

Binding of the recA Protein of *Escherichia coli* to Single- and Double-Stranded DNA*

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The recA protein of *Escherichia coli* binds both single- (SS) and double-stranded (DS) DNA; however, the optimal conditions differ for interaction with these DNA substrates. Binding of DS DNA by recA protein is pH dependent (optimum near pH 6.2) and requires a nucleoside triphosphate (ATP) and divalent cation. Substitution of the 5'-O-3'-thiotriphosphate (ATP(γS)) for ATP leads to formation of stable complexes of recA protein and DNA that dissociate very slowly. Formation of these complexes is extremely sensitive to ionic strength and pH. However, once formed, the complexes resist changes in pH and high salt concentrations. SS DNA binds to recA protein in the absence of a nucleoside triphosphate, but recA protein-SS DNA complexes are stabilized by ATP(γS). At high recA protein/DNA ratios (1 recA protein monomer/30 nucleotides), these complexes sediment in sucrose gradients as large protein-DNA aggregates. Although ATP(γS) blocks dissociation of recA protein from DNA, ATP stimulates the release of recA protein from SS DNA. Hydrolysis of the ATP is not required for dissociation since it is also enhanced by ADP and certain nucleoside triphosphates that are not hydrolyzed by recA protein.

recA protein binds with different affinities to ribohomopolymers and deoxyhomopolymers. It preferentially binds polydeoxythymidylate and polydeoxycytidylate but does not bind short oligonucleotides, indicating that there is a minimum size requirement for the binding step.

The recA protein exists as a heterogeneous aggregate at pH 7.5 and at low ionic strength. At pH 6.2 in the presence of Mg²⁺, the protein sediments homogeneously as a dimer. At pH 6.2, ATP or ATP(γS) promotes an oligomerization of the recA protein which can be observed as filamentous structures by electron microscopy. Oligomerization is not induced by UTP, a nucleoside triphosphate that is efficiently hydrolyzed by the recA protein, but fails to stimulate efficiently recA protein-promoted annealing and assimilation of single-stranded DNA.

The recA protein of *Escherichia coli* is one of several enzymes that catalyze the hydrolysis of nucleoside triphos-

phates in the presence of DNA (1-3). Recently, it has been shown that the recA protein couples ATP hydrolysis to the pairing of DNA molecules between complementary single-stranded (SS)¹ DNA chains (annealing) (2) as well as between SS DNA and homologous DS molecules (strand assimilation) (4, 5). The products of the annealing reaction are duplex regions several thousand base pairs in length (2),² whereas the products of strand assimilation are joint molecules or D-loops in which the SS DNA is base paired to its complementary strand in the duplex (4, 5). Assimilation, which requires the unwinding of a region of the DNA duplex in addition to pairing between complementary sequences, is implicated in the initiation of homologous recombination (6, 7). This argument is strengthened by the demonstration that recA protein purified from a conditionally recombination-deficient recA mutant strain fails to catalyze the DNA-pairing reactions under nonpermissive conditions *in vitro* (2).² Thus, these activities very likely reflect the function of recA protein in homologous recombination and DNA repair processes *in vivo*.

Our efforts to elucidate the mechanism of the recA protein-catalyzed annealing and assimilation reactions have demonstrated that the interaction of recA protein with DNA is markedly influenced by nucleoside triphosphates and their analogs (5, 8, 9). Moreover, analysis of the DNA-dependent UTP and ATP hydrolytic activities of recA protein indicates that either SS or DS DNA can serve as a cofactor for hydrolysis, although the optimal conditions for stimulation of nucleoside triphosphate activity by these DNA's are strikingly different (10).

We have reported that recA protein binds DS DNA in the presence of ATP or ATP(γS), and that these protein-DNA complexes can be detected by their binding to nitrocellulose filters (5). Although complexes formed in the presence of ATP eventually dissociate (and ATP is hydrolyzed), complexes formed with ATP(γS) are stable to prolonged incubation at high ionic strength. Furthermore, the DS DNA in these complexes is partially unwound (5, 8). Here we extend our analysis of the binding of recA protein to DNA by means of filter binding, agarose gel electrophoresis, sucrose gradient sedimentation analysis, and electron microscopy to characterize the binding reactions and their products. Other nucleoside triphosphates have been examined for their effects on recA protein-DNA interactions, and binding competition experiments have been performed to investigate binding of recA protein to various homopolymers.

¹ The abbreviations used are: SS, single stranded; DS, double stranded; BSA, bovine serum albumin; ATP(γS), adenosine 5'-O-3'-thiotriphosphate; UTP(γS), uridine 5'-O-3'-thiotriphosphate; GTP(γS), guanosine 5'-O-3'-thiotriphosphate; ADP[NH]P, adenylyl imidodiphosphate; ADP[CH₂]P, adenylyl-5'-yl methylenediphosphate.

² K. McEntee, G. M. Weinstock, and I. R. Lehman, unpublished results.

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EXPERIMENTAL PROCEDURES

Materials

Nitrocellulose filters (45 nm pore size) were obtained from Millipore; nucleoside triphosphates from P-L Biochemicals and Sigma; maleic acid from Sigma; calf thymus DNA from Calbiochem; ^3H -labeled poly(dT)₈₂₀ was obtained from Miles; agarose from Bio-Rad; ATP(γS) and GTP(γS) were from Boehringer Mannheim; homopolymers were obtained from Miles, Sigma, and P-L Biochemicals; and UTP(γS) was a generous gift of Dr. Fritz Eckstein, Max Planck Institute, Göttingen. Sodium borohydride was purchased from Amersham.

Bacteriophage P22 DNA was prepared as previously described (2). ^3H -labeled M13 DNA was generously provided by Joan Kobori of this department. ^3H -labeled SS P22 DNA was prepared by heating ^3H -labeled P22 DNA at 100 °C for 3 min and cooling quickly on ice. The average length of the single strands produced was approximately 3000 nucleotides. All DNA concentrations are expressed as concentrations of nucleotides. DNA concentrations were calculated using an A_{260} of 1 being equivalent to 50 $\mu\text{g}/\text{ml}$ of DS DNA and an A_{260} of 1 equivalent to 36 $\mu\text{g}/\text{ml}$ of SS DNA. Homopolymer concentrations were calculated using published extinction coefficients (11).

recA protein (Fraction II) was purified as previously described (2). This material was greater than 90% pure as judged by electrophoresis in a polyacrylamide gel. More highly purified *recA* protein (12) behaved identically in filter-binding studies. ^3H -labeled *recA* protein was prepared by sodium borohydride reduction (13). Approximately 70% of the ATPase activity remained following reductive methylation.

Methods

Filter-binding Assay—The filter-binding assay used for measurement of *recA* protein-DNA complexes has been described (5). Untreated filters or alkali-treated Millipore filters were soaked in buffer (50 mM Tris-HCl, pH 7.5, and 1 mM EDTA) for at least 30 min prior to use. Reaction mixtures (200 μl unless otherwise stated) contained 20 mM buffer ((Tris-HCl, pH 7.5 or 8.1, or sodium maleate, pH 6.2), 10 mM MgCl_2 , 1 mM dithiothreitol, and 0.5 mM EDTA), DNA, ATP(γS), and *recA* protein as indicated. Reactions in Tris-HCl buffer also contained 20 mM NaCl. Incubations were performed at the indicated temperature and time in plastic (Eppendorf) tubes to avoid adsorption of *recA* protein. Samples were removed and applied directly to the filter under suction and, as soon as the liquid passed into the filter, the sample was washed with 2 ml of high salt wash buffer (20 mM Tris-HCl, pH 7.5, 1 M NaCl, 10 mM MgCl_2 , and 0.5 mM EDTA) followed by washing with 2 ml of low salt wash buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl_2 , and 0.5 mM EDTA). In the experiments using only a low salt wash, filters were washed with 4 ml of low salt wash buffer, and the high salt wash was eliminated. Filters were dried and assayed for radioactivity. Untreated Millipore filters were routinely used for measurements of *recA* protein-DS DNA complexes, although comparable results were obtained using the alkali-treated filters. *recA* protein-SS DNA complexes were measured using the alkali-treated filters. In the absence of *recA* protein, less than 0.5% of the labeled SS DNA was retained even in the presence of 1 M NaCl. Untreated and alkali-treated Millipore filters have a high capacity for *recA* protein and *recA* protein-DNA complexes. Thus, the largest amount of *recA* protein and DNA used in these experiments failed to saturate the filters under the conditions described.

Dissociation of *recA* Protein-SS DNA Complexes—*recA* protein (18 ng) was incubated with 1.23 μg of ^3H -labeled M13 DNA in the absence of nucleoside triphosphates. After incubation at 25 °C for 20 min, excess unlabeled calf thymus SS DNA (25 μg) was added together with the indicated nucleoside triphosphate (250 μM). Samples were taken at the indicated times, filtered through alkali-treated Millipore filters, washed with low salt buffer, dried, and radioactivity measured.

Sucrose Gradient Sedimentation—Sedimentation analysis of *recA* protein-SS DNA complexes was performed using a linear 5–30% sucrose gradient containing 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 10 mM MgCl_2 , 1 mM EDTA, and 1 mM dithiothreitol. Centrifugation was at 4 °C for 70 min at 30,000 rpm or for 120 min at 46,000 rpm in a SW50.1 Ti rotor. Gradients (4.8 ml) were layered over a 150- μl shelf of 60% (w/w) CsCl in 30% sucrose. Fractions (5 drops) were collected from the bottom of the tube and radioactivity determined. Samples (100 μl) to be applied to the gradients contained the indicated amount of *recA* protein, 46.2 μM ^3H -labeled SS M13 DNA, 20 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 1 mM dithiothreitol, 0.5 mM EDTA, and either 100 μM ATP(γS) or UTP(γS). Incubation was at 37 °C for 15 min.

Agarose Gel Electrophoresis—Electrophoresis in 0.7% agarose gels was performed at room temperature as described (2). Reactions were stopped by the addition of loading buffer (15% glycerol, 0.1% bromophenol blue, and 10 mM EDTA) and a 50- μl sample was placed in each well of the gel for analysis. Gels were stained with ethidium bromide (0.5 $\mu\text{g}/\text{ml}$) and photographed under UV light.

Electron Microscopy—*recA* protein-DS DNA complexes were visualized by the method of Griffith (14) using tungsten shadowing. For electron microscopy of *recA* protein in the absence of DNA, a 15- μl drop of a 5 μM *recA* protein solution was applied to a carbon and Formvar-coated grid. After 1 min, excess fluid was removed with filter paper and the grid was stained for 30 s with 15 μl of a 2% uranyl acetate solution. Samples were visualized in a Philips 300 electron microscope at 60 kv.

RESULTS

Effect of Nucleoside Triphosphates on the Structure of *recA* Protein—Hydrolysis of both ATP and UTP is catalyzed by the *recA* protein, but the reactions display different characteristics (1–3, 10). As shown in Fig. 1, ATP and UTP have different effects on the structure of *recA* protein at pH 6.2. ATP caused a significant increase in the sedimentation rate of *recA* protein. With ATP(γS), the increase was somewhat greater. However, in both cases, the *recA* protein sedimented heterogeneously and a unique oligomeric form was not observed. In contrast, UTP had little effect upon the sedimentation of *recA* protein under the same conditions. At pH 7.5, *recA* protein sediments as a heterogeneous mixture of oligomers (1).²

When high concentrations (5 μM) of *recA* protein were incubated at pH 6.2 with ATP(γS) and examined with the electron microscope, long filamentous structures were readily observed (Fig. 2). Such structures were not observed when ATP(γS) was omitted. The filaments were approximately 0.018 μ in width and of variable lengths which could exceed 0.4 μ . These results indicate that at pH 6.2, the *recA* protein dimers can polymerize to form filaments in the presence of ATP or ATP(γS).

pH-dependent Formation of *recA* Protein-DS DNA Complexes—*recA* protein catalyzes the hydrolysis of ATP in the presence of DS DNA. Unlike SS DNA-stimulated ATP hydrolysis, which occurs over a wide range of pH values, stimulation by DS DNA shows an optimum near pH 6.2 (10). Binding of DS DNA by *recA* protein in the presence of ATP(γS) was also very sensitive to pH. Between pH 7.8 and 6.2, the rate of binding to duplex DNA increased by approximately 50-fold (Fig. 3). A nucleoside triphosphate is required

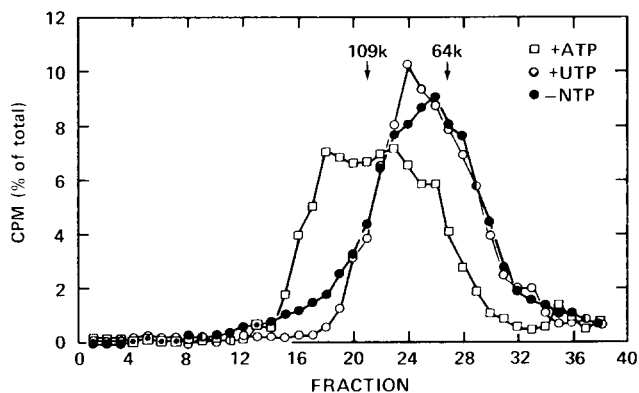


FIG. 1. Effect of ATP and UTP on the sedimentation of *recA* protein at pH 6.2. One-hundred μl of ^3H -labeled *recA* protein (16 μM) in 20 mM sodium maleate (pH 6.2), 1 mM dithiothreitol, 10 mM MgCl_2 , and 0.4 mg/ml of BSA was applied to a 10–30% sucrose gradient in the same buffer and sedimented in an SW50.1 rotor at 4 °C for 15 h at 45,000 rpm. ATP (1 mM) or UTP (2 mM), when present, were included in both the sample and the gradient.

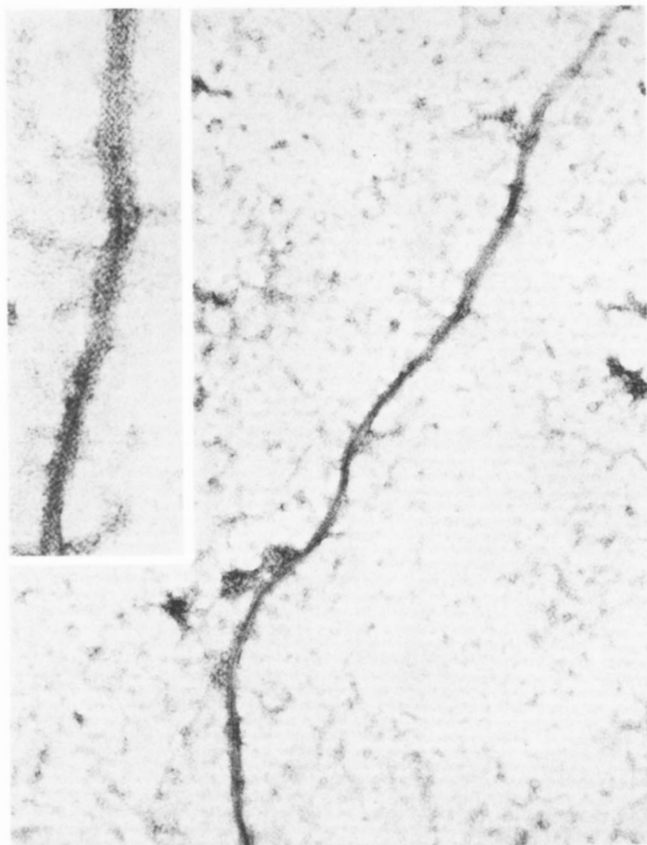


FIG. 2. Filamentous aggregate of *recA* protein. *recA* protein (5 μ M) was incubated in 20 mM sodium maleate (pH 6.2), 10 mM MgCl_2 , 1 mM dithiothreitol, and 400 μ M $\text{ATP}(\gamma\text{S})$ for 10 min at 30 $^\circ\text{C}$ and then mounted for electron microscopy as described under "Experimental Procedures."

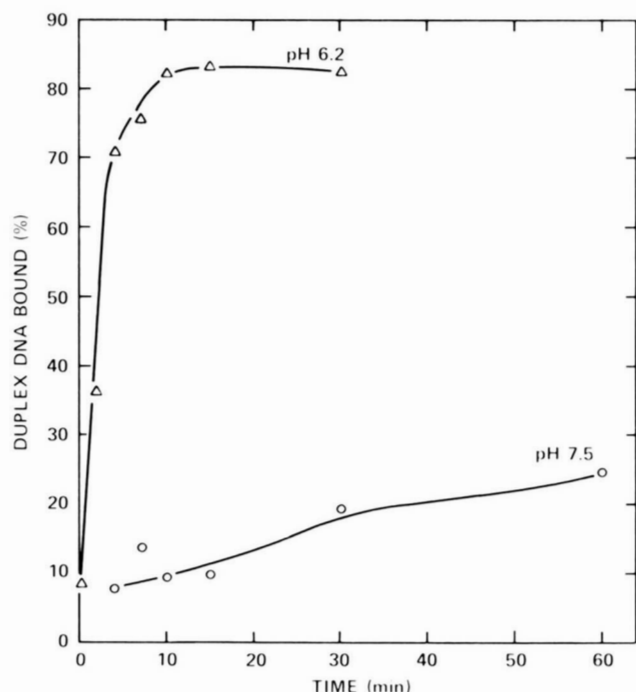


FIG. 3. Kinetics of *recA* protein-DS DNA complex formation. Reactions containing 0.43 μ M *recA* protein, 10.5 μ M ^3H -labeled DS P22 DNA (5.7×10^7 cpm/ μ mol), and 250 μ M $\text{ATP}(\gamma\text{S})$ in 20 mM maleate buffer (pH 6.2) or Tris-HCl buffer (pH 7.5) were incubated at 30 $^\circ\text{C}$. At the indicated times, samples (25 μ l) were taken for measurement of complexes by nitrocellulose filter binding as described under "Experimental Procedures."

for formation of these complexes at both pH values (5). When ATP was substituted for $\text{ATP}(\gamma\text{S})$, qualitatively similar results were obtained; binding was considerably faster at pH 6.2 than at pH 7.8 (data not shown). However, at the lower pH, ATP is rapidly hydrolyzed by *recA* protein, and the complexes

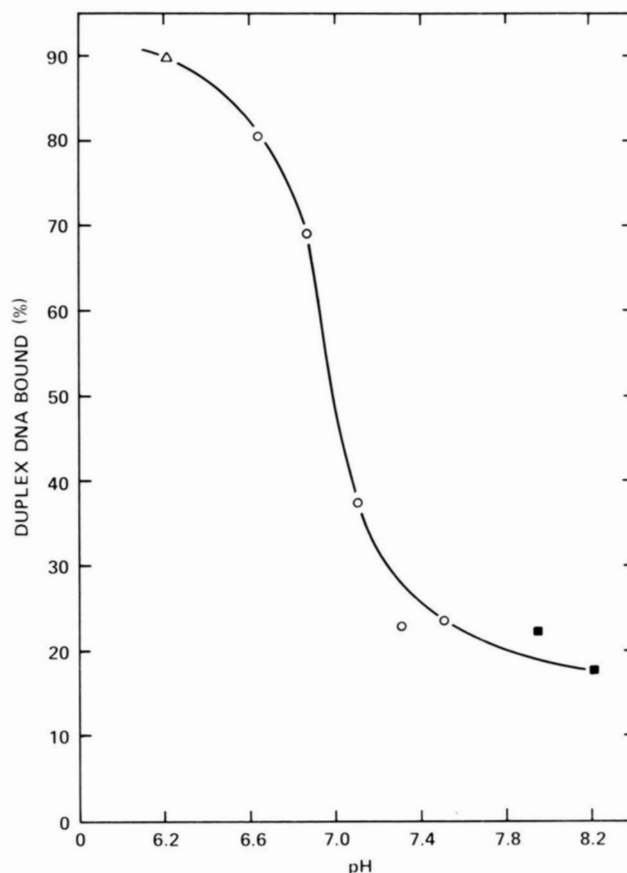


FIG. 4. pH dependence of *recA* protein-DS DNA complex formation. Reactions contained 0.43 μ M *recA* protein, 10.5 μ M ^3H -labeled DS P22 DNA, 250 μ M $\text{ATP}(\gamma\text{S})$, and 20 mM buffers at the indicated pH values. Incubations were performed at 30 $^\circ\text{C}$ for 30 min and complexes were determined by nitrocellulose filter binding as described under "Experimental Procedures." Δ — Δ , Na maleate; \circ — \circ , KPO_4 ; \blacksquare — \blacksquare , Tris-HCl.

TABLE I

Requirements for formation of recA protein-DS DNA complexes

Complete reactions (200 μ l) contained 0.35 μ M *recA* protein, 8.4 μ M ^3H -labeled DS P22 DNA, and the indicated nucleotide or analog in 20 mM Na maleate buffer (pH 6.2). Incubations were at 30 $^\circ\text{C}$ for 10 min and the amount of *recA* protein-DS DNA complex was determined by nitrocellulose filter binding as described under "Experimental Procedures."

	Complexes retained %
Complete	100
— <i>recA</i> protein	<3
— $\text{ATP}(\gamma\text{S})$	7.3
— $\text{ATP}(\gamma\text{S})$ + ATP (1 mM)	30.7
— $\text{ATP}(\gamma\text{S})$ + ADP[NH]P (0.5 mM)	6.8
— $\text{ATP}(\gamma\text{S})$ + ADP[CH ₂]P (0.5 mM)	7.3
— $\text{ATP}(\gamma\text{S})$ + CTP (1 mM)	7.3
— $\text{ATP}(\gamma\text{S})$ + dTTP (1 mM)	4.0
— $\text{ATP}(\gamma\text{S})$ + UTP (1 mM)	11.2
— $\text{ATP}(\gamma\text{S})$ + GTP (1 mM)	10.5
— $\text{ATP}(\gamma\text{S})$ + UTP(γS) (100 μ M)	75.0
— $\text{ATP}(\gamma\text{S})$ + GTP(γS) (100 μ M)	7.1
— Mg^{2+}	7.3

dissociate (5).² The binding of *recA* protein to DS DNA at pH 7.8 is stimulated by SS DNA and other polynucleotides in the presence of either ATP or ATP(γ S) (15).²

The rate and extent of complex formation decreased sharply as the pH was increased (Fig. 4). This finding is in agreement with results obtained for the DS DNA-dependent ATPase activity of *recA* protein which is also unusually sensitive to pH (10). Below pH 6.0, complexes of *recA* protein and DS DNA were formed; however, binding was independent of ATP(γ S). Because the ATPase associated with *recA* protein is inactive below pH 5.5 (10), DNA binding at this pH may represent another type of interaction between *recA* protein and DNA. We have made no attempt to study this protein-DNA association further.

Although the lower pH significantly stimulates binding of *recA* protein to DS DNA, *recA* protein remained bound to DS DNA (in the presence of ATP(γ S)) after shifting to pH 8.0. Thus, *recA* protein-DS DNA complexes were not dissociated by incubation at 37 °C for 60 min at pH 8.0 (data not shown). Although low pH is required only for the formation of complexes, the stimulation of ATP hydrolysis by DS DNA requires the low pH condition continuously since shifting to high pH during ATP hydrolysis inhibits the reaction immediately (10).

Requirements for *recA* Protein-DS DNA Complex Formation—Several nucleoside triphosphates and nucleoside triphosphate analogs were tested for their ability to promote formation of complexes of *recA* protein with DS DNA. At pH 6.2, ATP(γ S), and UTP (γ S) stimulated complex formation to

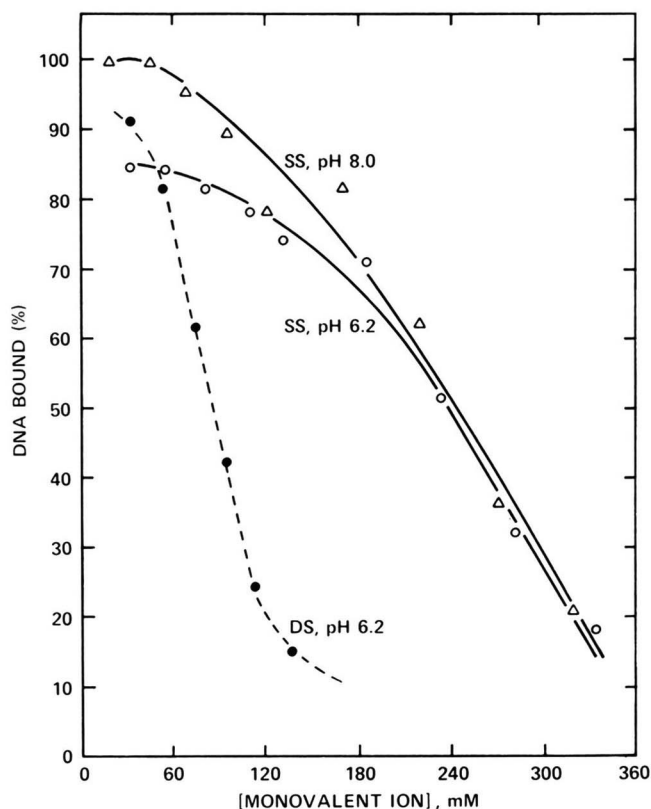


FIG. 5. Salt sensitivity of *recA* protein-DNA complex formation. Reactions contained either 0.22 μ M *recA* protein and 4.6 μ M ³H-labeled SS M13 DNA or 0.43 μ M *recA* protein and 10.5 μ M ³H-labeled DS P22 DNA in maleate buffer (pH 6.2) or Tris-HCl buffer (pH 8.0), 150 μ M ATP(γ S), and KCl as indicated. Incubations were performed for 15 min at 30 °C and *recA* protein-DNA complexes were measured using alkali-treated nitrocellulose filters as described under "Experimental Procedures."

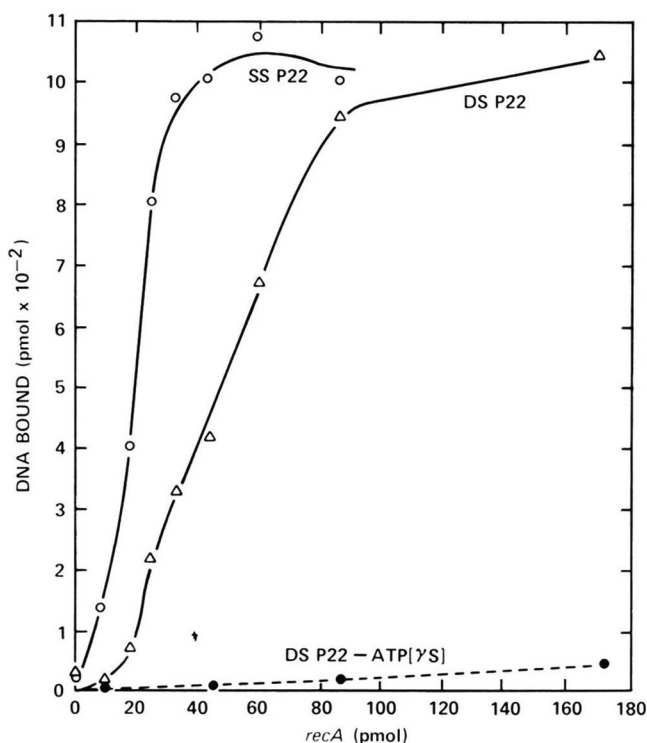


FIG. 6. Dependence on enzyme concentration of complex formation with SS and DS DNA's. Reactions contained the indicated amount of *recA* protein, 150 μ M ATP(γ S), and either 10.5 μ M ³H-labeled DS P22 DNA, 20 mM maleate buffer (pH 6.2), or 10.5 μ M ³H-labeled SS P22 DNA, and 20 mM Tris-HCl buffer (pH 8.1). *recA* protein-DNA complexes were measured after 30-min incubation at 30 °C with alkali-treated nitrocellulose filters as described under "Experimental Procedures."

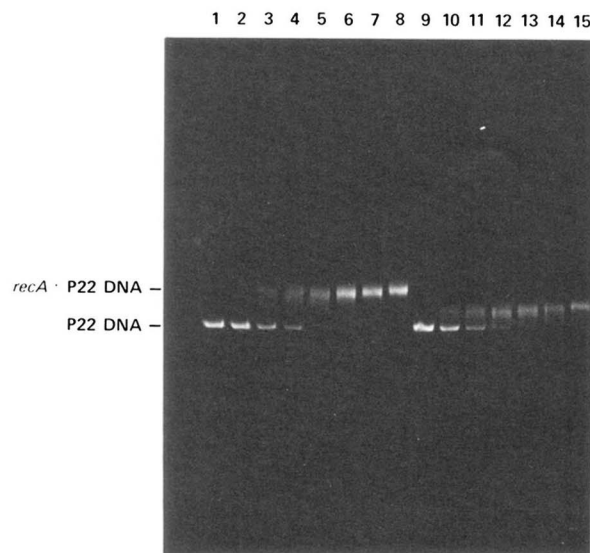


FIG. 7. Electrophoresis of *recA* protein-DS DNA complexes. Reactions (50 μ l) contained 20.1 μ M DS P22 DNA, 200 μ M ATP(γ S) (samples 1-8) or 300 μ M UTP(γ S) (samples 9-15), and the indicated amount of *recA* protein in 20 mM maleate buffer (pH 6.2) and were incubated at 30 °C for 30 min. Agarose gel electrophoresis was performed as described under "Experimental Procedures". 1, -*recA* protein; 2, 0.17 μ M; 3, 0.33 μ M; 4, 0.50 μ M; 5, 0.83 μ M; 6, 1.2 μ M; 7, 1.7 μ M; 8, 3.3 μ M; 9, 0.17 μ M; 10, 0.33 μ M; 11, 0.50 μ M; 12, 0.83 μ M; 13, 1.2 μ M; 14, 1.7 μ M; 15, 3.3 μ M.

slightly different extents (Table I). The relatively low level of complex formed with ATP may be due to hydrolysis of this nucleoside triphosphate and production of ADP, an inhibitor of DS DNA binding (data not shown). Other nucleoside

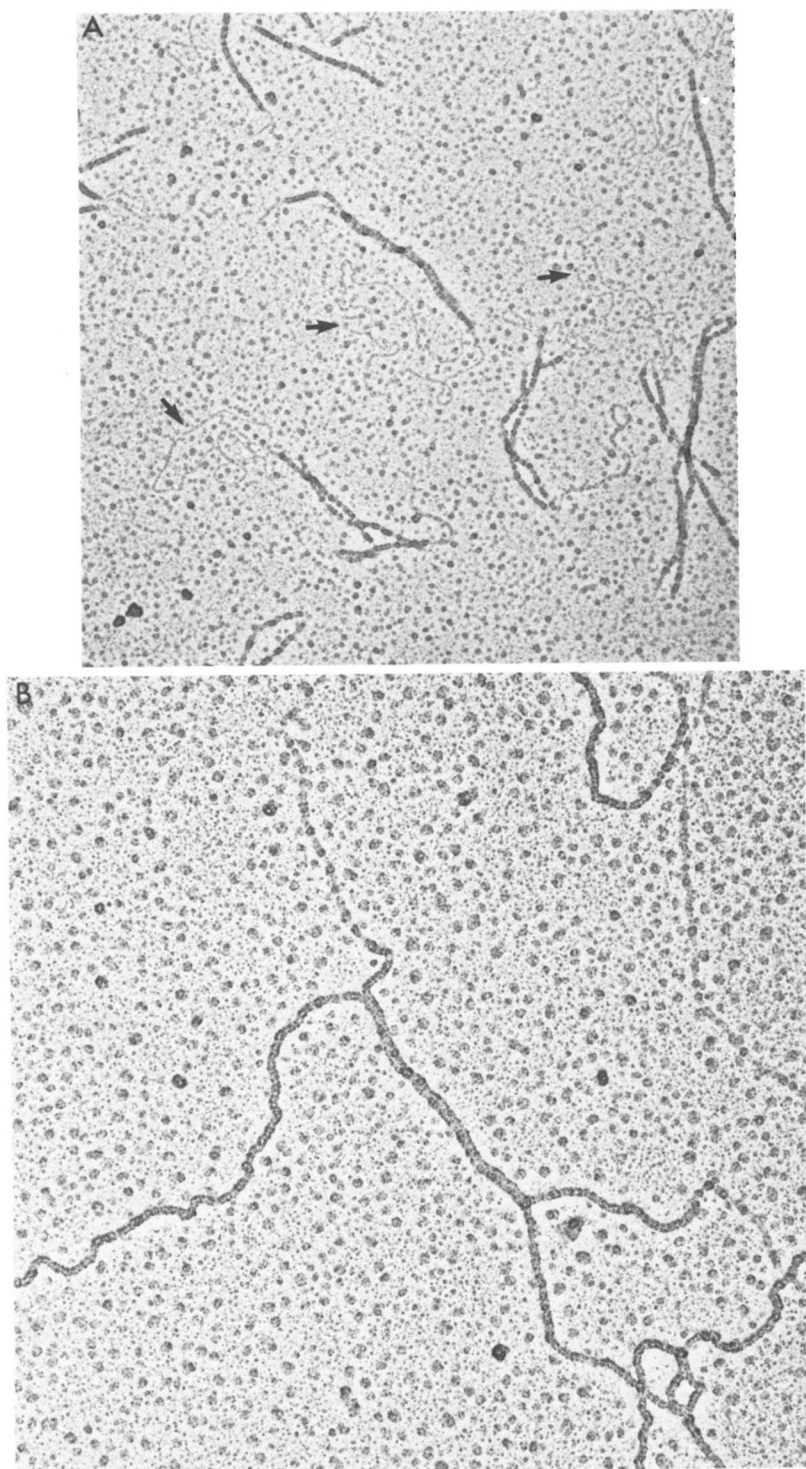


FIG. 8. Electron microscopy of *recA* protein-DS DNA complexes. Reactions (100 μ l) contained 3.5 μ M *recA* protein, 330 μ M ATP(γ S), 20 mM maleate buffer (pH 6.2), and either 25 μ M relaxed PM2 DNA (A) or 12 μ M P22 DNA (B). Samples were prepared and visualized as described under "Experimental Procedures." In A, the arrows indicate regions of duplex PM2 DNA which are not associated with *recA* protein.

triphosphates had either a slight stimulatory effect (UTP and GTP) or no effect (CTP).³ Binding was reproducibly lower in the presence of dTTP. Neither of the two ATP analogs, ADP[NH]P nor ADP[CH₂]P, stimulated complex formation; similarly, GTP(γ S) was ineffective in stimulating binding under these conditions. Complex formation with ATP(γ S) required a divalent cation; in the absence of Mg²⁺, only a very slow binding reaction could be detected. Mn²⁺ could substitute for Mg²⁺ in the formation of stable *recA* protein-DS DNA complexes.

³ At higher UTP and *recA* protein concentrations, UTP stimulated binding of *recA* protein to DS DNA.

Another distinguishing characteristic of the DS DNA-dependent ATPase of *recA* protein is its sensitivity to ionic strength. The rate of formation of *recA* protein-DS DNA complexes in the presence of ATP(γ S) was extremely sensitive to the monovalent ion concentration (Fig. 5) and appeared to be slightly more sensitive than the corresponding hydrolysis reaction (10). Under the same conditions, or at pH 8.0, *recA* protein-SS DNA complex formation was relatively salt resistant (Fig. 5), in agreement with the salt sensitivity of the SS DNA-stimulated ATPase activity (10).

Characterization of *recA* Protein-DS DNA Complexes—Formation of *recA* protein-DS DNA complexes shows a nonlinear dependence upon enzyme concentration (5). Similarly,

the DS DNA-stimulated ATPase activity of *recA* protein is nonlinearly dependent upon protein concentration (10). As shown in Fig. 6, the formation of *recA* protein-DS DNA complexes as measured by filter binding at pH 6.2 showed a sigmoidal dependence upon the *recA* protein concentration, whereas *recA* protein-SS DNA complexes increased linearly with *recA* protein. Under optimal conditions for binding, *recA* protein promotes unwinding of the DS DNA, and indirect measurements of the unwinding are consistent with cooperative binding of *recA* protein in the presence of ATP(γ S) (8). Further support for the cooperative interactions of *recA* pro-

TABLE II

Requirements for formation of *recA* protein-SS DNA complexes

Complete reactions (200 μ l) contained 0.22 μ M *recA* protein, 4.6 μ M 3 H-labeled SS M13 DNA, and the indicated nucleotide or analog in 20 mM Tris-HCl buffer (pH 8.1). Incubations were at 30 $^{\circ}$ C for 10 min and the amount of *recA* protein-SS DNA complex was determined by nitrocellulose filter binding as described under "Experimental Procedures."

	Complexes retained %
Complete	100
- <i>recA</i> protein	1.7
-ATP(γ S)	26.8
-ATP(γ S) + ATP (1 mM)	25.5
-ATP(γ S) + UTP	15.7
-ATP(γ S) + CTP	13.1
-ATP(γ S) + dTTP	16.2
-ATP(γ S) + GTP	27.1
-ATP(γ S) + ADP[NH]P (250 μ M)	17.3
-ATP(γ S) + ADP[CH ₂]P (250 μ M)	16
-ATP(γ S) + UTP[γ S] (100 μ M)	92
-ATP(γ S) + GTP[γ S] (100 μ M)	95
-Mg ²⁺	1.7
-Mg ²⁺ + Mn ²⁺ (20 mM)	88
-Mg ²⁺ + Zn ²⁺ (20 mM)	92
-Mg ²⁺ + Ca ²⁺ (20 mM)	72.1

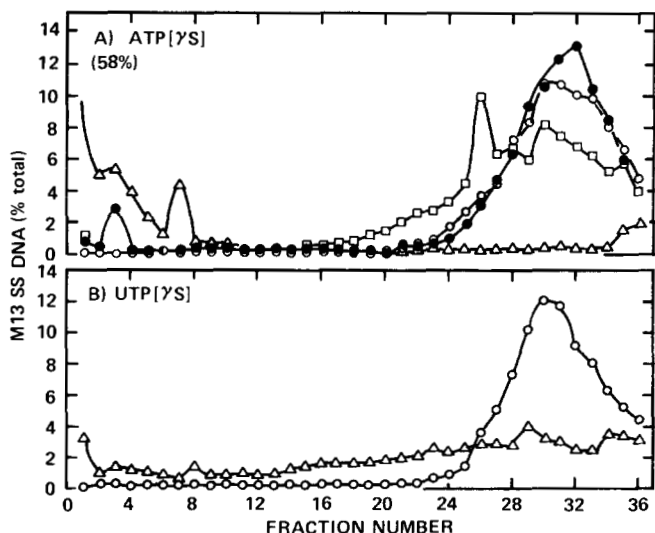


FIG. 9. Sedimentation properties of *recA* protein-SS DNA complexes. Reactions contained 3 H-labeled M13 SS DNA, the indicated amount of *recA* protein, and either 100 μ M ATP(γ S) (A) or 100 μ M UTP(γ S) (B). Incubations and sucrose gradient sedimentation analysis were performed as described under "Experimental Procedures." Greater than 85% of the radioactivity was recovered from each gradient. In A, the ratio of *recA* protein to SS DNA was 1/270 nucleotides (\circ — \circ); 1/134 nucleotides (\bullet — \bullet); 1/67 nucleotides (\square — \square); and 1/27 nucleotides (\triangle — \triangle) (Fraction 1 contained 58% of the 3 H-labeled M13 SS DNA). In B, the ratio of *recA* protein to SS DNA was 1/67 nucleotides (\circ — \circ) and 1/27 nucleotides (\triangle — \triangle).

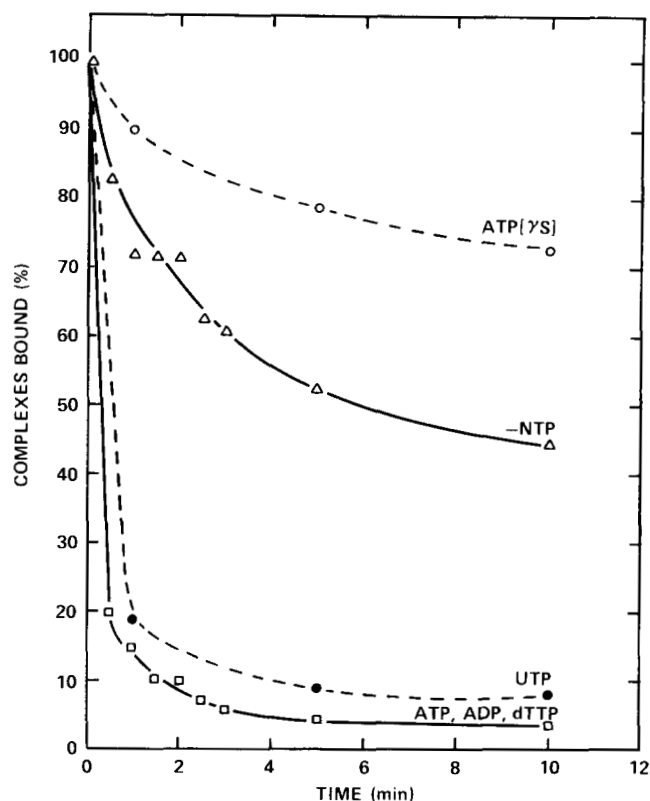


FIG. 10. Dissociation kinetics of *recA* protein-SS DNA complexes. Incubation and determination of *recA* protein-SS DNA complexes were done as described under "Experimental Procedures."

tein with DS DNA is shown in Fig. 7. Complexes of DS DNA, ATP(γ S), and varying amounts of *recA* protein were subjected to electrophoresis in an agarose gel without denaturing the protein. As the molar ratio of *recA* protein to DNA increased beyond 1:50, a fraction of the DS DNA appeared in a form that migrated more slowly in the agarose gel. Increasing the molar ratio of *recA* protein to 1:20 resulted in the conversion of all of the DNA into this slowly migrating complex. Little if any material was found at positions of intermediate mobility, arguing for cooperativity in the binding reaction. The more slowly migrating species is a complex of *recA* protein bound to DS DNA since labeled *recA* protein can be detected at this position in the presence of ATP(γ S). No *recA* protein migrated at the position of DS DNA (data not shown). When ATP(γ S) was replaced by UTP(γ S), cooperative binding of *recA* protein was also observed, although the mobility of the complexes was different from those formed with the ATP analog. The complexes detected in agarose gels survived treatment with 10 mM EDTA. This treatment did not remove *recA* protein from DS DNA (data not shown).

Electron microscopic examination of *recA* protein-DS DNA complexes further confirmed the cooperative mode of binding at low pH. As shown in Fig. 8A, *recA* protein is localized in "clusters" on the circular DS PM2 DNA under conditions where the DNA is approximately 30% saturated. Many of the DNA molecules bound to *recA* protein are extended and have the appearance of being twisted along an axis. Pairing of DNA molecules is also evident with linear DS P22 DNA and saturating amounts of *recA* protein (Fig. 8B). In this figure, 2 P22 DS DNA molecules are held together in an "H" form presumably through protein-protein interactions. The arms of this structure are duplex, whereas the pairing region should be 4 stranded. We have no evidence that the intermolecular pairing is at a region of homology.

Requirements for *recA* Protein-SS DNA Complex Formation—Filter binding was used to examine the requirements for formation of *recA* protein-SS DNA complexes (Table II). *recA* protein binds SS DNA in the absence of a nucleoside triphosphate cofactor. Retention on nitrocellulose filters of approximately 25% of the input DNA was observed in the presence of *recA* protein following a 1 M NaCl wash. A slight reduction in the amount of SS DNA bound to *recA* protein was seen when ATP, UTP, CTP, GTP, or dTTP was included in the reaction. All of the SS DNA could be retained on nitrocellulose filters in the presence of *recA* protein and ATP(γ S). Two additional phosphothiolate analogs, UTP(γ S) and GTP(γ S), also promoted formation of stable *recA* protein-SS DNA complexes. Enhancement of *recA* protein-SS DNA complex formation by GTP(γ S) is to be contrasted with the inability of this analog to promote formation of stable complexes of *recA* protein with DS DNA.

The requirement for a divalent cation in complex formation with SS DNA is satisfied by Mg^{2+} , Mn^{2+} , Zn^{2+} , and Ca^{2+} . In contrast, only Mg^{2+} is effective in the SS DNA-dependent hydrolysis of ATP by *recA* protein; Mn^{2+} is less than 10% as effective (10).

The requirements for *recA* protein-SS DNA complex formation were similar at pH 6.2 and pH 7.8. Furthermore, at both pH values, the binding reactions showed similar salt sensitivities (50% inhibition at 225 mM NaCl) (Fig. 5).

Characterization of *recA* Protein-SS DNA Complexes—Complexes of *recA* protein and SS DNA were analyzed by sucrose gradient sedimentation. In the absence of ATP(γ S), labeled SS DNA sedimented slightly faster in the presence of *recA* protein than in its absence (16).² However, incubation with ATP(γ S) led to the formation of fast sedimenting com-

plexes. The UTP(γ S) analog also promoted formation of fast sedimenting complexes when it replaced ATP(γ S).

The rate of sedimentation of the *recA* protein-SS DNA complexes depended upon the ratio of *recA* protein to SS DNA (Fig. 9). When *recA* protein was present at a ratio of 1 monomer/270 nucleotides, the labeled DNA sedimented in the upper third of the sucrose gradient. A slight increase in sedimentation rate was observed at a ratio of 1 *recA* protein monomer/134 nucleotides, and faster sedimenting material was seen at a ratio of 1 *recA* protein monomer/66 nucleotides. Increasing the ratio to 1 *recA* protein monomer/27 nucleotides resulted in complexes that sedimented to the bottom of the gradient. This ratio of *recA* protein to DNA corresponds to saturation as observed in filter binding experiments (see Fig. 6). In the presence of UTP(γ S), qualitatively similar results were obtained, except that a higher concentration of *recA* protein was needed to convert the DNA to rapidly sedimenting complexes. West *et al.* (16) have reported that at molar ratios of *recA* protein to DNA of approximately 1:2.5, *recA* protein-SS DNA complexes can be isolated as fast sedimenting material.

Dissociation of *recA* Protein from SS DNA—Complexes of *recA* protein and SS DNA are stabilized by the binding of ATP(γ S). As shown in Fig. 10, ATP(γ S) promotes stabilization at least, in part, by blocking dissociation of *recA* protein-DNA complexes. *recA* protein dissociated from SS DNA with a half-time of approximately 10 min at 28 °C. Addition of ATP(γ S) (200 μ M) at the time of addition of unlabeled competing DNA increased the half-life of the complexes to more than 60 min at 28 °C. Thus, a primary mechanism by which ATP(γ S) stabilizes *recA* protein binding to SS DNA is by preventing dissociation of the DNA. We have attempted to

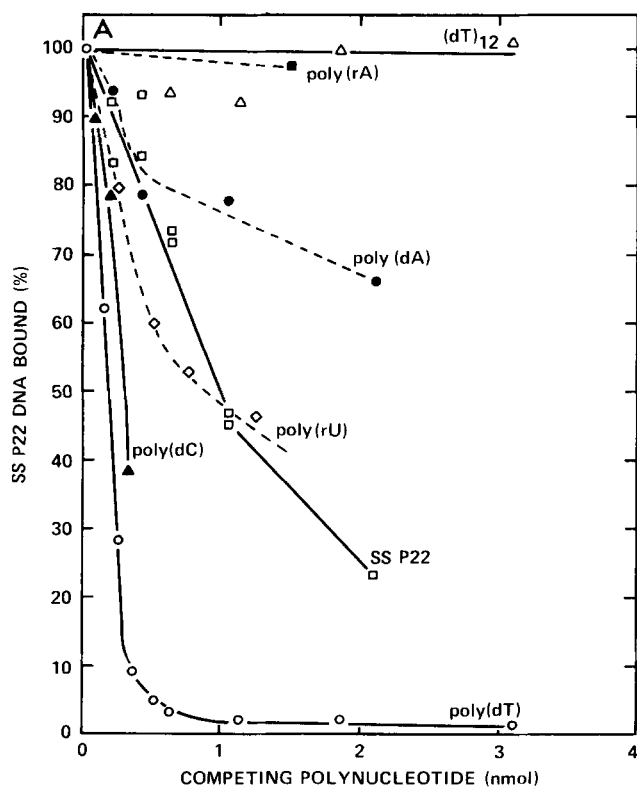
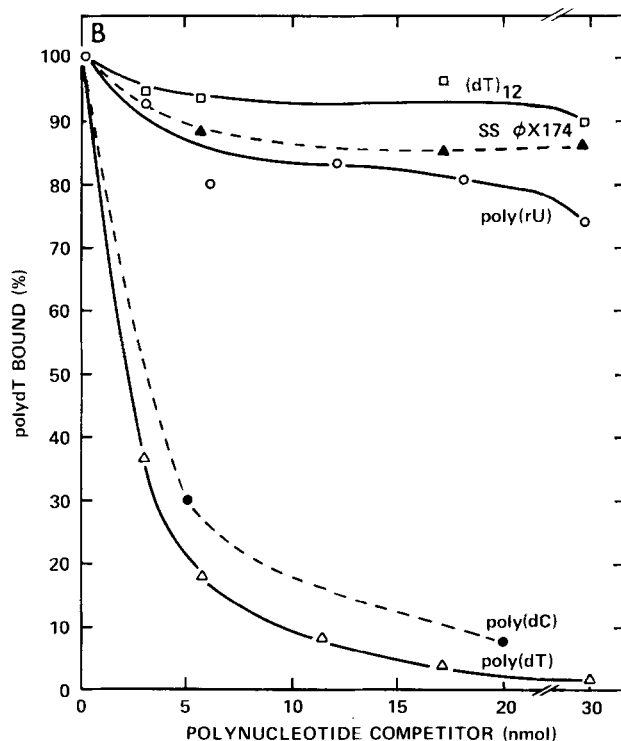


FIG. 11. Competition by homopolymers and DNA's for *recA* protein binding. A, reactions (200 μ l) contained 0.22 μ M *recA* protein, 5.3 μ M 3 H-labeled SS P22 DNA, 100 μ M ATP(γ S) in Tris-HCl buffer (pH 8.1), and the indicated amount of unlabeled competitor. Incubations were for 30 min at 30 °C. B, reactions contained 4.3 μ M *recA*



protein, 28.8 μ M 3 H-labeled (dT)₈₂₀, 100 μ M ATP(γ S) in Tris-HCl buffer (pH 8.1), and the indicated amount of unlabeled competitor. Incubations were for 30 min at 30 °C. Complexes were measured as described under "Experimental Procedures".

measure the effect of ATP(γ S) on the rate of binding of recA protein to SS DNA. However, at 28 °C, the rate was too rapid to permit an accurate "on" rate measurement even in the absence of ATP(γ S). Therefore, it is possible that ATP(γ S) stimulates the rate of recA protein binding as well as decreasing the rate of its dissociation. In the case of DS DNA, where a nucleoside triphosphate is required for the binding of recA protein, ATP(γ S) also acts to block dissociation (5).

In contrast to ATP(γ S), ATP stimulates dissociation of recA protein-SS DNA complexes. Addition of 200 μ M ATP to these complexes caused release of more than 90% of the DNA, as judged by nitrocellulose filter binding. The dissociation was extremely rapid with an estimated half-time of 20 s at 28 °C. UTP also stimulated release of recA protein from DNA. That this dissociation is not coupled to ATP or UTP hydrolysis is demonstrated by the finding that ADP efficiently stimulated dissociation, as did dTTP, a nucleoside triphosphate that is not hydrolyzed by recA protein (10). UTP(γ S) and GTP(γ S) also blocked dissociation of these complexes (data not shown).

Competition for recA Protein-DNA Complex Formation by Homopolymers—The ability of recA protein to bind to either ribo- or deoxyribopolymers was tested in binding competition experiments. As shown in Fig. 11A, there is a wide range in the ability of homopolymers to compete with labeled SS P22 DNA for binding to recA protein. However, several conclusions can be drawn concerning the interaction of recA protein with polynucleotides in the presence of ATP(γ S). (i) recA protein binds both ribo- and deoxypolymers. (ii) Poly(dT) competes most effectively with SS P22 DNA for recA protein. Homopolymers composed of pyrimidine residues (both ribo- and deoxypolymers) are generally more effective competitors for binding than homopolymers composed of purines, e.g. poly(dA), poly(rA), and poly(dG) (data not shown). (iii) Short oligonucleotides such as (dT)₁₂ fail to compete for recA protein. The latter finding is consistent with failure of (dT)₁₂ to stimulate the ATPase activity of recA protein (10).

A series of reciprocal competitive binding experiments using ³H-labeled poly(dT) and unlabeled DNA's and homopolymers was performed. As shown in Fig. 11B, only poly(dC) competed effectively with the labeled poly(dT) for binding to recA protein. Preferential binding to poly(dT) was not dependent upon ATP(γ S), since qualitatively similar competition curves

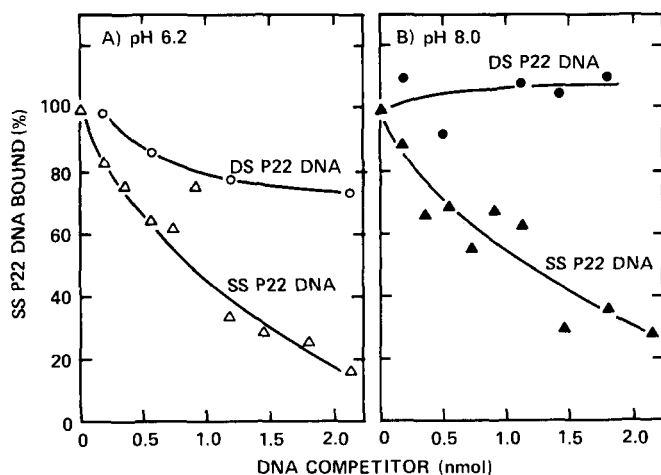


FIG. 12. Competition by DS DNA for recA protein binding to SS DNA. Reactions (200 μ l) contained 215 nM recA protein, 5.85 μ M ³H-labeled SS P22 DNA, 100 μ M ATP(γ S), and the indicated amount of unlabeled competitor DNA in 20 mM maleate buffer (pH 6.2) (A) or 20 mM Tris-HCl buffer (pH 8.1) (B). Incubations were for 15 min at 30 °C. Complexes were measured as described under "Experimental Procedures".

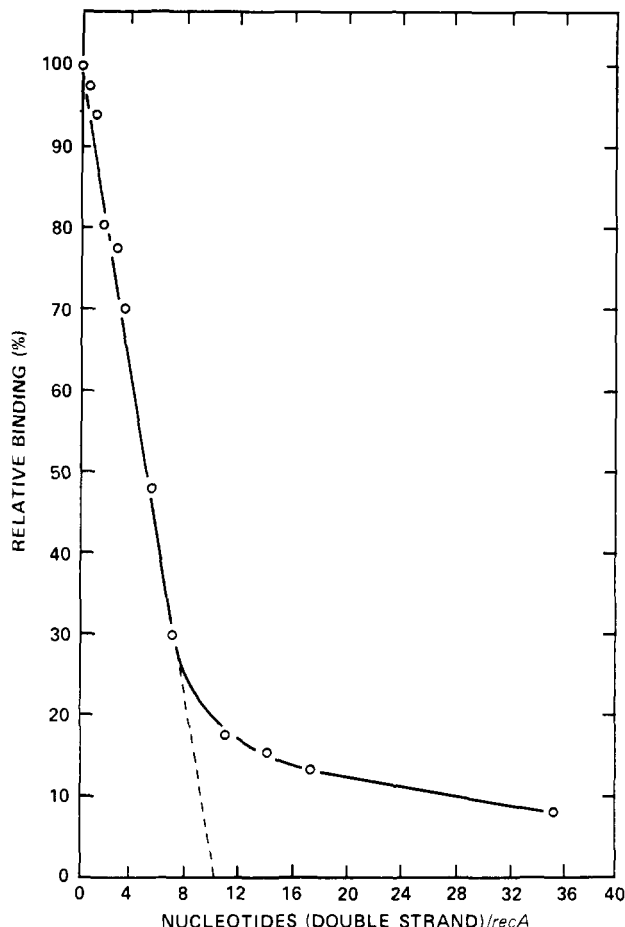


FIG. 13. Inhibition of SS DNA binding to recA protein by binding of DS DNA. Preincubation reactions (30 μ l) contained 7.1 μ M recA, 155 μ M ATP(γ S), 20 mM maleate buffer (pH 6.2), and the indicated amount of circular DS pZ66 DNA (13). After incubation at 30 °C for 30 min, 80 μ l of 20 mM Tris-HCl (pH 8.1), 10 mM MgCl₂, 1 mM dithiothreitol, 127 μ M ATP(γ S), and 18.2 μ M ³H-labeled P22 SS DNA were added, and incubation was continued for 30 min at 30 °C. recA protein-SS DNA complexes were measured using alkali-treated nitrocellulose filters as described under "Experimental Procedures".

were obtained when the analog was omitted (data not shown). Again, there was no competition by (dT)₁₂ when it was present in excess over the longer poly(dT).

Using DS DNA as competitor there was little, if any, reduction in the amount of SS DNA retained on filters at pH 8.1 (Fig. 12). Since in these competition experiments SS DNA is in excess over recA protein (1 recA protein/15–20 nucleotides), ternary complexes which might complicate the interpretation of these results are not formed. An identical competition experiment was performed at pH 6.2, where recA protein binding to DS DNA is optimal. As in the case of binding at pH 7.5, DS DNA did not effectively compete with SS DNA for binding to recA protein (Fig. 12). Preincubation of recA protein with DS DNA at pH 6.2 in the presence of ATP(γ S) inhibited the subsequent binding of labeled SS DNA (Fig. 13). This inhibition increased linearly with DS DNA and was saturated at approximately 10 nucleotide/recA protein monomer. Thus, although recA protein binds preferentially to SS DNA, the sites for binding to SS and DS DNA are identical or overlapping.

DISCUSSION

Binding of the recA protein to DNA is markedly affected by nucleoside triphosphates, diphosphates, and their analogs.

The optimal conditions for DS DNA binding are similar if not identical with those for the stimulation of ATP hydrolysis by DS DNA. Both the binding of DS DNA and the hydrolysis of ATP are optimal near pH 6.0, are sensitive to ionic strength, and depend nonlinearly on the concentration of *recA* protein (10). ATP(γ S) is not appreciably hydrolyzed by *recA* protein (9, 15) and is a potent competitive inhibitor of the DNA-dependent ATPase activities (9, 15). We, therefore, infer that complexes of *recA* protein and DNA formed in the presence of this analog are likely to represent intermediates in the ATP-dependent reactions. The similarities in pH, salt, and enzyme dependence of complex formation in the presence of ATP(γ S) and ATP hydrolysis suggest that these requirements are associated with the prehydrolytic binding of *recA* protein to DS or SS DNA. The *recA* protein-DS DNA complexes formed in the presence of ATP(γ S) are extremely stable and withstand incubation in high salt (1 M NaCl) at pH 8.0 for 60 min at 37 °C. In view of the salt and pH sensitivity of binding, we would suggest that the conformation of *recA* protein in these complexes is different from the unbound form of the enzyme.

recA protein forms a complex with DS DNA in the presence of UTP(γ S), another competitive inhibitor of *recA* protein-catalyzed ATP hydrolysis (9). The complex formed with DS DNA and UTP(γ S) is different from that formed with ATP(γ S) as judged by their different mobilities in agarose gels. The differences may be due to different DNA configurations in the two complexes or possibly to different amounts of *recA* protein that remain bound to the DNA. In both cases, the binding of *recA* protein shows a cooperative behavior that is consistent with filter binding and unwinding assays (5, 8). UTP(γ S) also promotes unwinding of duplex DNA by *recA* protein.² The differences observed between the two analogs are interesting in light of the finding that although UTP is rapidly hydrolyzed by *recA* protein, it is a poor cofactor for the annealing and assimilation reactions (2, 5). UTP and ATP also differ in their ability to promote oligomerization of *recA* protein. ATP binding induces the filamentation of *recA* protein, while UTP binding has little effect upon the structure of *recA* protein as judged by sedimentation in sucrose gradients or by electron microscopy. Possibly the oligomeric structure of *recA* protein is an important feature in the pairing of DNA molecules during the annealing reactions. A more detailed characterization of complexes formed in the presence of ATP(γ S) and UTP(γ S) may indicate how binding of ATP and UTP to *recA* protein differentially influences its structure and interaction with DNA.

The *recA* protein-DS DNA complexes visualized directly by electron microscopy indicate that under conditions that favor binding of *recA* protein, DS DNA molecules are paired for several thousand nucleotides. In the case of circular PM2 DS DNA, this pairing is intramolecular, whereas intermolecular pairing is evident with linear P22 DNA. The findings with PM2 DNA-*recA* protein complexes suggest that these paired regions are not homologous. Presumably the pairing is maintained by protein-protein or protein-nucleic acid interactions that are stabilized by the binding of ATP(γ S). In the presence of ATP, these paired structures may form and dissociate during the hydrolytic cycle, suggesting that *recA* protein may be capable of promoting homologous pairing of DS DNAs at low pH.

Although *recA* protein binds SS DNA in the absence of a nucleoside triphosphate, *recA* protein-SS DNA complexes are stabilized by binding ATP(γ S), UTP(γ S), and GTP(γ S). That GTP(γ S) stabilizes binding to SS DNA is surprising since it fails to stabilize complexes with DS DNA, and GTP is poorly hydrolyzed by *recA* protein. *recA* protein-SS DNA complexes

are formed equally well at pH 6.2 and pH 8.1 in relatively high salt (50% inhibition in 225 mM NaCl). The sedimentation properties of complexes formed in the presence of ATP(γ S) indicate that at a ratio of approximately 1 *recA* protein monomer/30 nucleotides, large protein-DNA aggregates are formed. This ratio corresponds to saturation in filter binding measurements and suggests that all of the DNA is bound to *recA* protein. Under these conditions, complexes formed with UTP(γ S) sediment more slowly, again indicating a difference between the phosphothiolate analogs of ATP and UTP in their interaction with *recA* protein.

The relationship of these fast sedimenting complexes of SS DNA and *recA* protein to the annealing reaction is not readily apparent. *recA* protein catalyzes the ATP-dependent annealing of single strands when the molar ratio of DNA to *recA* protein exceeds 50 to 1 (2).² Little or no fast sedimenting material is detected in the presence of ATP(γ S) at this ratio. Furthermore, ATP(γ S) inhibits the ATP-dependent annealing of single strands by *recA* protein (2). West *et al.* (16) have reported that ATP does not promote formation of large *recA* protein-DNA complexes as judged by sucrose gradient sedimentation. These results would suggest that if large complexes are intermediates in the renaturation reaction, they are short lived, forming and dissociating rapidly. Consistent with this idea, we have found that ATP stimulates the dissociation of *recA* protein from SS DNA. Hydrolysis is not required since both ADP and dTTP accelerate the dissociation. This property of *recA* protein is unusual and may reflect an important aspect of the mechanism of DNA annealing. We have, in fact, exploited this property in developing a rapid and highly specific column chromatographic method for purifying *recA* protein (12).

recA protein binds both ribo- and deoxyribohomopolymers, displaying the highest affinity for poly(dT) and poly(dC), two polynucleotides with little secondary structure. Purine-containing homopolymers such as poly(dA), which serve as effectors for ATP hydrolysis, only weakly compete with SS DNA for binding to *recA* protein. No competition was observed with poly(dG) which fails to stimulate ATPase activity. Ribohomopolymers (poly(rU) and poly(rC)) are generally poor effectors of the ATPase activity of *recA* protein (10) but compete effectively with SS DNA for binding to *recA* protein. Polynucleotide size is important for *recA* protein interaction since (dT)₁₂ is without effect in competition experiments and fails to stimulate the ATPase activity of *recA* protein. Preferential binding of *recA* protein to polypyrimidines (poly(dT) and poly(dC)) in the absence of ATP(γ S) has also been observed. Taken together, these results suggest that tight binding of *recA* protein to polynucleotides is a necessary but not sufficient condition for ATP hydrolysis.

These binding studies have illuminated certain features of the annealing and assimilation reactions. *recA* protein binds rapidly and preferentially to SS DNA. In the presence of ATP(γ S), where the dissociation of *recA* protein-DNA complexes is blocked, the distribution of *recA* protein bound to SS compared to DS DNA would be determined by the ratio of the binding rates. We estimate that *recA* protein binding to SS DNA is 10–20 times more rapid than binding to DS DNA at pH 6.2, and this ratio increases sharply at higher pH values. It is not surprising, therefore, that DS DNA is unable to compete with SS DNA for the binding of *recA* protein. Since preincubation with DS DNA at pH 6.2 does block subsequent binding of SS DNA, it is apparent that the DS and SS DNA binding sites on the *recA* protein are identical or overlapping.

Preferential binding of SS DNA by *recA* protein and inhibition of dissociation by ATP(γ S) explain the inhibitory effects of excess SS DNA on formation of ternary complexes

(17). At low SS DNA to recA protein ratios, the DNA chains act as a scaffold to bind recA protein in a complex (with ATP(γ S)) which can, in a second step, bind another DNA molecule. This structural role for SS DNA may be mimicked by low pH, where, in the presence of ATP or ATP(γ S), the protein oligomerizes in the absence of DNA. In the presence of ATP(γ S), binding of the 2nd DNA molecule, either DS or SS, occurs without dissociation of the 1st SS DNA molecule. A 2nd SS DNA molecule, binding preferentially to these recA protein-DNA structures, will block DS DNA binding and inhibit formation of ternary complexes. We have demonstrated that coating the SS DNA with SS DNA-binding protein reduces the inhibition by excess SS DNA (17), presumably by reducing the extent of SS regions that can bind in the second step. At the present time, we cannot rule out an alternative mechanism in which binding of SS DNA blocks the binding of DS DNA by affecting the distribution of recA protein along the DNA. In this model, recA protein binds to SS DNA noncooperatively. If binding of a duplex molecule in the second step requires a "cluster" of contiguous recA protein molecules on the SS DNA (cooperative binding), then excess SS DNA would reduce the frequency of contiguous binding by recA protomers, effectively reducing the number of DS DNA binding sites. Interestingly, the recA protein-directed proteolytic cleavage of phage λ repressor is also inhibited by excess SS DNA (18), suggesting that the mechanism of inhibition may be similar.

As noted above, strand assimilation in the presence of ATP is inhibited by excess SS DNA and this inhibition is reversible by SS DNA-binding protein (17, 19). Although the effect of ATP is to increase the dissociation of recA protein from SS DNA, we presume that SS DNA is first bound by recA protein in strand assimilation, and recA protein subsequently binds DS DNA. Possibly the SS DNA is released prior to binding of the protein to DS DNA. Since a nucleoside triphosphate is needed at low pH in order to bind DS DNA, it is reasonable that an enzyme-ATP complex interacts with the DS DNA

substrate. This model provides a two-step mechanism for the binding of recA protein to DS DNA under the conditions of strand assimilation. Experiments are in progress to test critical features of this sequential binding scheme.

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