of ^{32}P 2686 bp pUC18 (480 nM) DNA (open circles) in buffer E, was brought to 37°C. Increasing amounts of RecF protein were added (final volume 25 µl) and the incubation was continued for 15 min. The incubation mixture was diluted with 1 ml of ice-cold buffer E and filtered in a filter holder. The DNA retained on filter was corrected for the retention of [^{32}P]ssDNA and [^{32}P]dsDNA in the absence of RecF protein (2–3% of total input).

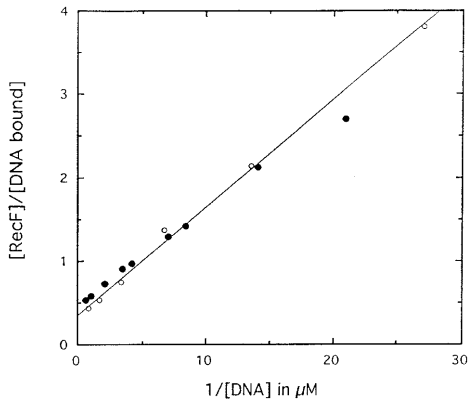

Figure 3. Determination of the relative affinity of RecF for ss- or dsDNA. Binding reactions containing 120 ng of RecF (112 nM) and increasing concentrations of ^{32}P 60 nt ssDNA (closed circles) or ^{32}P 2686 bp pUC18 DNA (open circles) in buffer E, was brought to 37°C (final volume 25 µl) and incubated for 15 min. The filtration and quantification of the binding products was as described in Figure 2.

Stability of the RecF–DNA complex

The stability of the RecF–DNA complex over time was determined. The RecF protein (110 nM) was incubated with a labeled 60 nt oligonucleotide (480 nM) or a 2686 bp pUC18 DNA fragment (480 nM) until equilibrium was reached (15 min). A 50-fold excess of specific non-labeled DNA was then added and samples analyzed at different times. As revealed in Figure 4, RecF–dsDNA complexes were stable, at least during the first 60 min. The decay rate of the RecF protein with ssDNA was biphasic. Two types of complexes were observed. In our standard reaction conditions at 37°C, the half-life of 30% of the RecF–ssDNA complexes was 35 ± 2 min, whereas the remaining 70% of the RecF–ssDNA complexes were stable, at least during

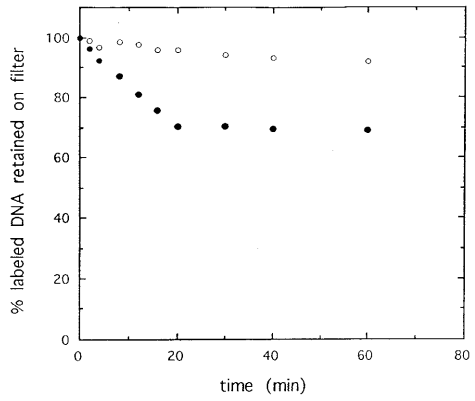

Figure 4. Stability of the RecF–DNA complex. Four ng of ^{32}P 60 nt (480 nM) ssDNA (closed circles) or 8 ng of of ^{32}P 2686 bp pUC18 (480 nM) DNA (open circles) was incubated with RecF (110 nM) in buffer E (final volume 25 µl) during 15 min at 37°C. After reaching the equilibrium, the reaction mixture was diluted 10-fold in the presence of 50-fold molar excess of specific unlabeled DNA and sampling began (Fig. 2). Under these reaction conditions, 40 ± 2% of the ssDNA and 45 ± 2% of the dsDNA was converted to protein–DNA complexes at time zero, and this amount of binding is indicated as 100%.

the time of our analysis (60 min). As suggested by Griffin and Kolodner (13) and Hedge *et al.* (22) for the *Eco*RecF protein–ssDNA complexes, two classes (type 1 and type 2) of RecF protein–ssDNA complexes are formed.

RecF–DNA complexes analysed using EMSAs

The binding of the RecF protein to DNA was further analyzed by means of EMSAs, that allow visualization of both the specificity of the complexes formed and the cooperative events. The RecF protein is unable to shift the mobility of a linear 60 or 50 nt ssDNA or 140 or 50 bp dsDNA when the reactions were electrophoresed in either Tris–borate (90 mM Tris–borate pH 8.0, 1 mM EDTA), Tris–glycine (50 mM Tris base, 190 mM glycine pH 8.0, 1 mM EDTA) or even at half of the strength (0.5×) of both buffers (data not shown). RecF–DNA complexes, however, could be detected when a low ionic strength buffer (7 mM Tris–HCl pH 7.9, 3 mM sodium acetate, 0.3 mM EDTA) was used.

As revealed in Figure 5A and B, when the 50 nt ssDNA (480 nM) or the 50 bp dsDNA substrate (480 nM) were incubated with various amounts of RecF protein prior to electrophoresis, two discrete species (indicated as types 1 and 2) with a retarded electrophoretic mobility were observed. Type 2 was preferentially formed at low protein concentrations, whereas at high protein concentration both types 2 and 1 were detected. Conversely, *Eco*RecF–DNA type 1 and 2 complexes were formed simultaneously and type 2 complexes were only obtained in the presence of ATPγS (22).

Stable RecF–ssDNA or RecF–dsDNA complexes were obtained at a similar input ratio to that reported above (Fig. 2). The formation of these complexes does not require any nucleotide cofactor and its presence does not alter their ratio (see lanes 7 and 9). Type 2 complexes, which migrate into the gel, might represent separated RecF–DNA complexes, whereas type 1 complexes are protein–DNA networks that remain in the well. The same type of complexes were observed when a 60 nt ssDNA and a 140 bp dsDNA was used (data not shown).

RecF substrate specificity

The RecF substrate specificity was analyzed by using the filter binding assay. The ability of non-labeled circular or linear DNA to act as competitor for the binding of ^{32}P-labeled linear ssDNA was tested. DNA binding reactions were performed in buffer E with a ^{32}P-labeled 60 nt ssDNA (480 nM), increasing concentrations of cold M13 phage DNA or a 60 nt ssDNA and the presence of saturating amounts of RecF (240 nM). Circular M13 ssDNA

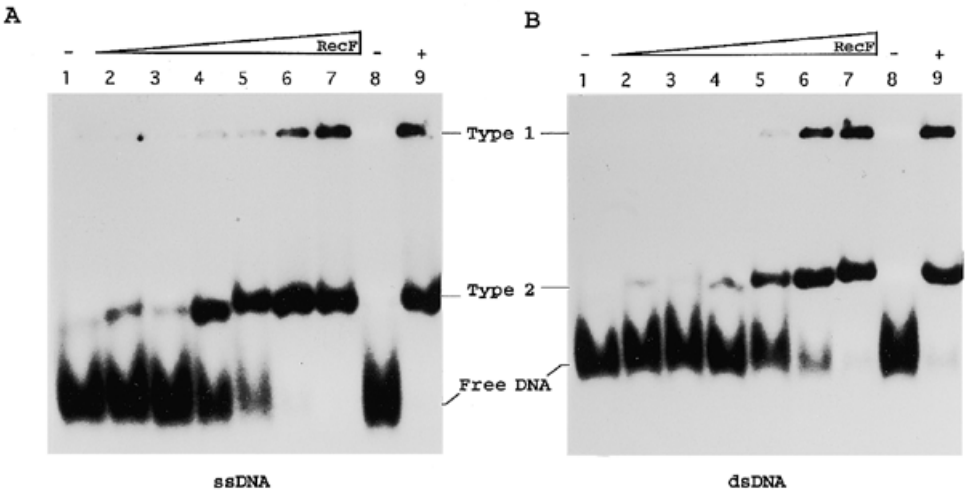

Figure 5. RecF–DNA complex formation. Different amounts of RecF (9, 27, 81, 243, 729 and 2187 nM, lanes 2–7, respectively) were incubated with 4 ng of ^{32}P-labeled 50 nt ssDNA (480 nM) (**A**) or 8 ng of ^{32}P-end-labeled 50 bp *Eco*RI–*Hin*dIII pUC18 dsDNA (480 nM) (**B**) for 15 min at 37°C in buffer F. Samples were transferred to ice and 3 µl of a solution containing 30% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol was added. The protein–DNA complexes formed were resolved on a 8% ndPAGE (80:1 acrylamide/bis), ran at 3 mA at 4°C and dried prior to autoradiography. In lane 9, 1.5 mM ATP, 2.1 µM RecF and 480 nM ssDNA (A) or 480 nM dsDNA (B) were present in the reaction mixture. In lanes 1 and 8 the RecF protein was omitted. The locations of protein–DNA complexes are indicated.

was half as efficient as the 60 nt long ssDNA in reaching 50% competition of the radiolabeled substrate.

When a filter binding assay was used to determine the specificity of dsDNA binding activity of RecF protein it was observed that non-labeled linear DNA competes for the binding of ^{32}P-labeled pUC18 linear DNA (480 nM) with a 1.5-fold higher efficiency than supercoiled pUC18 DNA (data not shown). Thus, RecF displays at best a low preference for linear ssDNA or dsDNA over circular ssDNA or supercoiled DNA.

The purified RecF protein does not seem to show an ATPase activity

The amino acid sequence alignment of 10 available RecF proteins (five of them from bacteria of Gram-negative origin) revealed a motif A (in the N-terminus) and a motif B (in the C-terminus) commonly associated with nucleotide triphosphate (NTP) binding and hydrolysis (23,24). The presence of the motif A or P-loop consensus sequence (residues 30-GXX$^{G}/_{A}$XGKT-37, where X can be any amino acid) and motif B (residues 312–327, an aspartate residue that participates in phosphate binding by binding a divalent cation) suggests that RecF could have NTPase activity (24,25).

To determine whether RecF protein has ATPase activity, we have measured ATP hydrolysis in the presence or absence of DNA. Under the experimental conditions in which RecF (up to 280 nM) binds to ssDNA (480 nM) or a dsDNA (480 nM) segment, a control protein (Mfd protein, 21) displays a modest ATPase activity (K_{cat} 1.9 min^{-1}, see 21) in the absence of ssDNA or dsDNA (data not shown). Under the experimental conditions used, RecF alone (up to 280 nM) or in combination with the RecR protein (up to 400 nM) is not able to hydrolyse ATP either in the presence or absence of ssDNA (M13 mp18) or dsDNA (pUC18) (data not shown). In both *E.coli* and *B.subtilis*, however, the change of lysine to arginine at position 36 (motif A, K36R) renders a *recF* allele impaired in DNA repair (26, our unpublished results).

The RecF protein does not significantly interact with the RecR, *Eco*RecA or *Eco*SSB proteins

To study a possible interaction between RecF and RecR, *Eco*RecA or *Eco*SSB, a protein affinity column was employed. The RecF (6 µM), RecR (6 µM) protein or bovine serum albumin (BSA) (6 µM), as non-specific control, were immobilized on the Affi10 matrix and then the RecR (1 µM), the *Eco*RecA (1 µM) or the *Eco*SSB (1 µM) proteins were loaded separately on the immobilized protein matrixes.

Neither *Eco*RecA nor *Eco*SSB binds to the RecF, RecR or BSA columns. A minor fraction (~25%) of the RecR was retained on the RecF affinity column and neither the presence of ATP (2 mM) or divalent cations [Zn^{2+} (1 mM) and Mg^{2+} (2 mM)] enhanced the amount of the retained fraction (data not shown). About 25% of the RecR protein was also retained on the BSA affinity column, hence raising some doubts about the specificity of binding. These data suggest that RecR binding to the RecF column is primarily non-specific. The RecF protein binds to an Affi10 column coupled with the BSA protein, hence the binding of RecF to RecR immobilized in a column was not tested (data not shown).

To analyze further the possible interaction between RecF and RecR, polyclonal antibodies raised against RecF were immobilized in a protein A–Sepharose column. The RecF (1 µM) and the RecR (1 µM) proteins were preincubated together at 37°C for 15 min under optimal conditions for protein binding to DNA, and then loaded onto the column. The RecF protein was retained in the column, whereas the RecR protein was present in the flow through volume. This result again suggests that there is no direct interaction between RecF and RecR proteins.

DISCUSSION

The 42 kDa RecF polypeptide (predicted molecular mass 42 304) was insoluble. The RecF protein was denatured, purified, and subsequently renatured. The N-terminal amino acid sequence of the purified polypeptide was consistent with a *recF* gene starting with the UUG codon.

The *Eco*RecF protein shows a weak ATP hydrolytic activity that is stimulated 2.5-fold by *Eco*RecR (7). Under our experimental conditions the RecF protein binds to ssDNA and dsDNA with a similar affinity than the *Eco*RecF protein (7,13), but under experimental conditions RecF does not seem to hydrolyze ATP. Furthermore, the presence or the absence of the RecR protein does not modify such a result (data not shown). However, a *B.subtilis* strain with a mutation in the putative phosphate binding loop (Walker's motif A, 23) renders a *recF* allele impaired in DNA repair (data not shown). The molecular role of the ATP binding domain in RecF is ill defined. An ATPase activity may become apparent upon addition of components not yet tested.

The K_{app} of RecF protein binding to ssDNA or dsDNA was of the order of 110–130 nM, and the reaction did not show cooperativity. This could be, however, an underestimation because the proportion of misfolded protein in our preparation is unknown. RecF–ssDNA complex formation is enhanced by the presence of divalent cations (Zn^{2+} or Mg^{2+}). The level of RecF protein required to convert 50% of the ssDNA to protein–DNA complexes was one RecF protomer for every 2 nt of ssDNA. This is consistent with some of the data obtained with the purified *Eco*RecF for ssDNA (13,28). The K_d of the *Eco*RecF for ssDNA is ~130 nM and the reaction did not show cooperativity (13). Since both proteins show a similar K_{app} it is likely that the denaturation and subsequent renaturation of the RecF protein has not markedly affected its DNA binding activity.

The amount of *Eco*RecF protein required to convert 50% of the ssDNA substrate to protein–DNA complexes was one *Eco*RecF monomer for every 4 (13) 15 (27) or 75 nt (22) of ssDNA. Unlike the RecF–ssDNA complex, the *Eco*RecF–ssDNA complex formation is insensitive to the addition of at least Mg^{2+} (13) and shows a marked preference for ssDNA ends (up to 85-fold) (13).

Both *Eco*RecF and RecF proteins bind specifically to ssDNA, their affinity (K_d 110–130 nM) is one order of magnitude lower than that of SSB of bacterial or phage (gene *32*) origin for ssDNA (28). However, the affinity *Eco*RecF and RecF proteins for ssDNA is one order of magnitude higher than that of the *Eco*RecA protein (29). Unlike the SSB protein, but similar to RecA, both RecF proteins form protein–DNA networks (22,28,29, Fig. 5).

The *Eco*RecF and RecF proteins form two classes of RecF–ssDNA complexes (13, this work). About 30% of the RecF–ssDNA complexes have a half-life of ~35 min, whereas the remaining 70% are stable (>60 min). In the *E.coli* case, the half-life of the unstable *Eco*RecF–ssDNA complex (~30% of the initial complex) is ~1 min and the half-life of the remaining 70% is ~60 min. Both complexes could by separated by EMSA.

In the *Eco*RecF case, in the presence of ATPγS, type 1 complexes are so large that they remain in the well, whereas type 2 complexes migrate into the gel. Both type of complexes are formed simultaneously. Furthermore, the type 2 complexes are very unstable and are rapidly converted into type 1 complexes (22). In the *B.subtilis* case, types 1 and 2 RecF–ssDNA complexes are formed in the absence of a nucleotide cofactor and type 2 complexes are formed first. Similar results were observed when the DNA substrate was duplex DNA. It is likely, therefore, that the stable protein–DNA complexes observed with both *Eco*RecF and RecF proteins could correspond to type 1 complexes.

The level of RecF protein required to convert 50% of the dsDNA substrate into protein–DNA complexes was one RecF protomer for every 2 bp. In stoichiometric amounts, and in the presence of ATPγS, there is one *Eco*RecF protomer bound to every 4–6 bp (7) or 75 bp (22).

Both RecF and *Eco*RecF proteins show some similarities as well as some differences. As in the case of the RecF protein, the *Eco*RecF DNA-binding reaction did not show any cooperativity (7) or very little (14). The major difference is that the *Eco*RecF binding to dsDNA requires a nucleotide cofactor. The *Eco*RecF binding to dsDNA in the presence of ATP is weak, but such a binding is markedly enhanced when ATP is replaced by ATPγS (22) or by the presence of ATP and the *Eco*RecR protein (7).

Recently it has been shown that (i) both *Eco*RecO and *Eco*RecR proteins promote the binding of *Eco*RecA to ssDNA in the presence of *Eco*SSB and facilitate homologous pairing by *Eco*RecA (6), (ii) the *Eco*RecF protein inhibits most of the activities of the *Eco*RecA protein *in vitro*, including ssDNA binding, joint molecule formation (11,28) and *Eco*LexA cleavage (7), (iii) the *Eco*RecO interacts with *Eco*RecR, *Eco*SSB (6) and *Eco*RecF (12), and (iv) *Eco*RecF interacts, in the presence of ATP and dsDNA, indirectly with *Eco*RecR (7). In this study we show that RecF does not interact with the RecR protein. Under our experimental conditions we cannot address any indirect interaction, in the presence of DNA and/or nucleotide cofactors, because both proteins bind DNA (8, this report). Furthermore, the RecF protein does not interact with the heterologous *Eco*RecA or *Eco*SSB proteins. This is consistent with the fact that the *Eco*RecF does not interact with the *Eco*RecA, *Eco*SSB or *Eco*RecR proteins (6,12). In the *B.subtilis* case it has been shown that (i) in *recF*, *recR* or *recL* mutants SOS induction is reduced and delayed (30), (ii) a high expression of a *B.subtilis* phage-encoded SSB protein, which competes for ssDNA with the host SSB protein, partially supresses the *recF*, *recR* and *recL* defect (31) and (iii) the RecF protein *in vitro* binds ssDNA and dsDNA with similar efficiency and in the absence of a nucleotide cofactor. Based on published results it can be inferred that the RecF could modulate the interaction of the SSB and/or RecA protein with ssDNA (6,7,13) or alternatively, could promote SOS induction by a direct protein–protein interaction (12). The possible role of the RecF protein in RecA-promoted DNA strand exchange remains to be determined.

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