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Homologous pairing in genetic recombination: Formation of D loops by combined action of recA protein and a helix-destabilizing protein*

(*Escherichia coli* single-strand binding protein/phage T4 gene 32 protein/renaturation versus D-loop formation)

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ABSTRACT *Escherichia coli* single-strand binding protein (SSB) or phage T4 gene 32 protein reduced the amount of recA protein required to catalyze the formation of D loops from double-stranded DNA and homologous single-stranded fragments. Neither SSB nor gene 32 protein alone catalyzed the formation of D loops, and excessive amounts of either protein, amounts that were sufficient to saturate the single strands, inhibited the formation of D loops completely. Both the stimulatory activity and the inhibitory activity of SSB resisted boiling, which is consistent with the known thermal stability of SSB, whereas the gene 32 protein was inactivated by heating. The formation of D loops in the presence of both recA protein and SSB required homologous DNA and ATP. Spermidine aided the combined action of SSB and recA protein in forming D loops, but Mg^{2+} alone was sufficient as a counterion.

Homologous molecules of DNA can pair in two ways: complementary single strands can join to form duplex DNA (1–3) or a linear single strand can pair with its complement in superhelical DNA to form a triple-stranded structure called a D loop (Fig. 1; refs. 8 and 9). Both reactions are interesting in relation to genetic recombination, the formation of a D loop particularly so because it helps to explain how a broken molecule might initiate an exchange with an unbroken one (see ref. 7). In the absence of any proteins, both reactions occur very slowly at 37°C. The discovery of proteins that accelerate these reactions by 3–4 orders of magnitude indicates that both kinds of pairing reaction are biologically significant. Alberts and Frey (4) discovered the first of a class of proteins, sometimes called helix-destabilizing proteins (14),† some of which catalyze the renaturation of complementary strands of DNA (for reviews, see refs. 6 and 7). Recently Shibata *et al.* (12) and McEntee *et al.* (13) found that *Escherichia coli* recA protein catalyzes the formation of D loops. Because recA protein is essential for recombination in *E. coli* (17, 18), these observations indicate that the pairing of a single strand with duplex DNA is especially important in recombination.

Experiments on the mechanism of formation of D loops by recA protein *in vitro* have shown that single-stranded DNA plays a key role in the reaction. Single strands signal recA protein to bind and partially unwind duplex DNA, thus presumably opening the recipient duplex molecule for a comparison of sequence homology and the possible formation of a joint molecule (10, 11, 19). The amount of single-stranded DNA also determines the amount of recA protein required to form D loops; the reaction is not detectable until approximately enough recA protein is added to saturate all of the single-stranded DNA (19), which suggests that recA protein may unfold single strands (Fig. 1). Because certain helix-destabilizing proteins, including gene 32 protein (4) and the *E. coli* single-strand binding protein (SSB) (5), appear to promote renaturation by unfolding single strands, we did experiments to determine whether these proteins might complement the action of recA protein and thus reduce the amount of recA protein required to form D loops. In addition, we were stimulated to explore the role of SSB by the report of Glassberg *et al.* (16), which implicates SSB in genetic recombination.

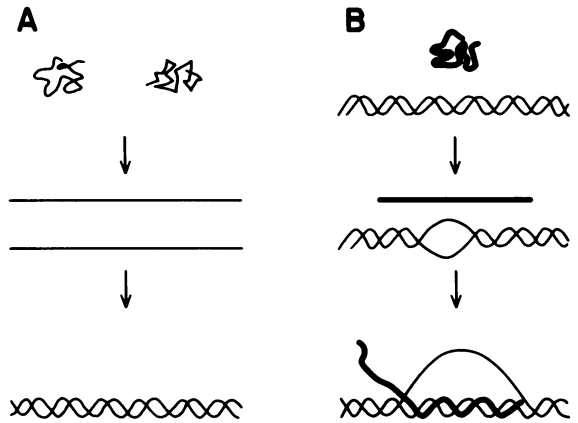

FIG. 1. The molecular basis of homologous pairing. Homologous molecules of DNA can pair in two ways. (*A*) Complementary single strands can join to form duplex DNA. Single strands must be unfolded by heat (1–3) or by the binding of helix-destabilizing proteins (4–7) to expose bases for interstrand pairing. (*B*) A linear single strand can pair with its complement in duplex DNA to form a triple-stranded structure called a D loop (8). This reaction requires the partial unwinding of the duplex molecule (9), and presumably the unfolding of single strands as well. Thus far, only the unwinding activity of recA protein (10, 11) has served effectively to promote the formation of D loops (12, 13).

METHODS

Enzymes. The recA protein was purified as described (12). SSB, a gift of Malcolm Gefter, was the DEAE-Sephadex fraction of the protein purified according to Molineux *et al.* (20). The preparation was free of exonuclease I, RNase H (M. Gefter, personal communication), and endonuclease activity on superhelical DNA. SSB that had been heated at 100°C was used in several experiments; it lacked any detectable ATPase activity.

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Abbreviations: SSB, single-strand binding protein. We use the following designations for the different forms of double-stranded DNA from phages φX174 and fd: form I, superhelical DNA; form II, nicked circular DNA.

* This paper is no. 4 in a series. Paper no. 3 is ref. 19.

† The helix-destabilizing protein from *E. coli* has recently been named single-strand binding protein (SSB) (15, 16), a term that we use here. We will use the term, helix-destabilizing protein (14), as a collective one for the class of proteins that destabilize double-stranded DNA by virtue of strong cooperative binding to single strands.

The T4 gene 32 protein was a gift of N. V. Sinha. The protein was purified by a procedure similar to that of Bittner *et al.* (21), involving successive steps of chromatography on DNA-cellulose, phenyl-Sepharose, and phosphocellulose. The preparation lacked detectable ATPase activity or endonuclease activity on superhelical DNA.

DNA. Circular forms of duplex DNA and fragments of single-stranded DNA of phages fd and ϕX174 were prepared as described or cited earlier (10–12). All concentrations of DNA are expressed as moles of nucleotide residue.

Formation and Assay of D Loops. The following conditions are modifications of procedures described before (9, 19): The standard reaction mixture contained, in 20.5 μl, 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, 88 μg of bovine serum albumin per ml, 4.4 or 8.8 μM double-stranded fd or ϕX174 [^{3}H]DNA, 12 or 29 μM single-stranded fragments of fd or ϕX174 DNA (average chain length about 600 nucleotides), and various amounts of purified recA protein (12) and SSB (15, 20) or T4 gene 32 protein (4, 21). To avoid precipitation of single-stranded DNA by spermidine (5), we incubated DNA, proteins, and ATP at 37°C for 4 min in the presence of 1.2 mM $MgCl_2$ before adding spermidine. To start the reaction, we added spermidine and increased the concentration of $MgCl_2$ to 6.7 mM. After an incubation of 30 min at 37°C, we stopped the reaction by diluting the mixture 1:3 in cold 25 mM EDTA at pH 9.4.

After stopping the reaction by adding EDTA, we treated the product with 0.5% Sarkosyl at 17°C for 5 min and then diluted the mixture about 15 times with 25 mM EDTA (pH 9.4). We took an aliquot of 50 μl to measure total radioactivity and an aliquot of 200 μl for the nicking assay of Kuhnlein *et al.* (22). Then, we diluted 200 μl of the mixture 1:6 with cold 1.5 M NaCl/0.15 M Na citrate, incubated it at 41°C for 4 min, and immediately diluted it 1:7 with cold NaCl/Na citrate. We filtered the sample at about 4 ml/10 sec through a nitrocellulose filter (Sartorious membrane filter, SM11306, pore size 0.45 μm) that had been washed with 2 ml of NaCl/Na citrate. We washed the filter successively with 1.5, 1.5, and 5 ml of cold NaCl/Na citrate. Radioactivity retained on the filter was measured in a scintillation counter by using Econofluor (New England Nuclear).

RESULTS

Helix-Destabilizing Proteins Reduce Amount of recA Protein Required to Form D Loops. The amount of recA protein required to form D loops is related stoichiometrically to the amount of single-stranded DNA (19). In a reaction mixture containing 2 mM spermidine, 6.7 mM $MgCl_2$, and form I (superhelical) DNA, the conditions of most of the experiments described here, no formation of D loops occurred when there was less than one molecule of recA protein per 20 nucleotide residues of single-stranded DNA, whereas optimal formation of D loops required one molecule per 5–8 nucleotide residues (ref. 19 and unpublished observations). Thus, when the concentration of single-stranded DNA was 12 μM and we added 1.8 μM recA protein, some 60% of form I DNA was converted to D loops at 37°C in 30 min, but when we added only 0.6 μM recA protein, no D loops were formed (Table 1, compare lines *A*2 and *B*1). The addition of 0.85 μM SSB to the mixture containing 0.6 μM recA protein restored the values measured by the D-loop assay to ⅔ of that seen with 1.8 μM recA protein alone (Table 1, compare lines *A*1 and *B*1). Electron microscopy confirmed that the product made in the presence of both recA protein and SSB contained D loops (data not shown). Controls showed that the requirements of the reaction were similar to those for the synthesis of D loops by recA protein alone: omission of single-stranded fragments or omission of ATP reduced the assay to the background level (Table 1, lines *A*4 and *A*6). Substitution of heterologous fragments of single-stranded ϕX174 DNA yielded no product (Table 1, line *A*5), whereas the same fragments yielded 39% D loops when incubated with ϕX174 form I DNA and recA protein.

Table 1. Formation of D loops by combined action of *E. coli* SSB and recA protein

Reaction mixture	D loops, %
(*A*) 0.6 μM recA protein	
1. Complete, including SSB	40
2. Without SSB	1
3. Without recA protein	1
4. Without single-stranded DNA	1
5. Heterologous single-stranded DNA	1
6. Without ATP	1
7. With 6.7 mM $MgCl_2$, without spermidine	22
8. With 25 mM $MgCl_2$, without spermidine	20
9. With 2 mM spermidine, without $MgCl_2$	1
(*B*) 1.8 μM recA protein	
1. Without SSB	64

Reaction mixtures contained 12 μM single-stranded fd DNA fragments, 8.8 μM fd form I DNA, and 0.85 μM SSB.

As indicated in Table 1*A*, neither 0.6 μM recA protein alone nor 0.85 μM SSB alone promoted the formation of D loops (lines 2 and 3). More D loops were formed by the combined action of SSB plus recA protein when spermidine was present as well as Mg^{2+}, but unlike the renaturation of complementary strands of DNA by SSB at neutral pH (ref. 5), the reaction was not completely dependent on the presence of spermidine (Table 1, lines *A*7 and *A*8). No reaction was detected in the presence of 2 mM spermidine alone without Mg^{2+} (Table 1, line *A*9).

In an experiment in which form II (nicked, circular) DNA was used to make D loops, we held the concentration of SSB constant at 1.1 μM and varied the amount of recA protein (Fig. 2). At concentrations of 0.5–4 μM recA protein, 1.1 μM SSB stimulated the formation of D loops. Stimulation was greatest at limiting concentrations of recA protein. For example, 1.2 μM recA protein alone produced only about 4% D loops above the background, but in the presence of 1.1 μM SSB the reaction yielded some 30% D loops, corresponding to a 7-fold increase in rate. This experiment also shows that SSB stimulates the formation of D loops from either form II or form I DNA.

In a similar experiment involving from II DNA as the recipient molecule, 1.5 μM phage T4 gene 32 protein also promoted the synthesis of D loops by a limiting concentration of recA protein (Table 2, lines *A*1 and *A*3).

Excess Helix-Destabilizing Protein Inhibits Formation of D Loops by recA Protein. To a reaction mixture containing 0.6 μM recA protein, a concentration insufficient to catalyze the formation of D loops, and to one containing 1.8 μM recA protein, which made 62% D loops in 30 min, we added increasing amounts of SSB. At the lower concentration of recA protein, the synthesis of D loops was stimulated as increasing amounts of SSB were added and reached a sharp maximum at 0.6–0.8 μM SSB (Fig. 3*A*). As in the experiment shown in Table 1, no stimulation of D-loop formation was seen in the absence of homologous single-stranded fragments. Higher concentrations of SSB sharply inhibited the formation of D loops at either concentration of recA protein. The fraction of nicked circular DNA, measured by the assay of Kuhnlein *et al.* (22), remained between 10% and 14% at all concentrations of SSB. Thus, because the preparation of SSB lacked either endonuclease or exonuclease activity (see *Methods*), its inhibitory action on the formation of D loops is not ascribable to degradation of the

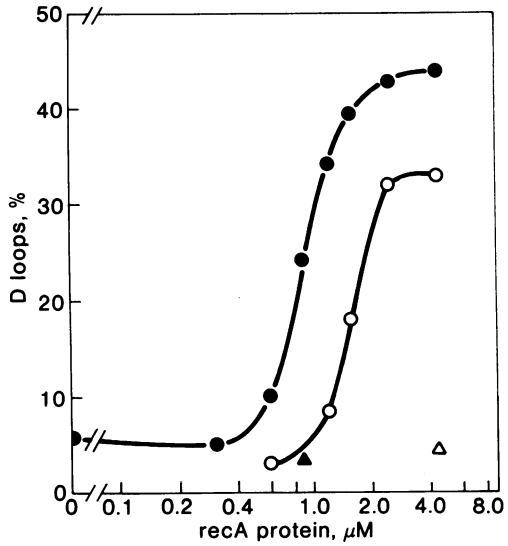

FIG. 2. Effect of SSB on the formation of D loops in form II DNA by various concentrations of recA protein. The concentration of form II [^{3}H]DNA of ϕX174 was 4.4 μM; the concentration of fragments of single-stranded ϕX174 DNA was 12 μM. ●, 1.1 μM SSB; ○, no SSB; ▲, 1.1 μM SSB, but no single-stranded DNA; △, no SSB or single-stranded DNA.

DNA. Similarly, boiled SSB, which retained both its stimulatory and inhibitory effects on the formation of D loops (see below), lacked any detectable ATPase activity which might be a source of inhibition.

In the absence of any recA protein, SSB did not promote the formation of D loops at concentrations of 0.5, 0.6, 0.85, or 1.0 μM (Table 1 and Fig. 3A) or at 1.3 or 3.0 μM (data not shown).

In the same kind of experiment, we substituted phage T4 gene 32 protein for SSB (Fig. 3B). Again, the action of gene 32 protein was virtually identical to that of SSB. When there was less than about one molecule of T4 gene 32 protein per 10 nucleotide residues of single-stranded DNA, gene 32 protein stimulated the formation of D loops by a suboptimal concentration of recA protein. Higher concentrations of gene 32 protein inhibited the formation of D loops. These concentrations of gene 32 protein had no detectable ATPase activity or endonucleolytic activity on form I DNA.

Thermal Stability of Stimulatory and Inhibitory Activities of Helix-Destabilizing Proteins. The activity of SSB in promoting replication *in vitro* is resistant to boiling (23). The data in Fig. 4A show a similar stability of both the stimulatory and inhibitory activities of SSB on the formation of D loops by recA protein. The enzyme used in the present experiments was purified without boiling (20). At a concentration of 0.6 mg/ml, SSB was heated for 3 min in a boiling water bath in a solvent containing 50 mM Tris·HCl (pH 8), 2 mM dithiothreitol, 40 mM NaCl, 1 mM EDTA, and 20% (vol/vol) glycerol. The samples was chilled quickly on ice and stored at 0–4°C at least overnight before use. The amounts of heated protein required either to stimulate or to inhibit the formation of D loops by recA protein were not detectably different from those of the unheated preparation (Fig. 4A and Table 3). By contrast, similar heating

Table 2. Formation of D loops by combined action of T4 gene 32 protein and *E. coli* recA protein

Reaction mixture	D loops, %
(A) 1.2 μM recA protein	
1. With 1.5 μM gene 32 protein	33
2. With 1.5 μM gene 32 protein, without ssDNA	3
3. Without gene 32 protein	4
4. With 2.0 μM gene 32 protein	10
(B) 4.4 μM recA protein	
1. Without gene 32 protein	25
2. Without gene 32 protein, without ssDNA	3

Mixtures contained 12 μM single-stranded fragments of ϕX174 DNA (ssDNA) and 4.4 μM ϕX174 form II DNA.

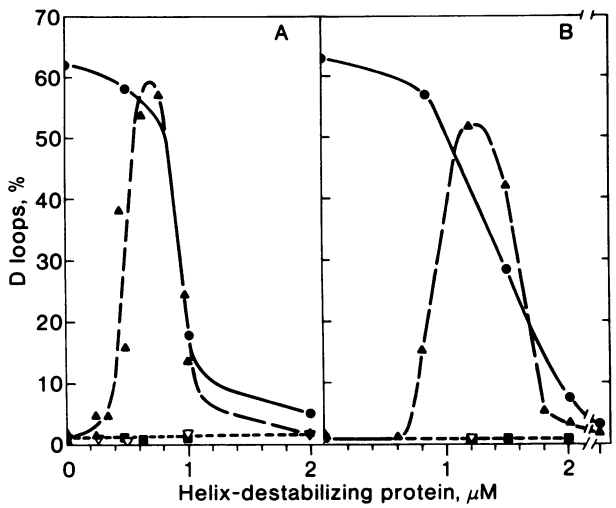

FIG. 3. Effect of helix-destabilizing proteins on the formation of D loops by recA protein. DNA in these experiments was from phage fd, 12 μM single-stranded fragments, and 8.8 μM form I DNA. (A) *E. coli* SSB at the concentrations indicated. (B) T4 gene 32 protein at the concentrations indicated. ●, 1.8 μM recA protein; ▲, 0.6 μM recA protein; ■, no recA protein; ▽, 0.6 μM recA protein, without single-stranded fragments of DNA.

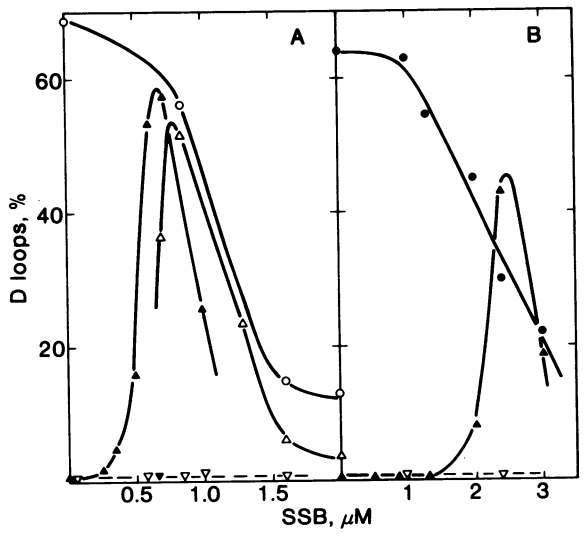

FIG. 4. (A) Thermal stability of stimulatory and inhibitory activities of SSB. The concentration of fragments of single-stranded fd DNA was 12 μM; that of fd form I DNA was 8.8 μM. ▲, 0.6 μM recA protein, untreated SSB at the concentrations indicated; △, 0.6 μM recA protein plus boiled SSB; ○, 1.8 μM recA protein plus boiled SSB; ▽, 0.6 μM recA protein, no fragments of single-stranded DNA; ▼, 0.6 μM recA protein plus fragments of heterologous single-stranded DNA (ϕX174). (B) Effect of concentration of single-stranded DNA on the amount of SSB required to stimulate or inhibit the formation of D loops by recA protein. The concentration of fragments of single-stranded fd DNA was 29 μM; that of form I fd DNA was 8.8 μM. The preparation of SSB was the same boiled sample used in A. ●, 4 μM recA protein; ▲, 0.6 μM recA protein; ▽, 0.6 μM recA protein, no single-stranded DNA. See Table 3 for stoichiometric relationships calculated from these and other data.

of gene 32 protein completely abolished both its stimulatory and inhibitory effects on the formation of D loops (Table 4). The contrasting behavior of the preparations of SSB and gene 32 protein shows that neither the stimulatory nor the inhibitory activity can be attributed to a nonprotein contaminant common to both preparations.

Stoichiometry. The data in Figs. 3 and 4 show that the amounts of SSB required to either stimulate or inhibit the formation of D loops are directly related to the concentration of single-stranded DNA. When the concentration of recA protein was held constant at 0.6 μM while the concentration of single-stranded DNA was increased 2.4 times, there was a comparable increase in the amount of SSB needed for optimal stimulation of D-loop formation (compare Fig. 4 *A* and *B*). By contrast, a 3-fold difference in the concentration of recA protein had no effect on the amounts of SSB required to inhibit the formation of D loops (Fig. 3*A*). Because of the narrow range of concentrations of SSB that stimulate the formation of D loops, it is easier to estimate the amount of SSB required for 50% inhibition. Data from several experiments, summarized in Table 3, show that one molecule of SSB per 10–13 nucleotide residues of single-stranded DNA decreased the synthesis of D loops by 50%. In one experiment, a similar ratio of T4 gene 32 protein to single-stranded DNA inhibited the formation of D loops whether the concentration of recA protein was 0.6 or 1.8 μM.

In terms of the mechanism of formation of D loops, we would like to know how little recA protein is sufficient to form D loops when SSB is present. In the experiment shown in Fig. 4*B* we varied the concentration of SSB and observed optimal synthesis of D loops at a ratio of 1 molecule of recA protein ($M_r \approx 40{,}000$, ref. 24) per 4 molecules of SSB ($M_r \approx 20{,}000$, refs. 20 and 23) per 48 nucleotide residues of single-stranded DNA. In a subsequent experiment, we started from the same concentrations of reactants that produced the ratios 1:4:48 and lowered the concentration of recA protein. Any reduction in the concentration of recA protein decreased the synthesis of D loops (Fig. 5); no synthesis at all occurred when the ratios of recA protein to SSB and nucleotide residues of single-stranded DNA fell below 1:11:126. The ratio of 1 recA protein per 4 SSB per 48 nucleotide residues represents a reduction in the amount of recA protein relative to single-stranded DNA of 1/6th–1/10th the original amount; we have previously observed optimal synthesis of D loops from form I DNA under the same conditions when there was 1 molecule of recA protein per 5–8 nucleotide residues of single-stranded DNA (ref. 19 and unpublished observations). We confirmed the magnitude of the reduced need for recA protein in the experiment shown in Fig. 5. In that experiment, 0.6 μM recA protein and 2.5 μM SSB together produced 53% D loops. That amount of recA protein alone yielded no D loops, but 4 μM recA protein alone, 7 times as much, yielded 51% D loops.

Table 3. Amounts of helix-destabilizing proteins (HDP) that inhibited formation of D loops by recA protein by 50%

Data from Fig.	HDP	recA, μM (a)	ssDNA, μM (b)	HDP, μM (c)	Nucleotide residues/HDP (d)
3*A*	SSB, native	0.6	12	0.9	13
3*A*	SSB, native	1.8	12	0.9	13
4*A*	SSB, boiled	0.6	12	1.25	10
4*A*	SSB, boiled	1.8	12	1.25	10
4*B*	SSB, boiled	4.0	29	2.5	12
3*B*	Gene 32 protein	0.6	12	1.6	8
3*B*	Gene 32 protein	1.8	12	1.4	9

In the experiments cited, we held constant the concentration of recA protein (column a) and single-stranded DNA (ssDNA) (column b) and we determined the amount of helix-destabilizing protein (column c) that decreased the formation of D loops by 50%. The last column (d) is the molar ratio of single-stranded DNA to helix-destabilizing proteins at 50% inhibition (b/c).

Table 4. Thermal inactivation of stimulatory and inhibitory activities of T4 gene 32 protein

Reaction mixture	D loops, %
(*A*) 0.6 μM recA protein	
Gene 32 protein	
1.2 μM	22
1.5 μM	38
1.2 μM, boiled	1
1.5 μM, boiled	1
Without gene 32 protein	1
(*B*) 1.8 μM recA protein	
Gene 32 protein	
2.4 μM	5
2.4 μM, boiled	58
Without gene 32 protein	54

Reaction mixtures contained 12 μM single-stranded fragments of fd DNA, 8.8 μM fd form I DNA, and the indicated amounts of recA protein and T4 gene 32 protein. Gene 32 protein was inactivated (boiled) by heating at 100°C for 3 min in 20 mM Tris·HCl (pH 8.1), 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 M NaCl, and 50% (vol/vol) glycerol.

DISCUSSION

These experiments show that helix-destabilizing proteins can aid recA protein in catalyzing the homologous pairing of a single strand with duplex DNA. Although neither SSB nor T4 gene 32 protein alone catalyzed the formation of D loops under any conditions observed so far, either of them reduced the amount of recA protein required to form D loops. When recA protein alone catalyzes the formation of D loops, the amount of single-stranded DNA stoichiometrically determines the amount of recA protein required (19). Similarly, when D loops were made by the combined action of SSB and recA protein, the amount of single-stranded DNA appeared to determine stoichiometrically the optimal concentration of SSB. Greater concentrations of SSB inhibited the formation of D loops, and in this case as well the amount of SSB required was determined by the amount of single-stranded DNA. Weinstock *et al.* (25)

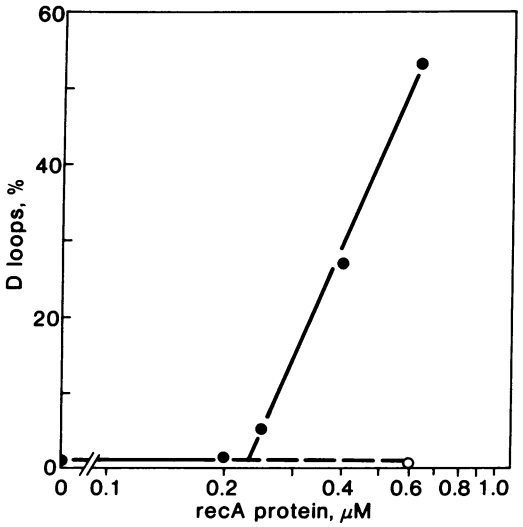

FIG. 5. Concentration of recA protein required to form D loops in the presence of 2.5 μM SSB. Concentrations of DNA were the same as in the legend to Fig. 4. In the absence of SSB, no D loops were formed under these conditions until the concentration of recA protein exceeded 1.4 μM, and 51% D loops were formed at a concentration of 4 μM recA protein. ●, 2.5 μM SSB; ○, no SSB.

observed that recA protein can promote the pairing of complementary single strands of DNA, although not nearly as well as certain helix-destabilizing proteins, including T4 gene 32 protein (4) and *E. coli* SSB (5). The simplest interpretation of these observations is that helix-destabilizing proteins or recA protein itself can unfold single strands preparatory to the action of recA protein in forming a D loop. However, because an excess of single-stranded DNA, in relation to recA protein, inhibits the formation of D loops (unpublished observation), it is possible that coating the single strands with protein not only unfolds them but also obviates the inhibitory effect of naked strands.

Helix-destabilizing proteins interact with DNA and other proteins in several ways that may be relevant to recombination (6, 7). In addition to catalyzing renaturation of complementary strands (4, 5), they protect single strands from digestion by a variety of nucleases *in vitro* (7, 26–29) and *in vivo* (29–31) and they promote strand displacement by other enzymes (32–34). The present observations show that helix-destabilizing proteins acting in concert with recA protein can favor the uptake of a homologous single strand by duplex DNA. Thus, like recA protein itself (35), helix-destabilizing proteins may play a role in both ends of a strand transfer—i.e., donation of a strand by a duplex molecule and uptake of that strand by a recipient duplex molecule. The existence of an enzymatic basis for donation and uptake of a strand, involving as one component recA protein, which is essential for recombination in *E. coli* (17, 18), supports the theory that recombination often begins asymmetrically with the transfer of a strand from one molecule to another (7, 36, 37).

The reduction by *E. coli* SSB in the amount of recA protein required to form D loops *in vitro* could mean that less recA protein might be needed *in vivo* than indicated by observations on purified recA protein acting alone (12, 13, 19). However, inhibition of D-loop formation by excess amounts of SSB raises problems for our understanding. If these proteins cooperate *in vivo*, additional factors must regulate their interaction or their relative abundance, particularly in view of the very small difference between the concentrations of SSB that stimulate or inhibit the formation of D loops. Our observation of this small difference between stimulation and inhibition resembles that of Yarranton and Gefter (34), who found that a narrow range of concentrations of SSB stimulates the unwinding activity of rep protein on a partially duplex molecule.

Finally, these observations support the view that the pairing of complementary single strands to form duplex DNA is a different reaction from the pairing of a single strand with duplex DNA to form a D loop (Fig. 1). Different proteins catalyze the reactions and, either catalyzed or not, the two reactions have different properties (ref. 9 and unpublished observations).

Note Added in Proof. While this paper was in press, McEntee *et al.* (38) reported related observations on *E. coli* SSB.

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