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Purified *Escherichia coli recA* protein catalyzes homologous pairing of superhelical DNA and single-stranded fragments

(genetic recombination/strand uptake/D-loops/enzymic formation of D-loops/ATPase)

TAKEHIKO SHIBATA, CHANCHAL DASGUPTA, RICHARD P. CUNNINGHAM, AND CHARLES M. RADDING

The Departments of Internal Medicine, and Molecular Biophysics & Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06510

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ABSTRACT **Purified *Escherichia coli recA* protein catalyzed ATP-dependent pairing of superhelical DNA and homologous single-stranded fragments. The product of the reaction: (*i*) was retained by nitrocellulose filters in 1.5 M NaCl/0.15 M Na citrate at pH 7, (*ii*) was dissociated at pH 12.3 but was not dissociated by heating at 55°C for 4 min or by treatment with 0.2% sodium dodecyl sulfate and proteinase K, (*iii*) contained covalently closed circular double-stranded DNA (form I DNA), (*iv*) contained single-stranded fragments associated with replicative form (RF) DNA, and (*v*) contained a significant fraction of D-loops as judged by electron microscopy. Linear and nicked circular double-stranded DNA did not substitute well for superhelical DNA; intact circular single-stranded DNA did not substitute well for single-stranded fragments. Homologous combinations of single-stranded fragments and superhelical DNA from phages φX174 and fd reacted, whereas heterologous combinations did not. The reaction required high concentrations of protein and $MgCl_2$. The ATPase activity of purified *recA* protein was more than 98% dependent on the addition of single-stranded DNA. In 1 mM $MgCl_2$, the ability of superhelical DNA to support the ATPase activity was two-thirds as good as that of single-stranded DNA.**

Two postulates guide current work on general genetic recombination: first, that breakage and reunion of DNA underlie recombination, and second, that reunion is accomplished by a molecular splice, called a heteroduplex joint, in which a strand from each parent pairs with its complement. While the heteroduplex joint ultimately effects reunion, we do not know how the joint is made. Because breakage by various means stimulates recombination, some investigators have favored the idea that breakage of one molecule generates a single strand that may pair with its double-stranded homolog and consequently provoke cleavage of the second molecule (ref. 1 and Fig. 1). Holloman *et al.* (5) observed the homologous pairing of single strands with superhelical DNA at high temperatures in the absence of any protein, and suggested that this reaction, which they called strand uptake, might be part of the molecular basis for the initiation of genetic recombination. When they transfected spheroplasts of *Escherichia coli* with mixtures of superhelical φX174 DNA and single-stranded fragments, Holloman and Radding (6) observed that the production of recombinant phage depended on both superhelicity and *recA*$^+$ function. The need for *recA*$^+$ was bypassed, however, when cells were transfected by complexes of superhelical DNA and single-stranded fragments, an observation that implicated recA$^+$ in the interaction of a single strand with double-stranded DNA. Given the pleiotropy of *recA*, its role in the interaction of single-stranded and double-stranded DNA might well have been indirect. Indeed, while *recA* has a profound effect on the frequency of recombination in *E. coli*, until recently no published evidence demonstrated that the *recA* protein acts directly in recombination. The discovery by Ogawa *et al.* (7) and by Roberts *et al.* (8) that *recA* protein has ATPase activity that depends upon single-stranded DNA suggested a direct involvement of the protein in recombination and repair of DNA. Recent observations of Kobayashi and Ikeda (9) on the effect of temperature-sensitive *recA* product on recombination in the absence of protein synthesis likewise argue for a direct role of *recA* protein.

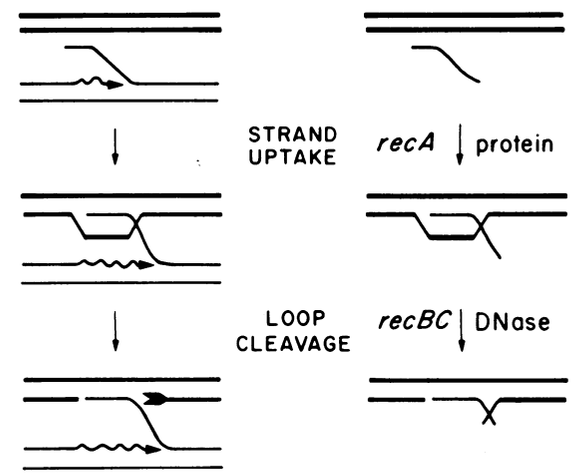

FIG. 1. Initiation of genetic recombination. On the left is a hypothetical sequence. Strand displacement by a polymerase produces a redundant strand, which is taken up by a homologous molecule. Uptake of a donor strand results in the cleavage and digestion of the strand displaced from the recipient molecule, opening the latter to propagation of a strand transfer. The model proposed by Meselson and Radding relates the last structure to reciprocal exchanges (2). On the right is a diagram of experiments which partially simulate the hypothetical sequence for initiation. This report describes the catalysis of strand uptake by an enzymatic activity associated with purified *recA* protein. Previous papers have described the enzymatic cleavage of D-loops by *recBC* DNase as well as several other endonucleases (3, 4).

The cloning of the *recA* gene by McEntee and Epstein (10) and the discovery of the DNA-dependent ATPase activity of *recA* protein (7, 8) enabled us to purify the protein highly and to examine its role in the uptake of a single strand by superhelical DNA. While our experiments were in progress, Weinstock *et al.* (11) kindly sent us their manuscript, prior to publication,

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Abbreviations: The so-called replicative forms (RF) of double-stranded DNA of phages φX174 or fd include DNA that is (*i*) superhelical (RFI or form I), (*ii*) nicked and circular (form II), (*iii*) linear (form III), and (*iv*) relaxed but circular and closed (form IV). $NaDodSO_4$, sodium dodecyl sulfate; 10 × NaCl/Cit, 1.5 M NaCl/0.15 M Na citrate at pH 7.

in which they reported that *recA* protein catalyzes the renaturation of complementary single strands of DNA.

METHODS

DNA. From phages ϕX174 and fd we prepared forms I, II, and III as described (12), and we prepared relaxed closed circular DNA (form IV) from form I by using an extract of nuclei from calf thymus as described by Pulleyblank and Morgan (13). Amounts of DNA are expressed in moles of nucleotide.

Strains of *E. coli*. The following strains are derivatives of KM4104 (14), which has a deletion in the *recA-srl* region. The plasmids in these strains are derivatives of pBR 322 (15). These strains were the generous gift of A. Sancar and W. D. Rupp*: (*i*) DR 1453. The plasmid, pDR 1453, carries a piece of the *recA-srl* region that contains 13,000 base pairs and makes the strain phenotypically Rec$^+$ and Srl$^+$. (*ii*) DR 1461. The plasmid, pDR 1461, has only part of *recA*, and the strain is phenotypically RecA$^-$.

Purification of *recA* Protein. From 100 g of DR 1453 induced by growth in 40 μg of nalidixic acid per ml we purified *recA* protein to apparent homogeneity by the steps indicated in Fig. 2. We will publish the details of the purification later.

Formation of D-Loops by *recA* Protein. In the experiments reported here, we made D-loops by a reaction divided into two stages. In the first, *recA* protein reacted with form I DNA; in the second, we added single-stranded DNA and raised the concentration of $MgCl_2$. The mixture for the first stage was composed of 13.5 μl of 43 mM Tris·HCl at pH 7.5, 1.6 mM $MgCl_2$, 2.7 mM dithiothreitol, 0.13 mg of bovine serum albumin per ml, 13.3 μM RFI [^{3}H]DNA, 2 mM ATP, and *recA* protein. After incubating the mixture at 37°C for 20 min, we chilled it on ice and added 5 μl of single-stranded fragments in 10 mM Tris·HCl at pH 7.5 and 0.1 mM EDTA, 1 μl of 1 M $MgCl_2$, and 1 μl of 26 mM ATP. Incubation for the second stage of the reaction was for 60 min at 37°C. (Subsequently, we observed that the reaction does not require two stages and that the order of addition of reactants is not critical.) We stopped the reaction by chilling the sample on ice and adding 0.5 ml of 25 mM EDTA at pH 7 or 9. We spotted 50 μl on a nitrocellulose filter to measure total radioactivity; we added 200 μl to 3 ml of 1.5 M NaCl/0.15 M Na citrate at pH 7 (10×NaCl/Cit) and heated at 50°C for 4 min before filtering the sample through nitrocellulose to detect D-loops as described (12). With another aliquot of 200 μl, we measured the fraction of nicked molecules by the method of Kuhnlein *et al.* (16).

ATPase. The standard reaction mixture, 18 μl, contained 35 mM Tris·HCl at pH 7.5, 6.7 mM $MgCl_2$, 2 mM dithiothreitol, 100 μg of bovine serum albumin per ml, 1.4 mM [^{3}H]ATP or [α-^{32}P] ATP, 50 μM single-stranded ϕX174 DNA, and *recA* protein. After an incubation at 37°C for 30 min, we added excess EDTA, and unlabeled ATP, ADP, and AMP as carriers, and assayed their labeled counterparts by thin-layer chromatography (17). We defined a unit of ATPase activity as the amount that hydrolyzes 1 nmol of ATP under the above conditions.

Electrophoresis. Proteins were subjected to electrophoresis in 10% polyacrylamide gels containing sodium dodecyl sulfate ($NaDodSO_4$) (Fig. 2) according to O'Farrell and Gold (18). Electrophoresis of DNA in 1.4% agarose gels was done substantially as described by Sharp *et al.* (19) except that we stained the gels with ethidium bromide only after electrophoresis.

RESULTS

Purification of *recA* Protein. By assaying DNA-dependent ATPase, we purified a protein of M_r 42,000 from a strain carrying the *recA* gene cloned in a plasmid. At each stage in the purification, we examined the polypeptides by electrophoresis in acrylamide gels containing 0.1% $NaDodSO_4$ (Fig. 2). A major band present in the extract corresponded to a polypeptide of molecular weight about 42,000, which is the size of *recA* protein reported by McEntee (20). This band was absent from extracts of: (*i*) the parental *recA*$^-$ strain lacking the *recA*$^+$ plasmid, (*ii*) the parental *recA*$^-$ strain carrying a plasmid with only part of the *recA* gene, and (*iii*) uninduced cells of the strain carrying the *recA*$^+$ plasmid.

In fraction III, only a third of the ATPase activity depended upon the addition of single-stranded DNA; in the most purified preparation, more than 98% of the activity depended on single-stranded DNA, and electrophoresis of 1 or 6 μg of protein revealed only a single band of polypeptide (Fig. 2, channels i, k, and l). Through five chromatographic steps there was no indication of more than one DNA-dependent ATPase activity; in each step half or more of the ATPase activity was recovered as a single peak. The specific activity of the DNA-dependent ATPase increased about 3-fold between fraction III and fraction VII, which corresponds qualitatively with the relative enrichment of the band of putative *recA* protein (Fig. 2, channel h vs. c). There was a 2-fold increase in specific activity between

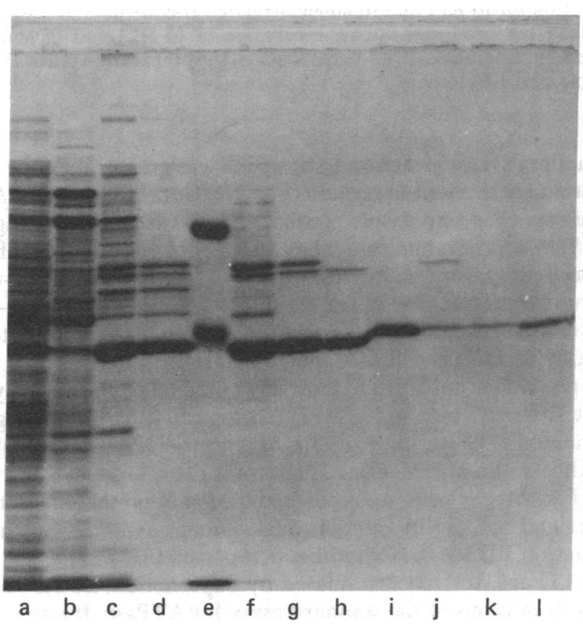

FIG. 2. Purification of *recA* protein. Electrophoresis in a polyacrylamide gel containing 0.1% $NaDodSO_4$. In channels c–h, we loaded about 100 units of ATPase activity from each fraction. a, Lysate, fraction I, 60 μg; b, 0.5 M NaCl extract of Polymin P precipitate, 50 μg; c, 1.0 M NaCl extract, precipitated by $(NH_4)_2SO_4$ at 50% saturation, fraction III, 29 μg; d, hydroxyapatite, fraction IV, 20 μg; e, standards for estimation of molecular weight, from top to bottom, bovine serum albumin (M_r 67,000), ovalbumin (M_r 45,000), and cytochrome *c* (M_r 12,000); f, phosphocellulose, fraction V, 22 μg; g, Sephacryl S200, fraction VI, 11 μg; h, DEAE-cellulose, fraction VII, 8 μg; i, peak fraction from ATP-agarose, fraction VIII, 6 μg; j, passthrough from ATP-agarose, 2 μg; k and l, other fractions from the peak of activity eluted from ATP-agarose, about 1 μg each. The apparent change in mobility of the polypeptides in i–l may be an artefact due to the buffer used to elute the protein from ATP-agarose.

* Sancar, A. & Rupp, W. D. (1979) *Abstracts of the Annual Meeting, American Society for Microbiology*, in press.

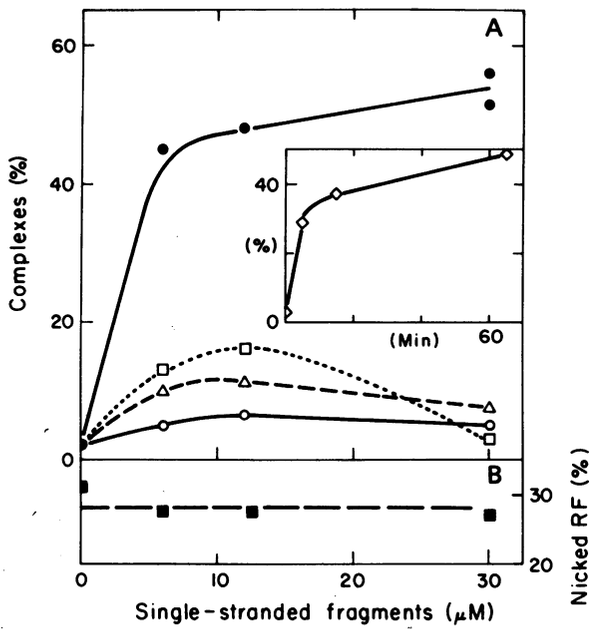

FIG. 3. Homologous pairing of single-stranded fragments and superhelical DNA catalyzed by *recA* protein. The reaction mixture contained 2 μg of fraction VII (Fig. 2). (*A*) Assay of complexes trapped on nitrocellulose filters in 10×NaCl/Cit. ●, fd RFI and fd single-stranded fragments; ○, fd RFI and φX174 single-stranded fragments; △, fd RFI and intact circular fd DNA; □, nicked fd RF and fd single-stranded fragments. This preparation of nicked DNA contained 71% form II, 8% form III, and 21% form I DNA. (*Inset*) Course of the reaction for fd RFI and fd single-stranded fragments. (*B*) Assay of nicked RF produced during incubation of fd RFI and fd single-stranded fragments. Prior to incubation, the preparation of RF DNA contained 70% form I.

fraction VII and fraction VIII, which was greater than the apparent enrichment in protein (Fig. 2, channel i vs. h). The ATPase specific activity of fraction VIII was 20,000 units/mg.

While other nuclease activities decreased sharply after chromatography on hydroxyapatite (fraction IV), ATP-dependent exonuclease in fractions V and VI still digested 4–7% of double-stranded DNA per μg of protein. After chromatography on DEAE-cellulose (fraction VII), as much as 0.22 mg of protein per ml failed to show any of the following nucleolytic activities: (*i*) nicking of RFI, (*ii*) digestion of linear single-stranded DNA, or (*iii*) digestion of linear double-stranded DNA either in the presence or absence of ATP.

Per 100 g of cells, we recovered 6.9 mg of protein in fraction VII and 2.1 mg in fraction VIII. Except as noted, we used fraction VII for the experiments reported below.

ATPase Activity Stimulated by Superhelical DNA. Under the conditions of the standard assay for ATPase, fraction VII *recA* protein had only 12% as much activity in the presence of double-stranded DNA as in the presence of single-stranded DNA. However, when the concentration of $MgCl_2$ was 1 mM, superhelical DNA supported two-thirds as much ATPase activity as single-stranded DNA. At the same concentration of $MgCl_2$, an equal mixture of nicked circular DNA (form II) and relaxed closed circular DNA (form IV) supported only one-fourth as much ATPase activity as single-stranded DNA. We concluded that the ATPase in fraction VII binds to superhelical DNA, presumably by virtue of the functional single-stranded character of such DNA (1).

ATP-Dependent Homologous Pairing of Single-Stranded Fragments and Superhelical DNA. To look for catalysis of strand-uptake by *recA* protein, we mixed it with superhelical

Table 1. Catalyzed pairing of RF DNA and single-stranded fragments

Exp.	Reaction mixture	Complexes, %
1 a	fd RFI + fd single-stranded fragments	54
b	fd RFI + φX174 single-stranded fragments	5
c	φX174 RFI + φX174 single-stranded fragments	66
d	φX174 RFI + fd single-stranded fragments	8
2 a	Complete, 50 mM $MgCl_2$	101
b	6 mM $MgCl_2$	26
c	1 mM $MgCl_2$	1
d	Minus *recA* protein	2
e	Minus single-stranded fragments	3
f	Minus ATP	2

The concentration of single-stranded fragments was 30 μM. The enzyme was 2 μg of fraction VII *recA* protein. In the experiments listed on lines 1c through 2f we removed protein by treatment with $NaDodSO_4$ and $CHCl_3$/isoamyl alcohol. Because this method sometimes resulted in poor recovery and possibly in overestimation of the fraction of complexes, for all other experiments we replaced it by the procedure described in *Methods*.

DNA and added single-stranded fragments. To detect a reaction, we used an assay that traps D-loops on nitrocellulose filters washed with 10×NaCl/Cit (12). Homologous combinations of superhelical DNA and single-stranded fragments from fd and φX174 yielded complexes, whereas the two heterologous combinations did not (Fig. 3; Table 1, lines 1 a–d). A preparation of nicked circular DNA was one-third as active as superhelical DNA (Fig. 3). Intact circular single-stranded DNA was one-fourth as active as single-stranded fragments. No reaction occurred in the absence of *recA* protein, single-stranded fragments, or ATP (Table 1, Figs. 3 and 4). The concentration

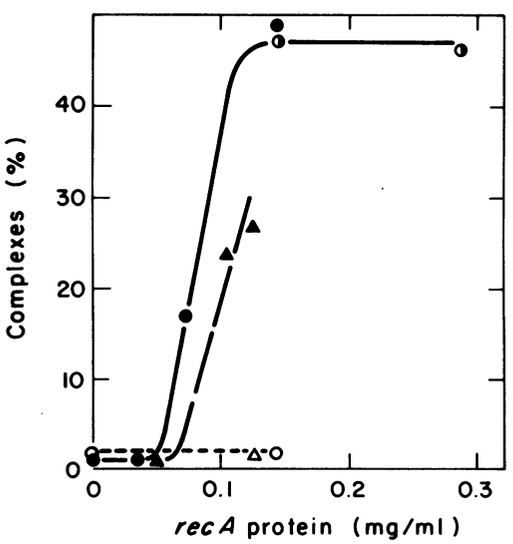

FIG. 4. Formation of complexes as a function of the concentration of *recA* protein. Fraction VII *recA* protein: ●, 9 μM fd RFI and 12 μM fd single-stranded fragments; ◑, 18 μM RFI and 12 μM fragments; ○, no single-stranded fragments. Fraction VIII *recA* protein: ▲, 9 μM RFI and 12 μM single-stranded fragments; △, no single-stranded fragments. In these experiments the preparation of form I DNA contained 30% form II DNA. The concentrations of *recA* protein are those existing in the first stage, before the addition of single-stranded fragments.

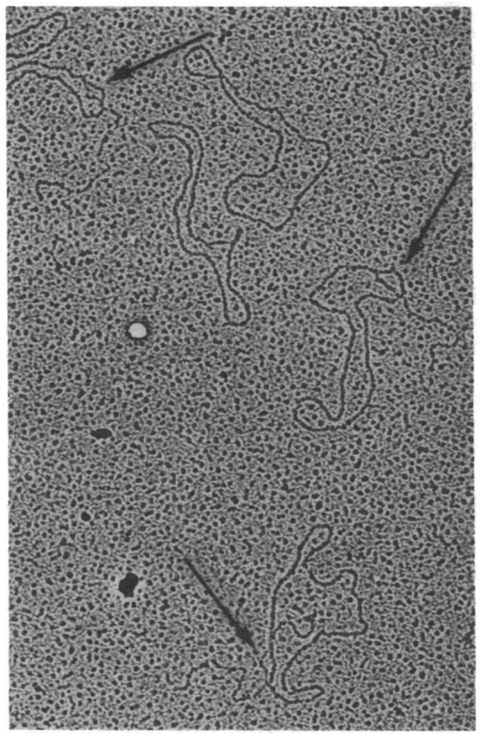

FIG. 5. Reaction product of fd RFI DNA, *recA* protein, and single-stranded fragments from fd. The DNA, in 50 mM Tris·HCl (pH 8.0)/5 mM EDTA/25% (vol/vol) formamide/0.01% cytochrome *c*, was spread on a hypophase of 10 mM Tris·HCl (pH 8.0)/1 mM EDTA. The samples were picked onto 3.5% parlodion-coated grids and rotary shadowed with Pt/Pd (4:1). The preparation contained 40% D-loops (arrows) according to the assay on nitrocellulose filters.

of $MgCl_2$ was important (Table 1). Ten minutes after the start of the second stage, the bulk of the reaction was over (Fig. 3 *inset*).

When there were about twice as many nucleotide residues of fragment as there were complementary sequences in superhelical DNA, the optimal concentration of *recA* protein corresponded to one polypeptide chain of M_r 42,000 per 5 nucleotide residues of fragment (Fig. 4). Per microgram, our most purified preparation of *recA* protein, fraction VIII, catalyzed strand uptake as well as fraction VII and showed the same sigmoidal relationship between the formation of complexes and the concentration of protein (Fig. 4). We used fraction VII for most experiments because it was more concentrated and more plentiful.

Characterization of Complexes. During the reaction there was no decrease in the fraction of covalently closed circular DNA (Fig. 3*B*). In most experiments the yield of complexes well exceeded the small fraction of form II and form III DNA present. Therefore the complexes contained covalently closed circular DNA, and trapping on the filters did not result from degradation of the RF DNA. Complexes were not dissociated by heating at 55°C for 4 min in 10×NaCl/Cit or by treatment with 0.2% $NaDodSO_4$ and 2 mg of proteinase K per ml at 37°C for 20 min. They were dissociated by incubation at pH 12.3 and room temperature for 3 min, but dissociation at alkaline pH was not accompanied by nicking, as might have occurred if protein were covalently linked to the circular DNA.

We prepared two reaction mixtures with *recA* protein and fd RFI DNA, one containing in addition single-stranded fragments from fd, the other single-stranded fragments from φX174. One of us (C.D.) examined the two samples by electron microscopy without knowing their identities. The samples were dilute. In the control that contained heterologous single-stranded fragments we found 65 identifiable circular molecules and no D-loops. In the other sample we found 45 identifiable molecules, 17 of which contained D-loops (Fig. 5).

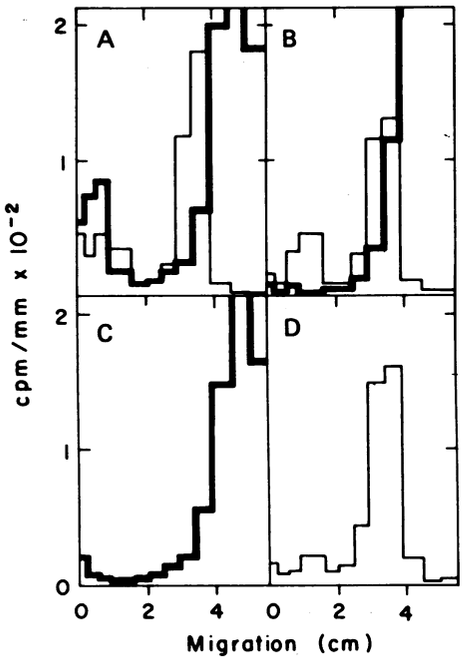

FIG. 6. —, φX174 RF [^{32}P]DNA; ▬, fragments of single-stranded φX174 [^{3}H]DNA. (*A*) Complete system: RF DNA, single-stranded fragments, and *recA* protein, (*B*) minus *recA* protein, (*C*) minus RF, (*D*) minus single-stranded fragments and *recA* protein. The complete reaction mixture, 83 μl, contained 10 μM φX174 form I [^{32}P]DNA (2.6×10^3 cpm/nmol), 12 μM φX174 single-stranded [^{3}H]DNA fragments (3.3×10^4 cpm/ml), 0.1 mg of fraction VII *recA* protein per ml, and other ingredients as described in *Methods*. The preparation of form I DNA contained only 6–12% nonsuperhelical DNA. The single-stranded fragments, prepared by boiling, were large, because they migrated near the position of intact circular single strands. After incubating the mixture as described in *Methods*, we added, per ml, 2 μg of $NaDodSO_4$ and 0.3 mmol of NaCl. We heated the sample at 55°C for 4 min, added 1 mg of proteinase K per ml, and incubated the mixture for 20 min at 37°C. We took one-fourth of the mixture to assay for D-loops and nicked molecules, and three-fourths to load on an agarose gel. In this experiment only 14% of form I DNA was converted into complexes according to the assay for D-loops, probably because many single-stranded circular molecules were intact and the fragments were large, on the average. After electrophoresis, we stained the gels with ethidium bromide and visualized the bands of DNA by illuminating the gel with UV light. With a razor, we cut the gel into pieces that were about 5 mm along the direction of migration, 7 mm wide, and 3 mm thick. We measured the length of each piece, which varied, in order to express the radioactivity as cpm per mm. We boiled the pieces for 2 min in 0.4 ml of 0.1 M HCl and neutralized the sample by adding 40 μl of 1 M NaOH. The radioactivity of the sample was measured in 4 ml of a Triton X-100 fluor (3) in a scintillation counter. The sample in D was not incubated prior to electrophoresis.

To look for direct evidence of the association of single-stranded fragments with RF DNA, we examined the product of the reaction by gel electrophoresis (Fig. 6). Only in the case of the complete reaction mixture did we find ^{3}H-labeled single-stranded fragments migrating with ^{32}P-labeled RF slightly behind the position of form II and form IV DNA. Without making any correction for nicked or relaxed RF present in the control sample of RF, we calculated that some 1300 residues of ^{3}H-labeled nucleotides were associated per molecule of RF DNA. This high value is consistent with the large size of the fragments used in this experiment (see legend to Fig. 6).

DISCUSSION

These experiments show that purified *recA* protein catalyzes the homologous pairing of single-stranded fragments with superhelical DNA, by a reaction that requires ATP and appears to produce a D-loop, the same structure that results from the uncatalyzed reaction under nonphysiologic conditions (3). Observations on the uncatalyzed reaction had revealed a large unfavorable change in enthalpy that may be explained by the need to unstack some base pairs in order to initiate strand uptake (12). Because it reacts with superhelical DNA (see *ATPase activity* above) and overcomes some rate-limiting step in the formation of D-loops, *recA* protein may act in part by unstacking base pairs.

We haven't yet excluded the possibility that the active principle in our preparation of purified *recA* protein is a trace contaminant. The evidence, however, supports the interpretation that *recA* protein itself is responsible for the observed strand uptake: (*i*) *recA* protein has DNA-dependent ATPase activity (7, 8), and the reaction that we observed requires ATP; (*ii*) Weinstock *et al.* (11) have shown by the use of a cold-sensitive *recA* mutant that the product of the *recA* gene is required for the renaturation of complementary single strands by purified *recA* protein; and (*iii*) the observations of Holloman and Radding implicated the *recA* gene in an interaction of superhelical DNA and homologous single-stranded fragments that leads to the formation of recombinants (6).

recA mutants are pleiotropic, and not all mutants have the same phenotype. The apparent multiple functions of the gene product may mirror the biological observations. Reported functions of the *recA* protein now include protease activity (21), ATPase activity dependent on single-stranded (7, 8) or superhelical DNA, renaturation of complementary single strands (11), and strand uptake by superhelical DNA.

As in uncatalyzed strand uptake (unpublished observation), circular single-stranded DNA works poorly as a donor, if at all, in the reaction promoted by fraction VII *recA* protein (Fig. 3), which suggests that the single strand must have a free end. According to the hypothesis illustrated in Fig. 1, two recombining molecules of DNA break at about the same place because the interaction of a broken molecule with an unbroken one provokes cleavage of the latter. Ross and Howard-Flanders (22) and Cassuto *et al.* (23) observed such provoked cleavage *in vivo* and *in vitro*. We have shown before that the *recBC* DNase and at least one other endonuclease from *E. coli* can cleave D-loops formed by strand uptake (3, 4).†

Demonstration of the existence in *E. coli* of an activity that catalyzes strand uptake supports the hypothesis that this reaction plays a role in the initiation of genetic recombination (5). The association of the activity with *recA* protein further strengthens the inference that the reaction observed *in vitro* is related to genetic recombination: Thus the products of two genes that are important for recombination in *E. coli* have been implicated in early steps of a pathway postulated by a number of investigators (Fig. 1, ref. 1). The ability to make joint molecules by using purified *recA* protein opens new possibilities for exploring *in vitro* both earlier steps in recombination, those that somehow initiate strand transfer, and later steps, those that resolve intermediate structures and sometimes effect reciprocal crossing-over (1, 2).

† The existence of more than one enzyme that can cleave D-loops may account in part for the dispensability of *recBC* for recombination in *E. coli*.

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