Homologous Pairing in Genetic Recombination

Purification and Characterization of Escherichia coli recA Protein*

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RecA protein, which is essential for genetic recombination in Escherichia coli, was extensively purified from a strain of E. coli which contained the recA gene cloned in a plasmid (Sancar, A., and Rupp, W. D. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 3144-3148). Using the DNA-dependent ATPase activity of recA protein as an assay, we obtained about 60 mg of purified recA protein from 100 g of cells. Ten μg or 1 μg of the purified protein exhibited only one detectable band with Mr = 40,000 upon sodium dodecyl sulfate-acrylamide gel electrophoresis. More than 99% of the ATPase activity of 25 μg recA protein was 37 min at 51°C. Purified recA protein binds to single-stranded and double-stranded DNA, unwinds duplex DNA by a mechanism that is stimulated by single-stranded DNA or oligonucleotides, and pairs homologous single strands with duplex DNA.

RecA protein is the product of a gene that was discovered by Clark and Marzluff (1965) on the basis of its indispensability for Escherichia coli recombination. Investigators have since uncovered many additional functions that depend upon this gene. These biological functions include repair of damaged DNA (Clark and Marzluff, 1965; Howard-Flanders and Boyce, 1966; Howard-Flanders and Theriot, 1966), UV-induced repair of UV lesions (Weigle, 1953; George et al., 1974; Defais et al., 1971), mutagenesis (Miura and Tomizawa, 1968; Witkin, 1969), prophage induction (Brooks and Clark, 1967; Hertman and Luria, 1967), cell division (Inouye, 1971; Witkin, 1967), inhibition of DNA degradation (Howard-Flanders and Theriot, 1966; Marsden et al., 1974; Pollard and Randall, 1973), and even replication, under certain circumstances (Lark & Lark, 1979). Inouye and colleagues observed that an active product of the recA gene was required for induction of extensive synthesis of a protein, called protein X. Mr = 40,000 (Inouye, 1971; Inouye and Guthrie, 1969; Inouye and Pardee, 1970). The cloning of the recA gene (McEntee and Epstein, 1977) led to the discovery that it is the structural gene for protein X and therefore that recA protein stimulates its own synthesis (Gudas and Mount, 1977; McEntee, 1977; Emmerson and West, 1977; Little and Kleid, 1977). Recently, Kenyon and Walker (1980) discovered that recA protein function is required for the induction by mitomycin C or ultraviolet light of gene expression at no less than five loci. Until recently, the striking pleiotropy of recA mutations made it appear likely that recA protein function is an indirect role in recombination; indeed, no published evidence indicated a direct role of the recA gene in recombination until Kobayashi and Ikeda (1978) demonstrated the effect of a thermosensitive recA mutant on molecular recombination in the absence of either RNA or protein synthesis. The discovery of the DNA-dependent ATPase activity of recA protein by Ogawa et al. (1979) and by Roberts et al. (1979) emphasized the interaction of recA protein with DNA (Gudas and Pardee, 1976; Satta et al., 1979). RecA protein was first purified by radiochemical methods (Ogawa et al., 1979) and as a protease that cleaved λ repressor (Roberts et al., 1978). The discovery of the ATPase activity of recA protein (see above) and the cloning of the gene on a multicopied plasmid (McEntee and Epstein, 1977; Ogawa et al., 1979; Sancar and Rupp, 1979) further facilitated the purification of the protein (Weinstock et al., 1979; Shibata et al., 1979a). Stimulated by the experiments of Holloman and Radding (1976), which suggested that the recA gene might play a role in the pairing of a single strand with duplex DNA to produce a D-loop (Beattie et al., 1977), and by the experiments cited above, which indicated that recA protein might act directly in recombination, we purified the recA protein and found indeed that it catalyzed the formation of D-loops (Shibata et al., 1979a). This discovery was made by McEntee et al. (1979).

This paper describes the purification and properties of recA protein.

Materials and Methods

DNA

In this paper, the amounts of DNA or oligodeoxynucleotides are expressed in moles of nucleotide residues.

Form I DNA and single-stranded circular DNA of phages fd and φX174 were prepared as described by Cunningham et al. (1980). As we described before (Beattie et al., 1977), form I DNA sometimes gives a high background in the D-loop assay (see below), which can be eliminated by heating the preparation of form I DNA at 68°C for 50-60 s in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA. Form IV DNA was prepared as described by Pulleyblank and Morgan (1975).

We prepared form II DNA by two methods. The first, digestion of form III DNA by S1 nuclease (Wiegand et al., 1975), produced some form III DNA as well. The reaction mixture contained 0.65 mM form I DNA, 50 mM Na-acetate buffer (pH 5.0), 0.5 mM ZnSO4, 100 mM NaCl, 1250 or 2500 units (Wiegand et al., 1975) of S1 nuclease (Miles Biochemicals) in a total volume of 0.2 ml. After an incubation of 30 min at 37°C, the sample was extracted twice with phenol and twice with chloroform to remove contaminating DNA. The second method was the endo-V digestion of form I DNA by endo-V nuclease (Robertson et al., 1977) as described by Watanabe and Radding (1981).

The duplex forms of DNA of the small DNA phages are designated as follows: form I, negatively superhelical DNA; form II, circular duplex DNA with one or more interruptions in either strand; form III, linear duplex DNA; form IV, closed circular duplex DNA that was fully relaxed under the conditions of closure.
with ether. We evaporated the ether by carefully bubbling N2 through the sample and then dialyzed overnight (0.3 l at 4°C for 2 l) against 7.7 mM Tris-HCl (pH 7.5), 1 mM EDTA. As judged by gel electrophoresis and counting of radioactivity extracted from the bands of form II and form III DNA (see below), 1250 units of S. nucleare (Wietz et al., 1975) produced a preparation containing 70% form II and 30% form III DNA; 2500 units of S. nucleare produced 62% form II and 38% form III DNA.

Form II DNA, with a single nick and free of any form III DNA, was prepared by treatment of form I DNA with pancreatic DNase in the presence of ethidium bromide, according to Shortle et al. (1979). The reaction mixture (100 ml), which contained 50 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 150 ml of ethidium bromide/ml, 50 ml form I DNA, 2 ml of pancreatic DNase/ml (Wortman, 3200 units/mg), was incubated at 25°C for 60 min. The reaction was terminated by the addition of 1/20 volume of 1 M Tris-HCl (pH 9) and 1 volume of cold phenol saturated with 1.5 M NaCl, 0.15 M Na-citrate; the phases were mixed by agitation at 4°C. After centrifugation, the upper aqueous phase was collected, and remaining ethidium bromide was extracted 4 times by isoamyl alcohol. The solution of DNA was dialyzed against 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA. The fractions of form II, form III, and form I DNA were estimated from the radioactivities in the bands corresponding to each form after electrophoresis through a 1.4-2% agarose gel slab as described previously (Shibata et al., 1979a). In order to confirm that our preparations of form II DNA consisted principally of circular DNA with a single nick, we denatured it by incubation at pH 12.3 at room temperature for 3 min before gel electrophoresis. After denaturation, form II DNA sedimented as a single nicked band on a 1.4-2% agarose gel slab as described previously (Cunningham et al., 1979).

Segments of single-stranded DNA were prepared by boiling 840 ml single-stranded phase DNA (Cunningham et al., 1979) in 40 ml of 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA for 15-10 min in a centrifuge tube (Eppendorf micro-test tubes, 1.5 ml), followed by quick chilling in ice water. The median chain length of the fragments was estimated by electrophoresis through a 2% agarose gel slab as described previously (Shibata et al., 1979). A boiled DNA I form I DNA was prepared as described previously (Cunningham et al., 1979).

DNA preparations were boiled with 1 ml 1 M Tris-HCl (pH 7.5), 1.8 mM dithiothreitol, 88 ml of bovine serum albumin/ml (4.4, 8.8, or 17.6 ml double-stranded fd or φX174 [3H]DNA, 6 or 12 ml single-stranded DNA (or fragments) of fd or φX174, various amounts of purified recA protein. To avoid precipitation of single-stranded DNA and spermidine (Christiansen and Baldwin, 1977), we incubated DNA, proteins, and ATP at 37°C for 4 min in the presence of 1.2 mM MgCl2 before adding spermidine. To start the reaction, we added spermidine, increased the concentration of MgCl2 to 6.7 ml, and incubated the reaction mixture at 37°C (Recently. The addition of ATP mixture with slight addition, adding ATP together with spermidine and more Mg++, after the precipitation of DNA with recA protein). Microtiter plates were used as described below (see ATPase assay). For the unwinding of duplex DNA or the formation of complexes of DNA and recA protein, 0.5 ml adenosine 5'-O-(3-thiotriphosphate, (Boehringer Mannheim GmbH) replaced ATP, and spermidine was omitted (Cunningham et al., 1979; Shibata et al., 1977).

**Assay C**—This assay measured the retention by nitrocellulose filters of complexes of protein and DNA (Shibata et al., 1977b). After DNA and recA protein were incubated at 37°C for 30 min, we diluted the reaction mixture 100-fold with cold BD buffer (200 ml 10% bovine serum albumin/ml with or without NaCl. We filtered the mixture through nitrocellulose filters (Millipore DAWP; pore size, 0.65 μm) which had been washed with 2 ml of BD buffer. The filter was then washed with 1 ml of BD buffer, dried, and put in a vial with Econofluor (New England Nuclear). Radioactivity was counted in a scintillation counter.

**Assay of ATPase Activity of RecA Protein**

The standard reaction mixture contained for ATPase activity contained, in 18 ml, 35 mM Tris-HCl (pH 7.5), 6.7 mM MgCl2, 2 mM dithiothreitol, 0.100 g of bovine serum albumin/ml, 1.4 mM ATP (total 26 mmol, 7.7 Ci/mol, Amersham), 10 μCi or plus minus 50 μCi single-stranded DNA of phage φX174. The reaction mixture was incubated at 37°C for 30 min in a well of a microtiter plate (Linbro/Titertek) floating on a water bath. The reaction was terminated by chilling to 0°C and by addition of 12 ml of 25 mM EDTA containing 3 ml each of unlabeled ATP, ADP, and AMP as carrier. Strips (1×10 cm) of polyethyleneimine were prepared by scoring the strips of polyethyleneimine on a plastic film (10×10 cm, Polygram Cel 300PEI, Macherey-Nagel Co.) in lines at intervals of 1 cm. Strips were soaked in 0.5 ml LiCl, 1 ml of 1 M formic acid, washed extensively with distilled water, and dried before use (Scott et al., 1977). An aliquot of 10 ml from each sample was placed on the strips and incubated for 4 hr at 37°C. The strips were developed by ascending chromatography at room temperature for about 40 min in 0.5 ml LiCl, 1 ml of formic acid. The spots of ATP, ADP, and AMP (in this order from bottom to top) were located under illumination by using a UV lamp (at 254 nm). Each spot was cut out and put in a vial for scintillation counting so that the layer of polyethyleneimine faced upward. (If the layer faced the bottom of the vial, the efficiency of counting H decreased 30%). Econofluor was added to the vials, and radioactivity was measured in a scintillation counter. One unit of ATPase is the amount that hydrolyzes 1 nmol of ATP under these conditions (Shibata et al., 1979b). The concentration of recA protein is expressed as moles of polypeptide of M, 40,000.

**Homologous Pairing Reaction, Unwinding of Duplex DNA, and the Formation of Complexes of RecA Protein and DNA**

The standard reaction mixture contained, in 20.5 ml, 35 mM Tris-HCl (pH 7.5), 6.7 mM MgCl2, 20 mM spermidine-HCl, 1.3 mM ATP, 1.8 mM dithiothreitol, 88 μg of bovine serum albumin/ml, 4.4, 8.8, or 17.6 μM double-stranded fd or φX174 [3H]DNA, 6 or 12 ml single-stranded DNA (or fragments) of fd or φX174, various amounts of purified recA protein. To avoid precipitation of single-stranded DNA and spermidine (Christiansen and Baldwin, 1977), we incubated DNA, proteins, and ATP at 37°C for 4 min in the presence of 1.2 mM MgCl2 before adding spermidine. To start the reaction, we added spermidine, increased the concentration of MgCl2 to 6.7 ml, and incubated the reaction mixture at 37°C. (Recently, the addition of ATP mixture with slight addition, adding ATP together with spermidine and more Mg++, after the precipitation of DNA with recA protein.) Microtiter plates were used as described above (see ATPase assay). For the unwinding of duplex DNA or the formation of complexes of DNA and recA protein, 0.5 ml adenosine 5'-O-(3-thiotriphosphate, (Boehringer Mannheim GmbH) replaced ATP, and spermidine was omitted (Cunningham et al., 1979; Shibata et al., 1979b).
incubated with recA protein and ATPyS at 37°C for 30 min in the mixture described above, the reaction mixture was chilled and diluted 2-fold with cold BD buffer. We added KCl, (NH₄)₂SO₄, and Tris, acetate buffer, 5 mM Na-acetate, 1 mM EDTA at pH 8.0. Current was collected by low speed centrifugation. The precipitate was resuspended in 12 ml NaC1. After the suspension was stirred for 30 min, the precipitate was finally dissolved in 12 ml 0.5 M KC1 solution and 8% Brij 58 (Sigma) to make the culture at 0°C for 30 min. We centrifuged the viscous cell lysate at 35 V for 16-18 h, and the buffer was reincubated between the reservoirs, following which we stirred the gel for 2 h in E buffer containing 0.5 μg of ethidium bromide/ml. We illuminated the gels from below with a short wavelength UV lamp (Ultra-Violet Products) and photographed them through a red filter (Kodak, Wratten 25A) with Polaroid Type 55 Land film.

Growth of Cells

We incubated 500 ml of K medium with cells of strain DR1453 which had grown on a 56-sol plate and incubated the culture at 37°C overnight with aeration. On the next morning, the 

\[ \text{growth} \times \text{incubation} \]

was dependent both on the presence of the recA+ gene in the cell and on the inducing treatment with nalidixic acid. We also examined fractions for the presence of a protein with \( M_r = 40,000 \) by electrophoresis through an acrylamide gel containing 0.1% sodium dodecyl sulfate (Fig. 2). The presence of a distinct band corresponding to a protein of \( M_r = 40,000 \) was dependent both on the presence of the recA+ gene in the cell and on the inducing treatment with nalidixic acid. We could not detect the protein when \( M_r = 40,000 \) in fraction II from induced cells of a strain containing only part of the recA gene (DR1461) or from a strain containing no recA gene (DR1432) (Sancar and Rupp, 1979).

The first chromatographic step in the purification was a column of hydroxyapatite (Fig. 1, A and B) which yielded two major peaks of protein and ATPase activity. We discarded the first peak which contained a significant amount of DNA-independent ATPase. The second peak contained one major protein of \( M_r = 40,000 \) (Fig. 2d) and little DNA-independent ATPase. Most but not all of the DNAse activity was removed by hydroxyapatite. The DEAE column removed proteins that

\[ \text{column (3.2 × 26.5 cm) of hydroxyapatite (Bio-Rad HTP) which previously had been equilibrated with buffer B (Fig. 1, A and B). The column was washed with 250 ml of buffer B and developed with a 3.6-liter linear gradient (0.02 to 0.5 M) of potassium phosphate in buffer B at 60 ml/h. RecA protein (DNA-dependent ATPase activity) was eluted at 0.06 M potassium phosphate. Protein in the pooled fraction (fraction III, 111 ml, 113 mg of protein) was precipitated by ammonium sulfate at 75% of saturation, collected by low speed centrifugation, and dissolved in 7.4 ml of buffer A containing 0.3 M ammonium sulfate (fraction IIIa, 10 ml).}

\[ \text{Sephacryl S200 Gel Filtration—Fraction IIIa was applied to a column (3.2 × 42 cm) of Sephacryl S200 (Pharmacia) which had been equilibrated with buffer A containing 0.3 M ammonium sulfate. The column was developed with the same buffer at 20 ml/h. Fractions with DNA-dependent ATPase but little DNA-independent activity were pooled and dialyzed against buffer B containing 1 mM EDTA (fraction IV, 28 ml, 64 mg of protein).}

\[ \text{DEAE-cellulose Chromatography—Ten ml of fraction IV were applied to a column (1 × 13.5 cm) of DEAE-cellulose (Whatman, DE52) previously equilibrated with buffer B containing 1 mM EDTA. The column was washed with 15 ml of the equilibration buffer and developed with a 110-ml linear gradient of KC1 (0 to 0.5 M) in the equilibration buffer, at 12 ml/h (Fig. 1, C and D). Active fractions were pooled (between 0.23 and 0.26 M KC1) and dialyzed against 50 mM Tris-HCI (pH 7.5) containing 0.3 mM EDTA, 5 mM dithiothreitol, 10% (v/v) glycerol (fraction V, 10 ml, 12 mg of protein). Samples were stored on ice in a coldroom for at least a year without loss of activity.}

\[ \text{RESULTS}

\[ \text{Purification of RecA Protein}

\[ \text{We purified recA protein from a strain of E. coli (DR1453) carrying the recA+ gene cloned on a plasmid (Sancar and Rupp, 1979). Cells had been treated with nalidixic acid in logarithmic phase of growth to induce extensive synthesis of recA protein (Inouye and Pardee, 1970; McIntee, 1977). To monitor the purification, we assayed the ATPase activity of recA protein, which requires single-stranded DNA as a cosfactor (Ogawa et al., 1979; Roberts et al., 1979). We also examined fractions for the presence of a protein with \( M_r = 40,000 \) by electrophoresis through an acrylamide gel containing 0.1% sodium dodecyl sulfate (Fig. 2). The presence of a distinct band corresponding to a protein of \( M_r = 40,000 \) in fraction II was dependent both on the presence of the recA+ gene in the cell and on the inducing treatment with nalidixic acid. We could not detect the protein when \( M_r = 40,000 \) in fraction II from induced cells of a strain containing only part of the recA gene (DR1461) or from a strain containing no recA+ gene (DR1432) (Sancar and Rupp, 1979).}

\[ \text{The abbreviation used is: ATPyS, adenosine 5'-0-(3-thiotriphosphate).}\]
A, ATPase activity in the absence of DNA; C and D, DEAE-cellulose. One ml of each fraction was assayed for ATPase activity under standard conditions. The volume of each fraction was 20 ml for hydroxyapatite and 2.5 ml for DEAE-cellulose.

- A, ATPase activity in the presence of ΦX174 single-stranded phage DNA; O, ATPase activity in the absence of DNA; △, A260. The straight lines in B and D indicate the concentration gradients of potassium phosphate and KCl, respectively.

![Graph showing ATPase activity](image)

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Fig. 1. Chromatography of recA protein. A and B, hydroxyapatite; C and D, DEAE-cellulose. One ml of each fraction was assayed for ATPase activity under standard conditions. The volume of each fraction was 20 ml for hydroxyapatite and 2.5 ml for DEAE-cellulose.

- A, ATPase activity in the presence of ΦX174 single-stranded phage DNA; C and D, ATPase activity in the absence of DNA; △, A260. The straight lines in B and D indicate the concentration gradients of potassium phosphate and KCl, respectively.

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![Graph showing ATPase activity](image)

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Fig. 2. Purification of recA protein. Preparations from each step of purification were examined by electrophoresis through a slab (14 cm × 15 cm × 1.6 mm) of 10% acrylamide gel containing 0.1% sodium dodecyl sulfate. Electrophoresis was at 20 mA for 5 h. Protein in the gel was stained by Coomassie brilliant blue. About 100 units of DNA-dependent ATPase activity of each fraction were loaded on channels c, d, e, and g. a, 14 μg of fraction II from hydroxyapatite column (Fig. 1). b, 60 μg of cell-free extract, fraction I. c, 23 μg of Polyvinyl P-(NH₄)₂SO₄, fraction II. d, 14 μg of the pooled fractions from the first peak from the hydroxyapatite column (Fig. 1), fraction III. e, 9.6 μg of pooled fractions from the Sephacryl S200 column, fraction IV. f and g, 1 and 11 μg, respectively, of pooled fractions from the DEAE-cellulose column (Fig. 1), fraction V. h, 10 μg of the fraction that contained the greatest activity of DNA-independent ATPase activity from the Sephacryl S200 column, i, 7 μg of fractions 13 and 14 from the DEAE-cellulose column (Fig. 1).

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Purification of RecA Protein

- The last column, were 25, 23, 24, and 28 units/10⁻³ A₂₆₀ units (A₂₆₀ unit = A₂₆₀ × volume in milliliters) (Fig. 1, C and D).

Under the conditions that we used to study the promotion of homologous pairing by recA protein (Shibata et al., 1979a), fraction V produced less than 0.2% acid-soluble material from either linear double-stranded or single-stranded DNA and caused the nicking of 2% or less of form I DNA. We detected neither topoisomerase nor ligase activity (Cunningham et al., 1979).

Since the major protein in fraction II was recA protein (Fig. 2, lane c), the specific activity of single-stranded DNA-dependent ATPase activity increased only 2-fold during further purification (Table I).

We could not detect the pairing activity of recA protein (see below) in fraction II, but we could detect that activity in fraction III and later fractions. In fraction II, the trapping of double-stranded DNA to a nitrocellulose filter by the D-loop assay was mostly independent of homologous single-stranded fragments.

From 100 g of induced cells, we got about 190 mg of recA protein in fraction III and 60 mg in fraction V (Table I).

Purified recA protein behaved as a single polypeptide with molecular weight of about 40,000 on electrophoresis through a 10% acrylamide gel containing 0.1% sodium dodecyl sulfate. The following proteins served as standards: bovine serum albumin (Mr = 67,000), ovalbumin (Mr = 45,000), and the α subunit of E. coli RNA polymerase (Mr = 39,000) (data not shown). On gel filtration through Sephacryl S200 (Pharmacia), recA protein exhibited a single peak near the void volume in a buffer containing 0.3 M (NH₄)₂SO₄, which suggests that the active protein is larger than the monomer of Mr = 40,000 (see Ogawa et al., 1979).

In 0.02 M K phosphate (pH 6.8), 2-mercaptoethanol, 10% glycerol, the half-life of the ATPase activity of 25 μM recA protein (fraction III) was 37 min at 51°C and 10–12 min at 52°C. Similarly, when fraction IV was incubated for 5 min in 30 mm potassium phosphate buffer (pH 7.6) containing 10% glycerol, ATPase activity was not affected below 50°C, but was completely inactivated above 55°C. RecA protein was stable at 0°C in phosphate buffer (pH 6.8) or Tris buffer (pH 7.5) containing 10% glycerol and either 5 mm 2-mercaptoethanol or 10 mm dithiothreitol, since no loss in DNA-dependent ATPase activity was detected during more than a year of storage in those buffers.
Activities of RecA Protein

DNA-dependent ATPase—Ogawa et al. (1979) and Roberts et al. (1979) discovered that recA protein has ATPase activity that depends upon the presence of single-stranded DNA. In our standard assay for DNA-dependent ATPase (Scott et al., 1977; Shibata et al., 1979a), hydrolysis of ATP was linearly dependent on the concentration of recA protein, up to 2 μM (Fig. 3).

Superhelical DNA (form I DNA) supported the ATPase activity of recA protein well in the presence of 1.2 mM MgCl₂ at pH 7.5, but relaxed double-stranded DNA, form II or form IV, did not work well under these conditions (Figs. 4 and 5). Superhelical DNA was a good cofactor only when the concentration of MgCl₂ was 1 to 2 mM (Fig. 4), whereas single-stranded DNA was effective in supporting ATPase activity in 1 to 7 mM MgCl₂.

Binding of RecA Protein to DNA—In order to assay rapidly the binding of recA protein to DNA, we sought conditions under which the retention of either single-stranded or double-stranded DNA by a nitrocellulose filter would depend upon the presence of recA protein. Two factors appeared to be important in the use of nitrocellulose filters to measure the binding of protein to DNA: these were the kind of filter and the ionic conditions (Table II). The Sartorius filter, SM11306 (pore size, 0.45 μm) trapped 24 to 29% of single-stranded DNA in the absence of recA protein or added NaCl (Table II, line 1). The addition of NaCl to the buffer used during the filtration increased the retention of single-stranded DNA either by the Millipore filter DAWP (pore size, 0.65 μm) or by the Sartorius filter, SM11306 (Table II, lines 1a and 1b). In 1 mM NaCl, SM11306 filters retained 100% of single-stranded DNA whereas DAWP filters retained only 13%. NaCl at 0.2 mM significantly increased the efficacy of trapping of single-stranded DNA by SM11306 filters but had no effect on the trapping by DAWP filters (Table II, line 1). Neither SM11306 filters nor DAWP filters retained double-stranded DNA alone in the absence or in the presence of NaCl up to 1 mM (Table II, line 4).

Accordingly, we studied the binding of recA protein to DNA using principally the Millipore filter DAWP with or without 0.2 mM NaCl. Under these conditions, recA protein caused the retention of some 20% of single-stranded DNA (Table II, line 2) but no double-stranded DNA (Table II, line 5). However, in the presence of the ATP analog, 5′-O-(3-thiotriphosphate), recA protein caused the retention of all of the single-stranded DNA (Table II, line 3) and 8 to 16% of double-stranded DNA (Table II, line 6). When we substituted the SM11306 filter for the DAWP filter, recA protein caused the retention of half of the double-stranded DNA in the presence of ATPyS (Table II, line 6). The effect of recA protein on the retention of DNA by nitrocellulose cannot be attributed to a nonspecific effect of protein, since all of the reaction mixture in these experiments contained 88 μg of bovine serum albumin/ml. We conclude that recA protein binds both to single-stranded and double-stranded DNA in the presence of ATPyS. When added to recA protein and single- or double-stranded DNA, ATPyS increased the retention of DNA by nitrocellulose filters, either by enhancing the binding of DNA to recA protein or by changing the size or shape of the protein-DNA complexes. On the basis of increased stability of complexes, we have argued elsewhere (Shibata et al., 1979a) that ATPyS enhances the binding of recA protein to DNA.

In the presence of ATPyS, single-stranded DNA, whether homologous or not, increases the retention of double-stranded DNA by nitrocellulose filters (Table II, lines 6-9). This observation, reported in more detail elsewhere (Shibata et al., 1979b), reveals the formation of a ternary complex of recA protein with single-stranded and double-stranded DNA. Elsewhere, we present kinetic evidence that such a ternary complex is an intermediate whose conversion to a D-loop limits the rate of the reaction (Radding et al., 1980).
DNA, 8.8 µm fd form I, or 12 µm fd single-stranded DNA or fragments thereof, was incubated with 2.4 µm recA protein (fraction V) at 37 °C for 30 min as described under "Materials and Methods," except that spermidine was omitted. After incubation, we measured the retention of labeled DNA by nitrocellulose filters (see Assay C under "Materials and Methods").

| TABLE II

| Binding of DNA by recA protein |
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For purposes of this table, the standard reaction mixture contained fd form II DNA and fragments of single-stranded fd DNA plus other components described under "Materials and Methods." Variations from this standard mixture are as noted. Double-stranded DNA (4.4 µm) and single-stranded DNA (6 µm) were incubated with 2.2 µm recA protein (fraction V) in the standard reaction mixture at 37 °C for 20 min, and D-loops were assayed as described under "Materials and Methods." Fd form II DNA was 62% form II and 38% form III; fd form I DNA contained more than 70% form I. φX174 form II DNA was free of detectable form I or form III DNA.

**Unwinding of Double-stranded DNA**—Since recA protein will catalyze the formation of a D-loop with nonsuperhelical DNA, it may be capable of unwinding duplex DNA to some extent. Initially, we were unable to find direct evidence of unwinding. The key to the unwinding reaction was provided by the observation that heterologous single strands stimulate the unwinding reaction. We depurinated the product prior to unwinding by treating it with 0.5% Sarkosyl at 75 °C for 5 min (see McEntee et al., 1979) followed by heating at 41 °C for 4 min in 1.5 M NaCl, 0.15 M Na-citrate. We also changed the standard reaction mixture, since we observed anomalous kinetics in the formation of D-loops from form I DNA under the conditions used in previous studies (Shibata et al., 1981). The standard reaction mixture used in the present experiments contained 6.7 mM MgCl₂, 2.0 mM spermidine, 1.3 mM ATP in addition to recA protein and DNA (see "Materials and Methods"). Under these conditions, the requirements for pairing single strands with form II DNA were much as seen before for form I DNA. The pairing reaction required homologous DNA, ATP, recA protein, and divalent cation (Table III). Spermidine, 2 mM, could only partly replace Mg²⁺ (Table III, lines h–j; and Shibata et al., 1981). The pairing reaction was inhibited by ATPγS (Table I, line 8) a competitive inhibitor of the ATPase activity of recA protein (Shibata et al., 1979b). In an accompanying paper (Shibata et al., 1981), we describe the characterization of D-loops formed by superhelical and nonsuperhelical DNA.
centrifugation in CsCl-ethidium bromide or by electrophoresis in agarose gels (Fig. 6). In these gels, unwound and ligated DNA appeared as a band at the same position as natural form I DNA. When DNA was ligated but not unwound, it appeared as a ladder-like set of bands between form III and II DNA (Fig. 6).

Like the formation of D-loops (Shibata et al., 1979a) no unwinding occurred until the concentration of recA protein exceeded some minimal amount. At a limiting concentration of recA protein, excess single-stranded DNA inhibited unwinding (Fig. 6, a-g). Optimal unwinding occurred when the ratio of single-stranded DNA to recA protein was 2.5 to 5 residues of nucleotide/molecule of recA protein (Fig. 6). Oligonucleotides could substitute for single-stranded DNA, but concentrations were needed to promote the same extent of unwinding (Fig. 6).

**DISCUSSION**

There are four potential assays for recA protein based on its ATPase (Ogawa et al., 1979; Roberts et al., 1979), protease (Roberts et al., 1978), unwinding (Cunningham et al., 1979), and pairing activities (Weinstock et al., 1979; Shibata et al., 1979a). Of these, the ATPase assay is the most suitable for purification of recA protein. Particularly in a strain in which the recA gene has been cloned on a plasmid, the recA protein is the principal ATPase activity that requires single-stranded DNA as a cofactor. The ATPase activity is readily assayed (Scott et al., 1977) and is directly proportional to the amount of recA protein (Fig. 3). The pairing activity is not readily detectable at early stages in the isolation and purification, and it is not a linear function of the concentration of recA protein (Shibata et al., 1979a; Shibata et al., 1981).

The assay for pairing does not specifically detect the formation of D-loops but rather detects the conversion of wholly duplex DNA to a partially single-stranded form. Controls are required to demonstrate in any particular instance that the assay is measuring the formation of a joint molecule. Since our preparations of recA protein lack nuclease activity, we have not encountered high assay values due to partial degragation of double-stranded DNA. The need to detach protein from the DNA prior to filtration through nitrocellulose imposes another limitation on the assay. As a function of the conditions for removing protein, some joint molecules do not survive the assay (Shibata et al., 1981). Nonetheless, the assay, which is fast and reproducible, has lent itself well to the study of the recA protein which pairs a number of topological variants of DNA thereby producing joint molecules that are sufficiently stable and that stick to nitrocellulose (Shibata et al., 1979a, 1981; DasGupta et al., 1980; Cunningham et al., 1980).

The recA gene plays a central role in the metabolism of DNA in E. coli, most notably in repair and recombination (see Introduction). In a general way, the importance of having the purified gene product and functional assays is evident. More particularly, since recA protein catalyzes the homologous pairing of DNA molecules, it provides a critical reagent for the study of recombination in vitro, a reagent with which we can hope to discover both steps that lead up to homologous pairing and steps that resolve the intermediates produced.

**Acknowledgments**—We gratefully acknowledge the expert technical assistance of Lynn Osterh. We thank Drs. Azz Sancar and Dean Rupp for the generous gift of the strain of E. coli containing the recA gene cloned on a plasmid.

**REFERENCES**

Beattie, K. L., Wiegand, R. C., and Radding, C. M. (1977) J. Mol. Biol. 116, 783-803
Blakesley, R. W., and Wells, R. D. (1975) Nature 257, 421-422
Brooks, K., and Clark, A. J. (1967) J. Virol. 1, 283-293
Christiansen, C., and Baldwin, R. L. (1977) J. Mol. Biol. 115, 441-454
Clark, A. J., and Margulies, A. D. (1965) Proc. Natl. Acad. Sci. U. S. A. 53, 451-459
Cunningham, R. P., Shibata, T., DasGupta, C., and Radding, C. M. (1979) Nature 281, 191-196, 282, 426
Cunningham, R. P., DasGupta, C., Shibata, T., and Radding, C. M. (1980) Cell 20, 223-235
DasGupta, C., Shibata, T., Cunningham, R. P., and Radding, C. M. (1980) Cell 22, 437-446
Defais, M., Fauquet, P., Radman, M., and Errera, M. (1971) Virology 43, 495-503
Emmerson, P. T., and West, S. C. (1977) Mol. Gen. Genet. 155, 77-85
George, J., Devoret, R., and Radman, M. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 144-147
Gudas, L. J., and Mount, D. W. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5280-5284
Gudas, L. J., and Pardee, A. B. (1976) J. Mol. Biol. 101, 459-477
Hartman, I., and Luria, S. E. (1967) J. Mol. Biol. 23, 117-133
Holloman, W. K., and Radding, C. M. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 3910-3914
Horui, K., and Zinder, N. D. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2555-2558
Howard-Flanders, P., and Boyce, R. P. (1966) Radiat. Res. 6, (suppl.) 156-184
Howard-Flanders, P., and Theriot, L. (1966) Genetics 53, 1137-1150
Inouye, M. (1971) J. Bacteriol. 106, 539-542
Inouye, M., and Guthrie, J. P. (1969) Proc. Natl. Acad. Sci. U. S. A. 64, 957-961
Inouye, M., and Pardee, A. B. (1970) J. Biol. Chem. 245, 5813-5819
Kenyon, C. J., and Walker, G. C. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 2819-2823
Kobayashi, I., and Ikeda, H. (1978) Mol. Gen. Genet. 166, 25-29
Kuhnlein, U., Penhoet, E. E., and Linn, S. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 1169-1173
Lark, K. G., and Lark, C. A. (1979) Cold Spring Harbor Symp. Quant. Biol. 43, 537-549
Little, J. W., and Kleid, D. G. (1977) J. Biol. Chem. 252, 6151-6252
Madsen, H. S., Pollard, E. C., Ginoza, W., and Randall, E. P. (1974) J. Bacteriol. 118, 465-470
Matsumoto, K., Ando, T., Saito, H., and Ikeda, Y. (1979) Agric. Biol. Chem. 43, 407-408
McEntee, K. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5275-5279
McEntee, K., and Epstein, W. (1977) Virology 77, 306-318
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McEntee, K., Weinstock, G. M., and Lehman, I. R. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 2615-2619

Miura, A., and Tomizawa, J. (1968) Mol. Gen. Genet. 103, 1-10

Monod, J., Cohen-Bazire, G., and Cohn, M. (1951) Biochim. Biophys. Acta 7, 585-599

Ogawa, T., Wabiko, H., Tsurimoto, T., Horii, T., Masukata, H., and Ogawa, H. (1979) Cold Spring Harbor Symp. Quant. Biol. 43, 909-915

Pollard, E. C., and Randall, E. P. (1973) Radiat. Res. 55, 265-279

Pulleyblank, D. E., and Morgan, A. R. (1975) Biochemistry 14, 5205-5209

Radding, C. M., Beattie, K. L., Holloman, W. K., and Wiegand, R. C. (1977) J. Mol. Biol. 116, 825-839

Radding, C. M., Shibata, T., Cunningham, R. P., DasGupta, C., and Osber, L. (1980) in Mechanistic Studies of DNA Replication and Genetic Recombination (Alberts, B., and Fox, C. F., eds) Vol. 19, pp. 863-870, Academic Press, New York

Roberts, J. W., Roberts, C. W., and Craig, N. L. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 4714-4718

Rupp, W. D., Wilde, C. E., III, Reno, D. L., and Howard-Flanders, P. (1971) J. Mol. Biol. 61, 26-44

Sancar, A., and Rupp, W. D. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 3144-3148

Sanger, F., Air, G. M., Barrell, B. G., Brown, N. L., Coulson, A. R., Fiddes, J. C., Hutchison, C. A., III, Slocombe, P. M., and Smith, M. (1977) Nature 265, 687-695

Satta, G., Gudas, L. J., and Pardee, A. B. (1979) Mol. Gen. Genet. 188, 69-80

Scott, J. F., Eisenberg, S., Bertsch, L. L., and Kornberg, A. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 193-198

Sharp, P. A., Sugden, B., and Sambrook, J. (1973) Biochemistry 12, 3055-3065

Shibata, T., DasGupta, C., Cunningham, R. P., and Radding, C. M. (1979a) Proc. Natl. Acad. Sci. U. S. A. 76, 1638-1642

Shibata, T., Cunningham, R. P., DasGupta, C., and Radding, C. M. (1979b) Proc. Natl. Acad. Sci. U. S. A. 76, 5100-5104

Shibata, T., DasGupta, C., Cunningham, R. P., and Radding, C. M. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 2606-2610

Shibata, T., DasGupta, C., Cunningham, R. P., Williams, J. G. K., Osber, L., and Radding, C. M. (1981) J. Biol. Chem. 256, 7565-7572

Shortle, D. R., Margalloske, R. F., and Nathans, D. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 6128-6131

Weigle, J. J. (1953) Proc. Natl. Acad. Sci. U. S. A. 39, 628-636

Weinstock, G. M., McEntee, K., and Lehman, I. R. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 126-130

Wiegand, R. C., Godson, G. N., and Lehman, I. R. (1975) J. Biol. Chem. 250, 8848-8855

Williams, J. G. K., Shibata, T., and Radding, C. M. (1981) J. Biol. Chem. 256, 7573-7582

Witkin, E. M. (1967) Proc. Natl. Acad. Sci. U. S. A. 57, 1275-1279

Witkin, E. M. (1969) Mutat. Res. 8, 9-14