Homologous Pairing in Genetic Recombination

THE PAIRING REACTION CATALYZED BY ESCHERICHIA COLI recA PROTEIN*

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Purified recA protein, which is essential for genetic recombination of *Escherichia coli*, catalyzed ATP-dependent homologous pairing of double-stranded DNA and single-stranded fragments to form D-loops. When the double-stranded DNA was nicked circular DNA (form II) or linear DNA (form III), the reaction proceeded nearly linearly during 30 min of incubation at 37 °C. When the double-stranded DNA was superhelical (form I), anomalous kinetics was observed. This anomaly was suppressed by the addition of spermidine without affecting the final yield of D-loops. The formation of D-loops required stoichiometric amounts of recA protein, which were proportional to the concentration of single-stranded DNA but which were not affected by the concentration of double-stranded DNA. With form II or III DNA as the recipient for the formation of D-loops, the rate of the reaction was greatest when there was one monomer of recA protein/2-3 nucleotide residues of single-stranded DNA; larger amounts of single-stranded DNA inhibited the reaction. The formation of D-loops was half inhibited by 30 mM NaCl and by 0.6 mM ADP, one of the products of the reaction. The thermal stability of D-loops made by recA protein was the same as that of D-loops made by annealing. In addition to pairing linear single strands with duplex DNA, recA protein made joint molecules from single-stranded circular DNA and homologous form II or III DNA. According to these and previous observations (Cunningham, R. P., DasGupta, C., Shibata, T., and Radding, C. M. (1980) *Cell* 20, 223-235), recA protein will stably pair two molecules of DNA if one of them is single-stranded or partially single-stranded and if either molecule has a free end.

Until recently, studies on genetic recombination in vitro were limited by the lack of suitable assays for recombination, either for the complete reaction or for early steps involved in synopsis. Assays have been devised that appear to measure the overall process of site-specific (Gottesman and Gottesman, 1975; Mizuuchi et al., 1978) or general genetic recombination (Roeder and Sadowski, 1979; Potter and Dressler, 1979; Kolodner, 1980). As an approach to studying synopsis, we explored the spontaneous uptake of homologous single strands by superhelical DNA (Holloman et al., 1975). To do so, we devised a rapid simple assay which detects the trapping by nitrocellulose of intact duplex DNA by virtue of its attachment to single-stranded DNA (Beattie et al., 1977). Using this assay, we discovered that purified recA protein will catalyze the formation of D-loops from single-stranded fragments and homologous duplex DNA (Shibata et al., 1979a). The D-loop assay has permitted a rapid characterization of the pairing reaction catalyzed by recA protein, as described here and in other papers (Shibata et al., 1979a and 1979b; McEntee et al., 1979; Cunningham et al., 1980). Moreover, the D-loop assay has proven useful in assaying the pairing of a variety of DNA substrates by recA protein because the efficient formation of joint molecules requires that one molecule be single-stranded or partially so (this paper; Cunningham et al., 1980; DasGupta et al., 1980) and because homologous pairing by recA protein does not require superhelical DNA (McEntee et al., 1979; Cunningham et al., 1979).

**MATERIALS AND METHODS**

The recA protein used in the experiments described here was the DEAE-cellulose fraction V described in the preceding paper (Shibata et al., 1981). All other methods and special terms are as described there as well. Concentrations of DNA are expressed in moles of nucleotide residues.

**RESULTS**

The *Time Course of Formation of D-loops*—In the standard reaction mixture (legend to Fig. 1; Shibata et al., 1981), the formation of D-loops from form II or III DNA was roughly proportional to the period of incubation at 37 °C for 30 min or more (Fig. 1A). The standard conditions described here, involving the use of 2.0 mM spermidine plus 6.7 mM MgCl₂, are conditions that we sought after we observed the anomalous kinetics illustrated in Fig. 1B. With superhelical DNA, in the absence of spermidine, complexes trapped by nitrocellulose filters were formed within 2 min and then decayed rapidly, giving way to a more regular time course in which the fraction of double-stranded DNA retained by the filter gradually reached a stable value. Although complexes formed at 2 min (hereafter called 2-min complexes) decayed during further incubation in the reaction mixture (Fig. 1B), paradoxically they had the same resistance to treatment with detergent and the same heat stability as D-loops formed at a later stage of incubation. Moreover, the formation of 2-min complexes depended upon the presence of homologous single-stranded fragments. The decay of 2-min complexes in the reaction mixture (Fig. 1B) was not due to nicking of superhelical DNA since, as we have observed before (Shibata et al., 1979a), superhelical DNA was not nicked in the course of the reaction. The formation of 2-min complexes required superhelical...
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Fig. 1. Time course of formation of D-loops. A, Fd form II and III \[^{3}H\]DNA (4.4 \(\mu M\); 61% form II, 38% form III) were incubated with 6 \(\mu M\) single-stranded fragments of fd \[^{35}P\]DNA and 2.2 \(\mu M\) recA protein (fraction V) for the indicated periods at 37 °C in the standard reaction mixture (Shibata et al., 1981) which contained 31 mM Tris-HCl (pH 7.5), 6.7 mM MgCl\(_2\), 2.0 mM spermidine-HCl, 1.3 mM ATP, 1.8 mM dithiothreitol, 80 \(\mu g\) of bovine serum albumin/ml. D-loops were assayed after treatment with 0.5% Sarkosyl for 3 min at 17 °C, followed by heating for 4 min at 41 °C (\(\bullet\)) or 50 °C (\(\bigcirc\)) in 1.5 M NaCl, 0.15 M Na-citrate. B, form I fd \[^{3}H\]DNA (8.8 \(\mu M\); more than 76% form I), 12 \(\mu M\) single-stranded fragments of fd \[^{35}P\]DNA were incubated with 4.4 \(\mu M\) recA protein (fraction V) for the indicated time periods at 37 °C. D-loops were assayed as described above. \(\bigcirc\), standard reaction mixture (2.0 mM spermidine, 6.7 mM MgCl\(_2\)); \(\bigtriangleup\), spermidine was omitted, and 25 mM MgCl\(_2\) was present during the incubation with recA protein.

DNA; form II or III DNA did not give rise to 2-min complexes even in the absence of spermidine. The 2-min complex does not appear to be an obligatory intermediate in the formation of D-loops catalyzed by recA protein since (a) 2.0 mM spermidine strongly suppressed the formation of 2-min complexes without a large effect on the yield of D-loops (Fig. 1B) and (b) form II or III DNA did not give rise to 2-min complexes.

Because of this abnormality in the time course of formation of D-loops from form I DNA, we have done many experiments with form II or III DNA, even though spermidine suppresses the kinetic anomaly. Since the time course of the reaction is approximately linear for 30 min with form II or III DNA, the assay at 20 or 30 min measures the rate of the reaction (Fig. 1).

The Stoichiometric Requirement for recA Protein—As in the renaturation of DNA by helix-disturbing proteins (Alberts and Frey, 1970; Christiansen and Baldwin, 1977), the formation of D-loops requires stoichiometric amounts of recA protein (Shibata et al., 1979a; McEntee et al., 1979). No D-loops are formed until recA protein reaches some minimal concentration, following which the yield of D-loops rises sharply to some maximal value (Fig. 2). The concentration of single-stranded DNA determines how much recA protein is required, while similar variations in the concentration of double-stranded DNA have no effect on the required amount of protein (Figs. 2 and 3; Shibata et al., 1979b).

Fig. 2 shows the effect of single-stranded DNA under the standard condition used in the experiments described here, namely 2.0 mM spermidine plus 6.7 mM MgCl\(_2\). The data from a number of experiments are summarized in Fig. 3. Estimated from the extrapolated intercept with the abscissa (Fig. 2), the minimal amount of recA protein needed to form D-loops was 1 monomer/8 nucleotide residues of single-stranded DNA when the double-stranded DNA was form II and III and 1 monomer of recA protein/20 residues when the double-stranded DNA was form I (Fig. 3). The least amount of recA protein needed to form D-loops appears to have been the same whether the reaction mixture contained 2.0 mM spermidine plus 6.7 mM MgCl\(_2\) or 25 mM MgCl\(_2\) without spermidine, since data obtained under both conditions could be plotted on the same curve (Fig. 3A).

Further stoichiometric relationships are illustrated in Fig. 4. In these experiments, we used one concentration of form II DNA, held the concentrations of recA protein constant at various levels, and measured the synthesis of D-loops at 30 min as a function of the concentration of single-stranded DNA. We determined that the time course of D-loop synthesis was linear, or nearly so, for 30 min for the lowest and highest concentrations of recA protein used. As a function of the concentration of single-stranded DNA, the rate of synthesis of D-loops increased, reached a maximal value, and then

![Graph](https://example.com/graph1.png)

**Fig. 1.** Time course of formation of D-loops. A, Fd form II and III \[^{3}H\]DNA (4.4 \(\mu M\); 61% form II, 38% form III) were incubated with 6 \(\mu M\) single-stranded fragments of fd \[^{35}P\]DNA and 2.2 \(\mu M\) recA protein (fraction V) for the indicated periods at 37 °C in the standard reaction mixture (Shibata et al., 1981) which contained 31 mM Tris-HCl (pH 7.5), 6.7 mM MgCl\(_2\), 2.0 mM spermidine-HCl, 1.3 mM ATP, 1.8 mM dithiothreitol, 80 \(\mu g\) of bovine serum albumin/ml. D-loops were assayed after treatment with 0.5% Sarkosyl for 3 min at 17 °C, followed by heating for 4 min at 41 °C (\(\bullet\)) or 50 °C (\(\bigcirc\)) in 1.5 M NaCl, 0.15 M Na-citrate. B, form I fd \[^{3}H\]DNA (8.8 \(\mu M\); more than 76% form I), 12 \(\mu M\) single-stranded fragments of fd \[^{35}P\]DNA were incubated with 4.4 \(\mu M\) recA protein (fraction V) for the indicated time periods at 37 °C. D-loops were assayed as described above. \(\bigcirc\), standard reaction mixture (2.0 mM spermidine, 6.7 mM MgCl\(_2\)); \(\bigtriangleup\), spermidine was omitted, and 25 mM MgCl\(_2\) was present during the incubation with recA protein.

**Fig. 2.** Stoichiometric requirement for recA protein. The formation of D-loops versus the concentration of recA protein for 3 different concentrations of single-stranded DNA. Conditions of the standard reaction and assay were as described in Shibata et al., 1981. Incubation was at 37 °C for 20 min. The concentration of form II fd \[^{3}H\]DNA was held constant at 4.4 \(\mu M\) (nucleotide residues). The concentrations of single-stranded fragments, expressed as nucleotide residues, were: ■, 2 \(\mu M\); ▲, 4 \(\mu M\); ●, 5.9 \(\mu M\).

**Fig. 3.** Stoichiometric requirement for recA protein. The least amount of recA protein required to form D-loops versus the concentration of single-stranded DNA (●, ○) or double-stranded DNA (▲). The least amount of recA protein was taken from the extrapolated intercept like those in Fig. 2. When the concentration of duplex DNA was varied (▲), the concentration of single-stranded fragments was kept constant at 12 \(\mu M\). When the concentration of single-stranded DNA was varied (●, ○), the concentration of duplex DNA was held constant at 8.0 or 8.8 \(\mu M\). A, experiments with form II DNA as recipient for the formation of D-loops. The open symbols indicate experiments done using the standard reaction mixture (see Fig. 1) which contains 6.7 mM MgCl\(_2\), 2.0 mM spermidine. The closed symbols represent experiments in which spermidine was omitted and the concentration of MgCl\(_2\) was 25 mM. B, experiments done with form I DNA as the recipient for the formation of D-loops. The concentration of MgCl\(_2\) was 25 mM, and no spermidine was present.
decreased. For each concentration of recA protein studied, from 0.8 to 2.4 µM, the optimal rate of synthesis occurred at a ratio of about 2 nucleotide residues of single-stranded DNA/monomer of recA protein. This value is about half of what we previously estimated when form I DNA was the recipient ratio of single-stranded DNA to recA protein and 10 min later no D-loops (Radding et al., 1980), the optimal rate of synthesis, i.e., the height of each peak in Fig. 4, was proportional to the concentration of recA protein.

The decreased rate of synthesis at high ratios of single-stranded DNA to recA protein could be due to a relative lack of recA protein or to an inhibitory effect of excess single-stranded DNA. When we started a reaction with an optimal ratio of single-stranded DNA to recA protein and 10 min later added four times as much single-stranded DNA, the rate of formation of D-loops promptly decreased (Fig. 5). This prompt inhibition presumably means either that single strands can inhibit by binding to a second site on recA protein or that complexes of recA protein and DNA dissociate and reassociate more rapidly than we could detect in this experiment.

Effects of Mg"++ and Spermidine on the Pairing Reaction—In the absence of spermidine, the range of optimal concentration of Mg"++ was broader when superhelical DNA was the substrate for the formation of D-loops (Fig. 6). In 6.7 mM Mg"++, form II DNA yielded few D-loops. The addition of 1 to 2 mM spermidine to a reaction mixture containing 6.7 mM Mg"++ restored the synthesis of D-loops with form II DNA (Fig. 7). Similarly, 1.1 mM Mg"++ alone did not support the formation of D-loops with either form I or II DNA (Fig. 6), but the addition of 2 mM spermidine restored the reaction to normal levels for both forms of DNA (Table I; Fig. 7). Spermidine alone did not support the formation of D-loops with either form I or II DNA (Table I). Thus, spermidine lowered the requirement for Mg"++ to very low levels and reduced the anomalous kinetics noted earlier (Fig. 1) but did not eliminate the requirement for Mg"++.

Reversible Formation of D-loops—In experiments on the effect of recA protein on the nucleolytic cleavage of DNA (Williams et al., 1981), we found evidence that recA protein can dissociate D-loops, which suggests that the formation of D-loops by recA protein is a reversible process. To demonstrate directly the reversibility of D-loop formation, we incubated ϕX174 form I [32P]DNA with recA protein and purified D-loops made from ϕX174 form I [3H]DNA. The percentage of [3H]- and [32P]-labeled D-loops was measured for 60 min (Fig. 8A). In the presence of recA protein, the [3H]-labeled D-loops decreased from 55 to 10%, while [32P]-labeled D-loops increased to 5%. In a control from which recA protein was omitted, both labeled D-loops were stable, and [32P]-labeled D-loops were not formed. The results of the previous experiment were repeated in the presence of 10 µM single-stranded fragments of ϕX174 DNA (Fig. 8B). [3H]-labeled D-loops were dissociated, and [32P]-labeled D-loops were formed, as in the previous experiment, but the final yield of D-loops of both labels was higher, at about 30%. In both experiments, which involved the reaction of superhelical DNA in the absence of spermidine, 2-min complexes were observed as described above (Fig. 1B). These experiments show that the formation of D-loops is reversible since 1) D-loops simultaneously dissociated from [3H]DNA and formed in [32P]DNA.

FIG. 4. Effect of the concentration of single-stranded DNA on the rate of formation of D-loops. Conditions of the reaction were as described in the legend to Fig. 1 except that spermidine was omitted and the concentration of MgCl2 was 12 mM. The double-stranded DNA was 4.2 µM ϕX174 form II DNA. The concentration of recA protein was: () 2.4 µM; (■) 1.8 µM; (▲) 1.6 µM; (□) 0.8 µM. At the least and greatest concentrations of recA protein, we verified that the reaction was linear for 30 min at 37 °C. ssDNA, single-stranded DNA.

FIG. 5. Prompt inhibition by added excess single-stranded DNA. The conditions of the reaction were as described for Fig. 1 except that spermidine was omitted and the concentration of MgCl2 was 12 mM. X, reaction mixtures contained 3.1 µM form II ϕX174 [3H]DNA, 6.3 µM fragments of single-stranded ϕX174 DNA, 2.4 µM recA protein. ( ), at the time indicated by the arrow, we increased the concentration of single-stranded DNA to 31 µM in a set of identical samples. △, a control in which single-stranded φ DNA replaced single-stranded ϕX174 DNA.

FIG. 6. Effect of concentration of MgCl2 on D-loop formation catalyzed by recA protein. ( ), 8.8 µM fd form I [32P]DNA (more than 70% form I), 12 µM fragments of fd single-stranded [32P]DNA were incubated for 30 min at 37 °C with 2.4 µM recA protein (fraction V) and the indicated concentrations of MgCl2 in the absence of spermidine. D-loops formed were assayed after heating for 4 min at 50 °C in 1.5 M NaCl, 0.15 M Na-citrate (see Shibata et al., 1979a). ( ▲ ) 4.4 µM fd form II (61%), form III (38%) [3H]DNA, and 6 µM fragments of fd single-stranded [32P]DNA were incubated for 20 min at 37 °C with 2.4 µM recA protein (fraction V) and the indicated concentrations of MgCl2 in the absence of spermidine. D-loops formed were assayed after treatment with 0.5% Sarkosyl for 5 min at 17 °C followed by heat treatment for 4 min at 41 °C in 1.5 M NaCl, 0.15 M Na-citrate (see Shibata et al., 1981).
in the absence of any added single strands, and 2) the addition of single strands decreased the apparent dissociation of D-loops and caused the fraction of D-loops in [32P]DNA and [3H]DNA to approach a higher common equilibrium value. 

Inhibition of D-loop Formation—The formation of D-loops catalyzed by recA protein was inhibited by NaCl; 20 mM NaCl inhibited about 20%, and 50 mM NaCl completely inhibited the reaction (Fig. 9). By contrast, the DNA-dependent ATPase activity of recA protein was hardly affected by 100 mM NaCl.

FIG. 8. Reversible formation of D-loops by recA protein. D-loops made by reacting superhelical φX174 [3H]DNA and unlabeled fragments of single-stranded DNA at 75 °C (Beattie et al., 1977) were purified by sedimentation in a sucrose gradient as described by Williams et al. (1981). In the experiments shown here, we incubated these purified [3H]-labeled D-loops with superhelical φX174 [32P]DNA in the presence of recA protein at 37 °C and measured [3H] and [32P] in D-loops as a function of time. Reaction mixtures, which were warmed to 37 °C prior to the addition of recA protein, contained 14 mM Tris-HCl (pH 7.5), 1.4 mM dithiothreitol, 1.0 mg of bovine serum albumin/ml, 10 mM MgCl2, 1.0 mM ATP, 5 μM superhelical φX174 [32P]DNA, 5 μM [3H]-labeled D-loops, and as indicated, 10 μM fragments of unlabeled single-stranded φX174 DNA, 3 μM recA protein. D-loops were assayed as described in Shibata et al. (1981) except that treatment with detergent was omitted. A, no single-stranded DNA was added. Open symbols, controls without recA protein; closed symbols, reaction with recA protein; circles, φX174 [3H]DNA; triangles, φX174 [32P]-DNA. B, fragments of unlabeled single-stranded φX DNA were added. Symbols are the same as in A.

FIG. 9. Inhibition of D-loop formation. Standard assay mixtures included 4.4 μM fd double-stranded [3H]DNA (81% form II, 7% form I), 6 μM fd single-stranded fragments, 2.2 μM recA protein, and the indicated concentrations of inhibitors: ○, NaCl; △, ADP; □, AMP. Incubation was at 37 °C for 30 min.

ADP, a product of hydrolysis of ATP by recA protein, significantly inhibited the formation of D-loops; 12 mM ADP

1J. Roberts and A. J. Clark, personal communication.

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**TABLE I**

Partial replacement of Mg2+ by spermidine in the pairing reaction

D-loops were made by incubation at 37 °C for 30 min and assayed as described in Shibata et al., 1981. The reaction mixture contained 2 mM spermidine (see Fig. 1A). The concentration of double-stranded DNA was 4.4 μM and that of single-stranded fragments was 6 μM. Fd form II, III DNA was 61% form II and 38% form III; fd form I DNA contained more than 70% form I.

| Reaction mixture               | D-loops formed (%) |
|--------------------------------|--------------------|
| Form II, III DNA              |                     |
| Complete (including 6.7 mM MgCl2 and 2 mM spermidine) | 34 |
| Minus single-stranded fragments | 4                |
| Minus MgCl2                   | 1                  |
| MgCl2 reduced to 1.1 mM        | 35                 |
| MgCl2 increased to 25 mM       | 38                 |
| Minus spermidine              | 4                  |
| Minus spermidine, MgCl2 increased to 25 mM | 39 |

| Form I DNA                    |                     |
|--------------------------------|--------------------|
| Complete                      | 41                 |
| Minus MgCl2                   | 4                  |
| Minus single-stranded fragments | 3                |
inhibited the reaction about two-thirds (Fig. 9). On the other hand, 1.2 mM AMP had no effect on the reaction (Fig. 9). During the incubation of 8.8 μM fd form I DNA and 12 μM fd single-stranded fragments at 37 °C for 30 min in the presence of 1.8 μM recA protein under standard conditions, 45% of ATP was hydrolyzed. Since this corresponds to 0.57 μM ADP, the pairing reaction is presumably inhibited by one of the products of the reaction.

The Structure of D-loops—Electron micrographs of D-loops made by recA protein (Shibata et al., 1979a; Cunningham et al., 1980) have shown that they have the same appearance as D-loops made by the uncatalyzed reaction at high temperatures (Wiegand et al., 1977). Previously, we have observed that the average length of the heteroduplex region in D-loops made by annealing is the length expected on the basis of the average superheliix density of the recipient duplex molecule (Beattie et al., 1977; Wiegand et al., 1977). A similar relationship appears to hold for D-loops made in superhelical DNA by recA protein. In superhelical fd DNA, the mean size of D-loops made by recA protein was about 400 nucleotides long, corresponding to 40 superhelical turns (Fig. 10). By contrast, the size of D-loops in nicked circular duplex fd DNA showed a broader distribution, as one would expect (Fig. 10). The observed narrow distribution of sizes of D-loops made by recA protein in superhelical DNA supports the conclusion that recA protein has no topoisomerase activity under the conditions studied (Cunningham et al., 1979). RecA protein appears neither to increase nor decrease the superheliix density of closed circular DNA in which D-loops are made.

The stability of D-loops diluted in 1.5 M NaCl, 0.15 M Na-citrate was examined at various temperatures after treatment with Sarkosyl to remove recA protein. D-loops made from form I DNA by recA protein were as stable as form II or III DNA itself; the latter melted above 80 °C (Fig. 11A) and D-loops in form I DNA dissociated above 80 °C (Fig. 11A). This behavior is identical with that of D-loops made in form I DNA by annealing (Wiegand et al., 1977). D-loops made from form II or III DNA were much less stable; in 1.5 M NaCl, 0.15 M Na-citrate, they dissociated at temperatures above 25 °C (Fig. 11A). D-loops made from form I DNA by recA protein and then nicked by pancreatic DNase had exactly the same stability as D-loops made from form II or III DNA by recA protein (Fig. 11A). This behavior is also the same as that of D-loops made by annealing (Radding et al., 1977). We have
90% of molecules were circular. The preparation of form incubated for 30 min (form other components of the standard reaction mixture. Mixtures were to that after treatment at 25 °C. Examination of the preparation of contained 89% form double-stranded [3H]DNA in complexes after heat treatment at 65 °C 6 recA protein to unwind duplex DNA partially (Cunningham et al., 1979) prompted us to explore the specificity of the pairing reaction. Elsewhere, we and others have reported that recA protein will make joint molecules from closed circular duplex DNA and circular duplex DNA with a gap in one strand (Cunningham et al., 1980; Cassuto et al., 1980). To characterize further the pairing activity of recA protein, we studied the possible reaction of circular single-stranded DNA with several kinds of duplex DNA (Table II).

According to examination by gel electrophoresis, our preparation of single-stranded phage DNA consisted predominantly of circular DNA (data not shown), and according to examination by electron microscopy, about 90% of single-stranded molecules were circular (DasGupta et al., 1980). As measured by the D-loop assay, circular single-stranded DNA does not form a detectable fraction of stable joint molecules with form I DNA (Shibata et al., 1979a). The preparation of circular single-stranded DNA used in the present experiments made a small fraction of complexes with a preparation of form I DNA, and these complexes had the same thermal stability as D-loops made with form I DNA (Table II). Some of these at least are attributable to contamination of the single-stranded circular DNA with 10% of linear single strands.

When we reacted the circular single-stranded DNA with form II or III DNA, the yield of complexes exceeded the amounts made by fragments of single-stranded DNA (Table II). The yield of complexes made by circular single-stranded DNA relative to complexes made by linear single-stranded fragments was four times greater for form II or III DNA than for form I DNA. The complexes made by circular DNA were also more stable than complexes formed by single-stranded fragments that were about 600 nucleotides long. Circular single-stranded DNA also paired with fragments of double-stranded DNA made by digestion of fd duplex DNA by endonuclease R Hae III. Complexes formed with these restriction fragments had the relative heat stability of D-loops in form I DNA, which is very similar to the stability of duplex DNA itself (Table II, Fig. 11, and Wiegand et al., 1977). Controls with circular single-stranded DNA of ϕX174 showed that the formation of all of the complexes described required homologous DNA (Table II).

These observations suggested that recA protein stably pairs circular single-stranded DNA with either nicked circular duplex DNA or linear duplex DNA. We have confirmed that inference and characterized the structures of the products by electron microscopy, as described elsewhere (DasGupta et al., 1979b).

### Table II

**Substrate specificity of the pairing reaction**

| Duplex fd [3H]DNA | Single-stranded DNA | Ratio* | Index of heat stability |
|-------------------|---------------------|-------|-------------------------|
| Form I            | a. None             | 0.0   |                         |
|                   | b. fd fragments     | 68.7  | 0.28                    |
|                   |                     | 19.3  | 0.80                    |
| Form II           | a. None             | 3.4   |                         |
|                   | b. fd fragments     | 33.3  | 1.2                     |
|                   |                     | 40.1  | 0.64                    |
|                   | c. fd circles       | 6.8   |                         |
| Form III          | a. None             | 0.9   |                         |
|                   | b. fd fragments     | 38.2  | 1.4                     |
|                   |                     | 55.1  | 0.63                    |
|                   | c. fd circles       | 2.0   |                         |
| Hae III digest    | a. None             | 2.8   |                         |
|                   | b. fd fragments     | 17.7  | 1.6                     |
|                   |                     | 27.7  | 0.83                    |
|                   | c. fd circles       | 1.0   |                         |

*Complexes formed by fd circles/complexes formed by fd fragments.

used the relative stability of joint molecules at 65 °C versus 25 °C as a criterion for characterizing products of the pairing reaction (see below and Table II).

Under our previous condition for assaying D-loops, which included heating at 50 °C for 4 min in 1.5 M NaCl, 0.15 M Na-citrate, more than two-thirds of D-loops made with form II or III DNA dissociated. This explains in part why we previously observed a low yield of D-loops with form II DNA (Shibata et al., 1979a; Cunningham et al., 1979). In our assays for D-loops, we nonetheless heated the product of the reaction at 41 °C for 4 min (except when stated otherwise) in 1.5 M NaCl, 0.15 M Na-citrate where one-third of D-loops of form II or III DNA dissociated, since we usually obtained more reproducible results than without heating or by heating at lower temperatures.

When the product of the reaction was treated with Sarkosyl at a temperature above 17 °C, a significant fraction of D-loops made with form II DNA dissociated. Under the conditions used in the experiments described in this paper, including incubation with 0.5% Sarkosyl at 17 °C for 5 min, recA protein appears to be inactivated and detached from DNA, since almost all of the ternary complexes of double-stranded DNA, recA protein, and single-stranded DNA formed in the presence of adenosine 5′-O-(3-thiotriphosphate) dissociated when treated in the same way (see Shibata et al., 1979b).

### Table III

**Activation of recombination activities and repressor cleavage by single-stranded DNA**

| Substrate | Form I DNA plus single-stranded fragments | Form II DNA plus single-stranded fragments | Form II DNA | Cleavage of repressor |
|-----------|------------------------------------------|-------------------------------------------|-------------|----------------------|
| At the minimal amount of recA protein required | 20                                      | 8                                         | 5–10        | 16–17                |
| At the optimal amount of recA protein | 5–8                                     | 2–3                                       | 3–5         | 6–7                  |

* Shibata et al. (1979b) and present observations.
  * Shibata et al. (1981).
  * Craig and Roberts (1980).
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1980). The present results, together with other observations (Cunningham et al., 1980), show that the D-loop assay can be used to measure a variety of joint molecules that can be made by recA protein.

**DISCUSSION**

The energy of superhelix formation drives the uncatalyzed formation of D-loops by stabilizing the product. The energy of superhelix formation is not sufficient, however. The uncatalyzed reaction has a temperature threshold, which can be rationalized by supposing that heat partially unwinds the duplex DNA, thereby permitting it to pair with a third strand (Beattie et al., 1977; Radding et al., 1977). Using the energy of ATP, recA protein is able to promote this pairing reaction at 37 °C, possibly by means of its ability to partially unwind duplex DNA (Cunningham et al., 1979; Shibata et al., 1981). RecA protein makes D-loops in duplex DNA whether it is superhelical or not (Cunningham et al., 1979; McEntee et al., 1979). Nonetheless, as one might expect, there are distinct differences between superhelical and nonsuperhelical DNA as substrates for recA protein: 1) although the amount of recA protein needed is independent of the amount of duplex DNA (Fig. 3) but rather is a function of the concentration of single-stranded DNA, the kind of duplex DNA affects the requirement for recA protein; about half as much recA protein is needed for a given amount of single-stranded DNA when the recipient molecule is superhelical (Fig. 3; Table III). 2) The time course of D-loop synthesis with form I DNA showed an anomaly that was suppressed by spermidine (Fig. 1). 3) In the absence of spermidine, form I DNA formed D-loops at lower concentrations of MgCl₂ than form II (Fig. 6). Elsewhere we have reported that gapped circular molecules preferentially pair with superhelical DNA versus nicked circular DNA (Cunningham et al., 1980). In addition, form I DNA in 1-2 mM MgCl₂ was a good cofactor for the ATPase activity of recA protein, whereas form II DNA was not (Shibata et al., 1981). These observations suggest that the energy of superhelix formation can help to make D-loops, but they also indicate that superhelicity may significantly alter the mechanism of the reaction (Fig. 1B).

In the presence of 1 mM MgCl₂ and single-stranded DNA, or form I DNA, recA protein hydrolyzes ATP without spermidine (Shibata et al., 1981). Under this condition, neither the pairing reaction (Fig. 6) nor the formation of ternary complexes occurs.² The pairing reaction was inhibited completely by 70 mM NaCl (Fig. 9), but ATP hydrolysis was only slightly affected by even 100 mM NaCl.³ Thus, the hydrolysis of ATP can be uncoupled from the complete reaction.

Despite the functional dissimilarity between the proteolytic inactivation of a molecule of repressor and the homologous pairing of DNA molecules, striking similarities exist between the requirements for three relevant reactions in vitro, namely cleavage of repressor, pairing of single- and double-stranded DNA, and unwinding of double-stranded DNA: 1) all three reactions require single-stranded DNA as a cofactor, and the single-stranded DNA governs the properties of all three reactions in similar ways. At a limiting concentration of recA protein, increasing amounts of single-stranded DNA first stimulate the reactions and then inhibit (Craig and Roberts, 1980; Shibata et al., 1979b; Fig. 4). Optimal reactions occur at ratios of 2-8 nucleotide residues of single-stranded DNA/monomer of recA protein (Table III). Similarly, none of the three reactions occur until a certain minimal amount of recA protein is present (Fig. 2; Craig and Roberts, 1980; Shibata et al., 1979a and 1979b; McEntee et al., 1979).² The minimal amount of recA protein required is proportional to the concentration of single-stranded DNA, both for synthesis of D-loops (Fig. 3) and for inactivation of repressor (Craig and Roberts, 1980) and corresponds to 1 molecule of protein monomer/8 to 20 nucleotide residues (Table III). 2) All three reactions require ATP as a cofactor (Craig and Roberts, 1980; Shibata et al., 1979a; McEntee et al., 1979; Cunningham et al., 1979). In the cases of the cleavage of repressor and the unwinding of the double helix, adenosine 5'-O-(3-thiotriphosphate), an analog of ATP which competitively inhibits the ATPase activity of recA protein (Shibata et al., 1979b), is much more effective than ATP (Roberts et al., 1978; Cunningham et al., 1979). 3) In the cases of cleavage of repressor and unwinding of duplex DNA, single-stranded DNA can be replaced by oligonucleotides. In both cases, the reaction requires a larger molar amount of oligonucleotides than that of single-stranded DNA.³ These observations show that in vitro a common mechanism activates recA protein as a specific protease and as a DNA enzyme. By analogy, we infer that in vivo a common mechanism activates the direct participation of recA protein in the recombinination and repair of DNA and its indirect participation via the derepression of other genes (Witkin 1976; Oishi et al., 1979; McPartland et al., 1980; Little et al., 1980; Kenyon and Walker, 1980). The relative extent of direct and indirect action of recA protein in various aspects of

² T. Shibata and R. P. Cunningham, unpublished data.

³ J. W. Roberts, personal communication.
recombination and repair remains to be determined.

The experiments reported here and elsewhere (Cunningham et al., 1980; DasGupta et al., 1980; Cassuto et al., 1980) have revealed a broader specificity of recA protein in the synthesis of joint molecules than indicated by the formation of D-loops. RecA protein will form joint molecules of circular single strands and either linear or nicked circular duplex DNA (Table II; this paper; DasGupta et al., 1980), as well as joint molecules of gapped circular DNA and form I DNA (Cunningham et al., 1980; Cassuto et al., 1980). Stable complexes that were detectable by the D-loop assay did not form between superhelical DNA and circular single-stranded DNA (Table II; this paper; DasGupta et al., 1980) or between superhelical DNA and nicked circular DNA (Cunningham et al., 1980). The substrates that formed joint molecules are summarized on the right of Fig. 12. All of these combinations produced joint molecules with approximately equal efficiencies. From these observations, we deduce a rule that describes the specificity of the reaction. RecA protein will catalyze the formation of joint molecules when one of them is single-stranded, or partially so, and when either one has a free end. We interpret the requirement for single-stranded DNA to reflect not only the need for a donor strand but also the specific role that single-stranded DNA plays as an effector that stimulates recA protein to bind and unwind duplex DNA, as described above. We interpret the need for a free end as topologic and thermodynamic; in the absence of topoisomerase activity (cf. Cunningham et al., 1979), a free end is necessary to form a heteroduplex region that has the normal Watson-Crick duplex structure (Fig. 12 step b). In Fig. 12, we have presented a model that relates all of these observations, and elsewhere we have discussed further inferences derived from the electron microscopic study of the various joint molecules (DasGupta et al., 1980).

REFERENCES

Alberts, B. M., and Frey, L. (1970) Nature 227, 1313-1318
Beattie, K. L., Wiegand, R. C., and Radding, C. M. (1977) J. Mol. Biol. 116, 783-803
Cassuto, E., West, S. C., Mursalin, J., Conlon, S., and Howard-Flanders, P. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3962-3966

Christiansen, C., and Baldwin, R. L. (1977) J. Mol. Biol. 118, 441-454
Craig, N. L., and Roberts, J. W. (1980) Nature 283, 26-30
Cunningham, R. P., Shibata, T., DasGupta, C., and Radding, C. M. (1979) Nature 281, 191-195, 282, 426
Cunningham, R. P., DasGupta, C., Shibata, T., and Radding, C. M. (1980) Cell 20, 233-235
DasGupta, C., Shibata, T., Cunningham, R. P., and Radding, C. M. (1980) Cell 22, 437-446
Gottesman, S., and Gottesman, M. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2188-2192
Holloman, W. K., Wiegand, R., Hoessli, C., and Radding, C. M. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2394-2398
Kenyon, C. J., and Walker, G. C. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 2819-2823
Kolodner, R. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 4847-4851
Little, J. W., Edmiston, S. H., Facelli, L. Z., and Mount, D. W. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3225-3229
McIntee, K., Weinstock, G. M., and Lehman, I. R. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 2615-2619
McPartland, A., Green, L., and Echols, H. (1980) Cell 20, 731-737
Mizuuchi, K., Gellert, M., and Nash, H. A. (1978) J. Mol. Biol. 121, 375-392
Oishi, M., Smith, C. L., and Friefeld, B. (1979) Cold Spring Harbor Symp. Quant. Biol. 43, 897-907
Potter, H., and Dressler, D. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1084-1088
Radding, C. M., Beattie, K. L., Holloman, W. K., and Wiegand, R. C. (1977) J. Mol. Biol. 116, 825-838
Radding, C. M., Shibata, T., Cunningham, R. P., DaGupta, C., and Osber, L. (1980) in Mechanistic Studies of DNA Replication and Genetic Recombination (Alberts, B., and Fox, C. F., eds) Vol. 19, pp 883-870, Academic Press, New York
Roberts, J. W., Roberts, C. W., Craig, N. L., and Phizichy, E. M. (1979) Cold Spring Harbor Symp. Quant. Biol. 43, 917-920
Roeder, G. S., and Sadowski, P. D. (1979) Cold Spring Harbor Symp. Quant. Biol. 43, 1023-1032
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, 5180-5184
Shibata, T., Cunningham, R. P., and Radding, C. M. (1981) J. Biol. Chem. 256, 7557-7564
Williams, J. G. K., Shibata, T., and Radding, C. M. (1961) J. Biol. Chem. 256, 7573-7582
Wiegand, R. C., Beattie, K. L., Holloman, W. K., and Radding, C. M. (1977) J. Mol. Biol. 116, 805-824
Witkin, E. M. (1976) Bacteriol. Rev. 40, 869-907