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On the Role of Single-stranded DNA Binding Protein in recA Protein-promoted DNA Strand Exchange*

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Michael M. Cox‡, Daniel A. Soltis, Zvi Livneh§, and I. R. Lehman

From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

RecA protein-promoted DNA strand exchange is greatly stimulated by the single-stranded DNA binding protein (SSB) of *Escherichia coli*. Stimulation is not a consequence of the binding of SSB to excess single-stranded DNA. It results instead from stabilization of recA protein-single-stranded DNA complexes formed in the presence of ATP and SSB. In the presence of SSB, recA protein does not measurably dissociate from these complexes for up to 90 min. However, in its absence, recA protein moves rapidly between two populations of single-stranded DNA, and complete equilibration occurs with $t_{1/2}$ of 17 s. Rapid transfer of recA protein to single-stranded DNA occurs during all stages of DNA strand exchange and does not require ATP. The transfer involves an equilibrium between free and bound recA protein rather than a direct redistribution between single-stranded DNA molecules. Thus, SSB prevents dissociation of recA protein from single-stranded DNA, rendering the binding of the recA protein to single-stranded DNA irreversible. Under these conditions, the pairing phase of the strand exchange reaction is accelerated to the point that it is no longer rate-limiting. These results can explain the relative inefficiency of DNA strand exchange in the absence of SSB.

The recA protein of *Escherichia coli* promotes the complete exchange of strands between full length linear duplex and homologous circular single-stranded DNA, yielding a circular duplex with a single interruption and a linear single strand (1-4). The reaction can be divided kinetically into two phases. The first consists of all steps preceding and including the formation of the first base pairs of heteroduplex DNA to yield a structure termed a D-loop. In the second phase, the heteroduplex is extended via branch migration to yield products (1, 2). D-loop formation is rapid and requires ATP but not its hydrolysis (1, 5). In contrast, branch migration is relatively slow, has a constant requirement for ATP hydrolysis (1), and proceeds with a unique polarity (2, 6-8). The reaction requires stoichiometric amounts of recA protein and is greatly stimulated by SSB[1] (1, 3, 4). Polarity is a property of the reaction whether or not SSB is present. However, the relative rates and the ATP requirement of the two phases have been examined only in the presence of SSB.

A number of genetic studies have suggested that SSB is a factor in homologous genetic recombination and in recombinational repair *in vivo* (9-11). *In vitro*, SSB stimulates D-loop formation under conditions in which insufficient recA protein is present to saturate the ssDNA (5, 12), suggesting that it may titrate excess ssDNA that would otherwise inhibit the reaction. Other observations have, however, pointed to a more direct role for SSB in recA protein-associated reactions. Thus, SSB permits formation of D-loops in the presence of adenosine 5′-*O*-(3-thiotriphosphate) (1-3, 5); and, in fact, the ability to detect such D-loops only in the presence of SSB led to the initial suggestion that SSB influences the dissociation of recA protein from DNA (13). It has also been shown that filaments of recA protein (14-16) are formed more rapidly in the presence of SSB than in its absence (17). Finally, recA protein-promoted strand exchange is greatly stimulated by SSB even when recA protein is present in saturating amounts (1, 3, 4). Taken together, these observations suggest that there may be a direct interaction between the two proteins.

We have recently shown that SSB and ATP are required for the formation of a highly stable complex of recA protein and ssDNA (4). This complex has been defined kinetically as an early intermediate in DNA strand exchange in the presence of SSB. Subsequent steps occur within this complex, which contains up to 1 monomer of recA protein/2 nucleotides of ssDNA. Moreover, recA protein does not dissociate from this complex for up to 90 min. This finding suggests that stimulation of the reaction by SSB is a result of stabilization of these complexes. Correspondingly, the relative inefficiency of strand exchange in the absence of SSB may reflect an instability of recA protein·DNA complexes which should be kinetically demonstrable. We show here that in the absence of SSB, recA protein does, in fact, move rapidly from one population of ssDNA molecules to another during all stages of DNA strand exchange. Such movement does not occur in the presence of SSB.

EXPERIMENTAL PROCEDURES

Materials—RecA protein was purified to homogeneity as described (18). Its concentration was calculated from the extinction coefficient $E^{1\%}_{280} = 5.9$ (19). SSB was purified by a modification of a published procedure (20). Its concentration was calculated from the extinction coefficient $E^{1\%}_{280} = 6.2$ (21). Nuclease S1, phosphoenolpyruvate, and pyruvate kinase were purchased from Sigma. Restriction endonucleases were purchased from New England Biolabs. To reduce the salt content of the pyruvate kinase preparation, an aliquot of the ammonium sulfate suspension was centrifuged and the pellet was resuspended in reaction buffer to the original volume.

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‡ Fellow of the American Cancer Society and the A.P. Gianinni-Bank of America Foundation. Present address, Department of Biochemistry, University of Wisconsin, Madison, WI 53706.

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[1] The abbreviations used are: SSB, *E. coli* single-stranded DNA binding protein; φX, bacteriophage φX174; ssDNA, single-stranded DNA; RFII, closed circular duplex DNA with a single strand interruption.

Linear duplex and circular single-stranded φX DNA (+strand) were prepared, and their concentrations were determined as described (1, 4). *Pst*I-cut linear duplex DNA replaced *Ava*I-cut linear duplex unless otherwise indicated. ^{3}H-labeled φX DNA preparations had specific activities ≥100,000 cpm/μg (nucleotide). DNA concentrations are given in nucleotides.

^{3}H-labeled nucleotides were purchased from Amersham Corp. Adenosine 5′-*O*-(3-thiotriphosphate) was purchased from Boehringer Mannheim and purified on DEAE-Sephadex before use. Nitrocellulose filters (HAWP, 0.45-μm pore diameter) were from Millipore.

Methods—Reaction mixtures contained 25 mM Tris·HCl, 80% cation (final pH = 7.2), 5% (v/v) glycerol, 10 mM $MgCl_2$, 1 mM dithiothreitol, and DNA, recA protein, SSB, and ATP as described in the text and figure legends. In experiments involving an ATP-regenerating system, the Tris·HCl buffer concentration was reduced to 20 mM and 10 mM KCl was added.

ATP-regenerating systems contained sufficient phosphoenolpyruvate and pyruvate kinase to convert all ADP present to ATP with a $t_{1/2}$ of 5–10 s and to maintain a negligible ADP concentration for at least 60 min.

Unless noted otherwise, reactions were initiated by the addition of ATP or ATP and SSB as a mixture after preincubation of other components. Where measurements of extent are reported, the values represent the average of at least three determinations taken between 40 and 60 min after start of the reaction. When both labeled and unlabeled φX ssDNA were present, "per cent heteroduplex" refers to the fraction of ^{3}H-labeled DNA incorporated into heteroduplex rather than the fraction of the total DNA that had reacted. Similarly, when both labeled and unlabeled duplex DNA were present, "per cent D-loops" refers to the fraction of labeled DNA incorporated into D-loops rather than the fraction of total DNA that had reacted.

Nitrocellulose Filter Binding Assay for D-loop Formation—This assay, which measures and defines the product of the first phase in strand exchange,[2] was carried out as described previously (1) except for two minor modifications. The aliquot size was decreased to 25 μl, and the high salt mixture (1 ml of 2 M NaCl, 0.15 M sodium citrate) was added at 15 s rather than 20 s after the reaction was stopped. These changes increased the efficiency of the assay approximately 10% but had no effect on controls with heterologous ssDNA and duplex DNA.

Assay for Heteroduplex Formation—This assay measures the incorporation of ^{3}H-labeled ssDNA into S1 nuclease-resistant material. Procedures used in the assay and the treatment of data have been described previously (4).

Agarose-Gel Electrophoresis—This was carried out as described previously (1).

RESULTS

Strand Exchange in the Absence of SSB—The progress of both D-loop and heteroduplex formation during strand exchange in the absence of SSB is shown in Fig. 1. After a lag of about 2 min, D-loop formation increased linearly for 10 min and then leveled off. The net formation of heteroduplex DNA proceeded more slowly. A similar relationship between D-loop formation and net heteroduplex formation was observed previously in reactions containing SSB (1). Although several interpretations of this result are possible, the simplest is that D-loop formation is fast relative to the subsequent extension of the heteroduplex by branch migration. For reactions carried out in the presence of SSB, this interpretation has been shown to be correct (1, 2). However, as demonstrated below, the results presented in Fig. 1 reflect a different kinetic situation.

[2] The terms used in this paper are defined as follows. "Strand exchange" refers to the complete conversion of linear duplex and circular (+) single-stranded DNA to RFII and displaced linear (+) single-stranded DNA. "D-loop formation" refers to the first phase of strand exchange and includes all steps between the first interaction of recA protein with ATP or DNA to the formation of a homologously paired D-loop detectable in the nitrocellulose filter binding assay. "Branch migration" or "heteroduplex extension" refers to the second phase of strand exchange as described in the Introduction. "Heteroduplex formation" refers to the experimental quantity measured in the S1 nuclease assay prior to kinetic interpretation and thus does not necessarily denote a step or phase in the reaction.

SSB Affects an Early Step in DNA Strand Exchange—As with any stimulatory effect, SSB must act at the step in the reaction sequence that is rate determining in its absence. The effect of SSB on the progress of heteroduplex formation during strand exchange is shown in Fig. 2. Stimulation was greatest in the first 10 min, the result of a 5–10-min lag observed consistently in the absence of SSB. We demonstrated previously that the branch migration phase of the reaction is rate-limiting when SSB is present and that the S1 nuclease assay for heteroduplex formation reflects this phase of the reaction (1, 2). The experiment of Fig. 2 thus shows that the step that is affected by SSB occurs at or before the branch migration phase of strand exchange.

As shown in Fig. 3, SSB also has a strong stimulatory effect on D-loop formation. The magnitude of this effect is influenced by the order of addition of reaction components. Stimulation was greatest when SSB was the last component added, as observed previously for heteroduplex formation (3, 4). Thus, when saturating amounts of SSB (1/10 nucleotides of ssDNA) were added last, essentially all of the duplex DNA

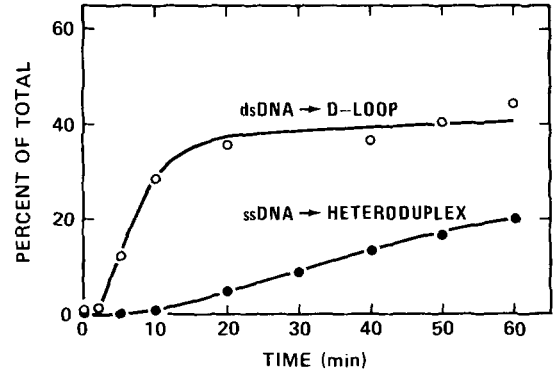


FIG. 1. **RecA protein-promoted DNA strand exchange in the absence of SSB.** Reactions were carried out as described under "Methods." Reaction mixtures contained 3.3 μM circular φX ssDNA, 5.6 μM linear φX duplex DNA (cleaved with *Pst*I), 1.3 mM ATP, 1.9 μM recA protein, and an ATP-regenerating system. O——O, D-loop formation measured by the nitrocellulose filter binding assay; ●——●, net heteroduplex formation measured with the S1 nuclease assay. *dsDNA*, double-stranded DNA.

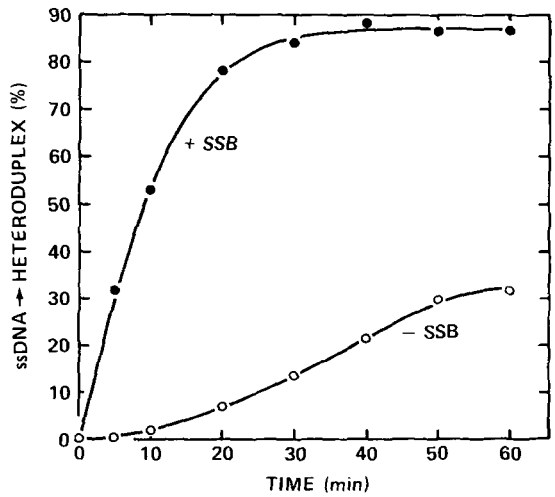


FIG. 2. **The effect of SSB on recA protein-promoted heteroduplex formation.** Reactions were carried out as described under "Methods." Reaction mixtures contained 11.2 μM linear duplex φX DNA, 6.6 μM ^{3}H-labeled circular φX ssDNA, 3.8 μM recA protein, 2.6 mM ATP, and an ATP-regenerating system. SSB to 0.63 μM was added as indicated.

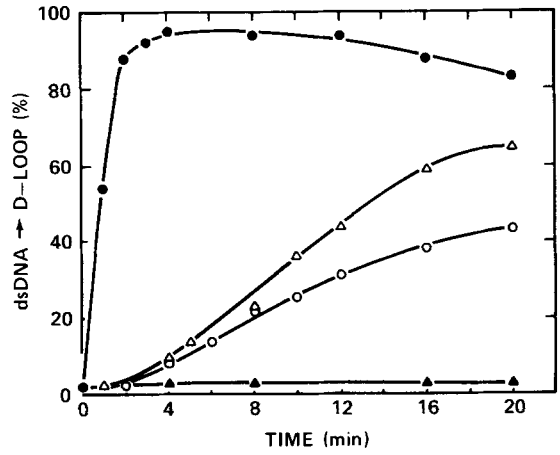


FIG. 3. **The effect of SSB on recA protein-promoted D-loop formation.** Reactions were carried out as described under "Methods." Reaction mixtures contained 5.6 μM linear duplex φX DNA, 3.3 μM circular φX ssDNA, 3.2 μM ATP, 1.9 μM recA protein, and 0.31 μM SSB as indicated. ●——●, reaction initiated with ATP and SSB; ○——○, reaction initiated with ATP, no SSB added; △——△, reaction initiated with ATP after preincubation of all other components including SSB; ▲——▲, bacteriophage M13 ssDNA substituted for φX ssDNA. *dsDNA*, double-stranded DNA.

molecules were converted to D-loop-containing structures within 2 min, reflecting an increase in rate of 15–20-fold over the reaction in the absence of SSB. When SSB and recA protein were both added to the reaction mixture 10 min before the addition of ATP, only 50% stimulation was observed. Intermediate levels of stimulation were obtained by varying the concentration of SSB or the order of its addition (data not shown). Thus, the phase of the reaction that is stimulated by SSB and, therefore, is rate-limiting in its absence is D-loop formation.

This conclusion is supported by the results presented in Fig. 4. Full length linear duplex φX DNAs were prepared with the use of three different restriction endonucleases, *Pst*I, *Stu*I, and *Xho*I. These enzymes produce termini that contain four unpaired bases at the 3′ termini, no unpaired bases, and four unpaired bases at the 5′ termini, respectively. Since branch migration proceeds 3′ → 5′ relative to the (−) strand of the duplex (2, 6–8), the DNA cleaved with the *Pst*I enzyme might be expected to be more reactive than the others if D-loop formation is rate-limiting. As shown in Fig. 4, the structure of the ends significantly affected the rate of heteroduplex formation in the absence of SSB, with linear duplex DNA produced by *Pst*I cleavage showing the greatest activity. Inasmuch as the branch migration phase of the reaction should be independent of the structure of the ends, the phase of strand exchange that is rate-limiting in the absence of SSB is again D-loop formation. Heteroduplex formation in the presence of SSB, in contrast, was unaffected by the structure of the ends. Thus, the phase of the reaction that is rate-limiting in the presence of SSB is branch migration, a result that is consistent with our earlier findings (1, 2).

Stimulation of Strand Exchange by SSB Does Not Result from Binding Excess ssDNA—In the experiments described thus far, recA protein was present in amounts sufficient to saturate the ssDNA (≥1 monomer/2 nucleotides). It is thus unlikely that SSB is acting simply to bind excess ssDNA. To examine this question further, increasing amounts of SSB were added in the presence of subsaturating or saturating concentrations of recA protein, and initial rates of D-loop or heteroduplex formation were measured (Figs. 5 and 6). At subsaturating concentrations of recA protein (1 monomer/8

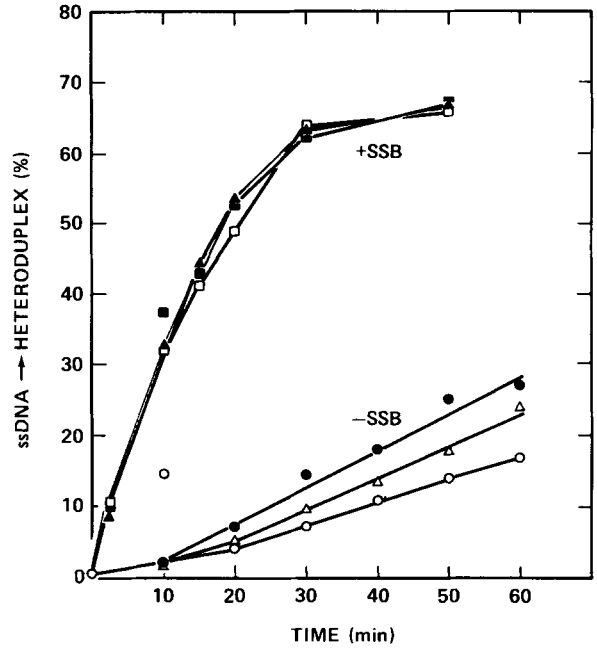


FIG. 4. **Effect of the structure of ends of duplex DNA on the rate of heteroduplex formation.** Reactions were carried out as described under "Methods." Reaction mixtures contained 11.2 μM linear φX duplex DNA, 6.6 μM ^{3}H-labeled circular φX ssDNA, 1.9 μM recA protein, 2.0 mM ATP, and an ATP-regenerating system. SSB to 0.3 μM was added as indicated. Linear duplex DNA was generated by cutting with the restriction enzymes *Pst*I (□——□ and ●——●); *Stu*I (▲——▲ and △——△); and *Xho*I (■——■ and ○——○).

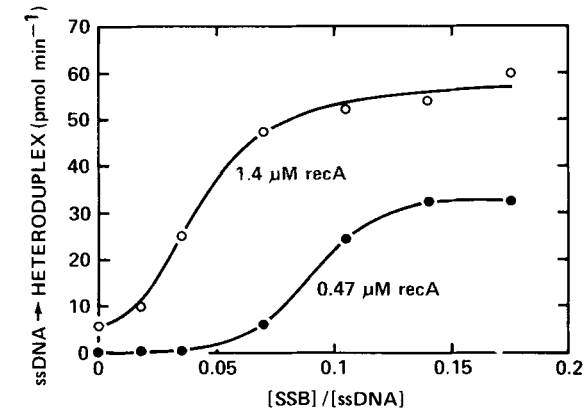


FIG. 5. **RecA protein-promoted heteroduplex formation: dependence on SSB concentration.** Reactions were carried out as described under "Methods." Initial rates of heteroduplex formation were determined from at least five time points taken in the first 6 min of reaction. Reaction mixtures contained, in 314 μl, 3.3 μM ^{3}H-labeled circular φX ssDNA, 5.6 μM linear duplex φX DNA, 1 mM ATP, and SSB and recA protein as indicated.

nucleotides), very little reaction was observed in the absence of SSB. This amount of recA protein should bind up to one-half of the ssDNA (14). Addition of enough SSB (1/20 nucleotides) to bind one-half of the ssDNA failed to produce significant stimulation of either D-loop or heteroduplex formation. Stimulation was observed only as the concentration of SSB approached that required to saturate the ssDNA (1 SSB tetramer/10 nucleotides). In contrast, when 3-fold *higher* concentrations of recA protein were employed, the reaction was stimulated by *lower* levels of SSB (Fig. 5). A plausible interpretation of these findings is that SSB binds open regions of ssDNA more readily than regions to which recA protein is

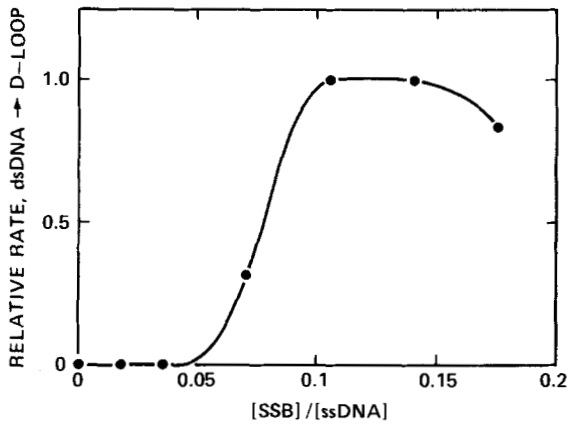


FIG. 6. **RecA protein-promoted D-loop formation: dependence on SSB concentration.** Reactions were carried out as described under "Methods." Initial rates of D-loop formation were obtained from 4–5 time points taken in the linear portion of a full time course (the first 2–8 min, depending on SSB concentration). Reaction mixtures contained 5.6 μM ^{3}H-labeled linear duplex φX DNA, 3.3 μM circular φX ssDNA, 0.47 μM recA protein, 3.2 mM ATP, and SSB as indicated. *dsDNA*, double-stranded DNA.

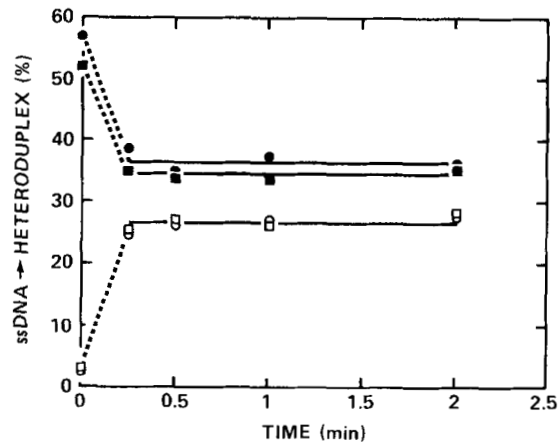


FIG. 7. **Equilibration of recA protein between two populations of ssDNA molecules.** Reactions were carried out as described under "Methods." The following experimental conditions were used.

	Addition at			x
	−20 min	−10 min	0	
●—●	recA	[^{3}H]SS	SS	ATP, DS 2 SSB
○—○	recA	SS	[^{3}H]SS	ATP, DS 2 SSB
■—■	recA	[^{3}H]SS ATP	SS	DS 2 SSB
□—□	recA	SS ATP	[^{3}H]SS	DS 2 SSB

Buffer components and an ATP-regenerating system were present in all cases before the first addition listed. The extent of heteroduplex formation was measured and taken as the average of three measurements between 50 and 60 min after $t = 0$. Final concentrations resulting from the indicated additions are unlabeled circular φX ssDNA (SS), 6.6 μM; ^{3}H-labeled φX circular ssDNA ([^{3}H]SS), 6.6 μM; full length linear φX duplex DNA (DS), 11.2 μM; recA protein (recA), 1.8 μM; SSB, 0.64 μM; and ATP, 2.5 mM. Where the number 2 precedes the indicated addition (*i.e.* 2 SSB), the final concentration resulting from that addition is doubled (to 1.3 μM in the case above). Components added at the same time were added as a mixture. Where $x = 0$, DS and SSB (±ATP) were added at $t = -2$ min, either SS or [^{3}H]SS was added at $t = 0$, and SSB again at $t = 0.5$ min. Reaction mixtures were kept at 37 °C for 5 min before the first indicated addition.

bound. Furthermore, stimulation of the reaction does not occur until the open regions of ssDNA are saturated with recA protein and additional SSB can promote stable complex formation. Thus, little if any stimulation occurs simply by binding SSB to excess ssDNA.

Rapid Movement of recA Protein in the Absence of SSB— The observation that stable complexes of recA protein and ssDNA are formed in the presence of ATP and SSB provides a kinetic tool with which to examine movement of recA protein in the absence of SSB. Presumably, recA protein remains associated with the ssDNA molecule to which it is bound at the time at which SSB is added. As in the previous study (4), a competition was set up between two populations of φX ssDNA molecules. RecA protein is incubated with one population, designated Reaction 1, and another is added as a challenge, designated Reaction 2. Enough recA protein is present to promote an almost maximal strand exchange with Reaction 1 alone. However if it is distributed between Reactions 1 and 2, a submaximal reaction will occur in both cases. Reaction 1 or 2 can be followed independently by using ^{3}H-labeled ssDNA for one or the other. At various times after the challenge, any movement of recA protein from Reaction 1 to Reaction 2 is halted by the addition of ATP and SSB. Duplex DNA is added at the same time to permit strand exchange with those ssDNA molecules to which recA protein is bound. The final extent of heteroduplex formation in Reaction 1 or Reaction 2 is measured 40–60 min after the addition of SSB and duplex DNA. The results of such an experiment are presented in Fig. 7. When ATP, SSB, and duplex DNA were added before the Reaction 2 ssDNA, efficient strand exchange occurred with Reaction 1, while the ssDNA of Reaction 2 was inert. This is the result to be expected from formation of a complex in Reaction 1 that prevents further interaction of recA protein with Reaction 2. When the Reaction 2 ssDNA was added before the SSB/duplex DNA/ATP mixture, recA protein apparently equilibrated between Reactions 1 and 2 within 15 s ($t_{1/2} \leq 5$ s), resulting in a partial reaction in both cases. Since some movement of recA protein can presumably occur in the time required for stable complex formation (4), a correction must be made for this apparent rate of equilibration. In a separate experiment, carried out as described previously (4), it was determined that the $t_{1/2}$ for complex formation was 12 ± 5 s under these conditions. Adding this value to the apparent $t_{1/2}$ noted above yields a value of ≤17 s for the half-time of the equilibration process. Employing the appropriate assumptions,[3] this value yields a rate constant $k_2 \geq 1 \times 10^4$ M^{-1} s^{-1} for the transfer process:

$$D\text{-recA} + R \overset{k_2}{\rightleftarrows} D + R\text{-recA}$$

where D and R are donor and recipient DNA sites, respectively, defined as stretches of 4 nucleotides of ssDNA. Recent studies have demonstrated the cooperative binding of recA protein to DNA (14). If filaments of recA protein are transferred rather than monomers, the DNA sites required will be larger, their concentration lower (in a constant total pool of

[3] Taking 20 s as the upper limit for the $t_{1/2}$ for equilibration yields an apparent first order rate constant $k_1 \geq 3.4 \times 10^{-2}$ s^{-1}. This is a rate constant for the approach to equilibrium and is thus equivalent to the sum of the constants for the forward and reverse processes (22). The assumption is made that the forward and reverse rate constants are equivalent so that the k_1 above is simply corrected by a factor of 2 to yield $k_1 \geq 1.7 \times 10^{-2}$ s^{-1} for a unidirectional transfer of recA protein. The concentration of reaction 2 ssDNA is 6.6×10^{-6} M, making the concentration of 4-nucleotide-recA protein binding sites 1.65×10^{-6} M. Dividing by this number yields $k_2 \geq 1 \times 10^4$ M^{-1} s^{-1}.

DNA), and k_2 correspondingly larger. This rate constant is only a lower limit and should thus be viewed as a starting point for a more detailed physical analysis of this phenomenon.

The result shown in Fig. 7 was unaffected by the addition of ATP prior to Reaction 2. This finding does not imply that ATP is without effect on the binding of recA protein to ssDNA. For example, the limitations of the measurement may not permit its detection. In fact, a substantial stimulation of dissociation of recA protein from ssDNA was previously observed in the presence of ATP and a number of other nucleotides at 28 °C (14). It is clear, in any case, that on the time scale of the strand exchange reaction, movement of recA protein between ssDNA molecules is rapid unless SSB is present.

Movement of recA Protein during Ongoing Strand Exchange—This process was examined by challenging the reaction with an equivalent amount of unlabeled φX ssDNA. The levels of recA protein were again sufficient to promote maximal strand exchange with only one set of ssDNAs. The effect of a ssDNA challenge to a reaction carried out in the presence of SSB is shown in Fig. 8. When 1 eq of unlabeled φX ssDNA was added 4 min after the beginning of the reaction, the rate of heteroduplex formation was decreased by 50%, but the extent of the reaction was unaffected. No reaction of the challenging DNA was observed (determined by labeling the challenging DNA instead of the DNA in the initial reaction). When more SSB was added after the challenge, the inhibition was relieved. Previous work indicates that neither recA protein nor SSB is transferred to the challenging DNA under these conditions (4), although transfer of small amounts of either protein may have gone undetected. If inhibition of the rate resulted from movement of recA protein to the challenging DNA, then the extent of reaction should decrease, the challenging DNA should participate in the reaction, and the additional SSB would be expected to stimulate the reaction of the challenging DNA. Since at 4 min stable complexes and D-loops have already been formed (Ref. 4 and Fig. 3), the inhibition is most likely a consequence of competitive interaction with the reacting complexes that results, in turn, in the inhibition of branch migration. Inasmuch as recA protein can promote a reciprocal exchange involving four strands of DNA (23, 24), sites may exist that can accommodate four DNA strands in these complexes. Since only three are occupied in this reaction, the additional φX ssDNA could competitively inhibit branch migration by binding transiently to the putative fourth site. Since any interaction of extraneous ssDNA with the complex might also interfere with branch migration, the four-site model for inhibition is not the only one possible. Additional SSB could, in any case, relieve the inhibition by preventing the unproductive interaction.

The decrease in reaction rate could also be explained by a partial transfer to the challenging DNA of SSB rather than recA protein. However, this possibility is made unlikely by the observation (4) that if additional recA protein and SSB are added in sequence after the challenge, a maximally efficient reaction occurs with the challenge DNA. If SSB were transferred to the challenge DNA before the second recA protein addition, a submaximal reaction of the challenge DNA would be expected depending on the amount of SSB transferred (4).

Challenging an ongoing strand exchange with a 10-fold excess of heterologous ssDNA results in complete inhibition of heteroduplex formation (1). However, this effect very likely reflects the type of inhibition described above rather than resulting from the sequestering of free recA protein. A similar challenge with heterologous duplex DNA had no effect on the reaction (4).

Similar challenge experiments were carried out on reactions in the absence of SSB (Figs. 9 and 10). SSB was added 2 min after the challenge in each case to halt movement of recA protein. Addition of SSB 12 min after the beginning of the reaction resulted in an immediate stimulation of heteroduplex formation to a rate and extent equivalent to a reaction initi-

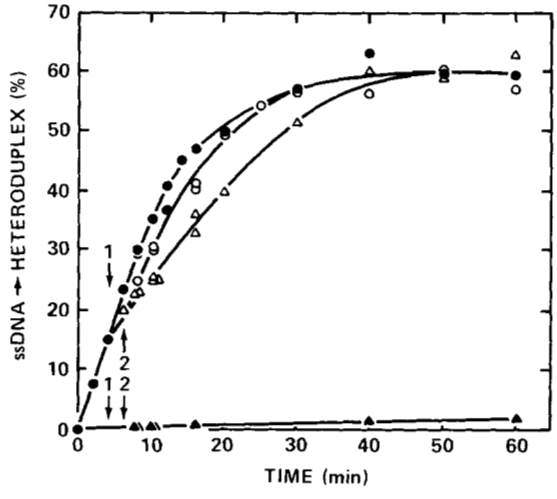


FIG. 8. **RecA protein-promoted heteroduplex formation in the presence of SSB: inhibition by added ssDNA.** Reactions were carried out as described under "Methods" and contained 6.6 μM circular φX ssDNA (^{3}H-labeled or unlabeled), 11.2 μM linear duplex φX DNA, 1.8 μM recA protein, 0.33 μM SSB, and 2.5 mM ATP. △——△, ^{3}H-labeled ssDNA in reaction challenged with 6.6 μM unlabeled circular φX ssDNA at t = 4 min (*arrow 1*); ○——○, ^{3}H-labeled φX ssDNA in reaction challenged with 6.6 μM unlabeled φX ssDNA at t = 4 min and 0.33 μM SSB at t = 6 min (*arrow 2*); ▲——▲, unlabeled φX ssDNA in reaction challenged with 6.6 μM ^{3}H-labeled ssDNA at t = 4 min and 0.35 μM SSB at t = 6 min; ●——●, no challenge.

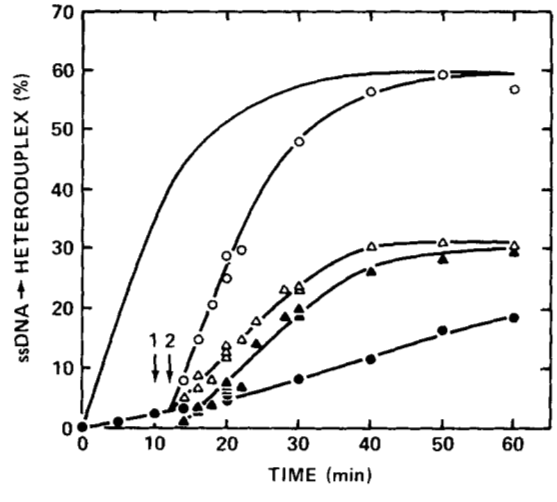


FIG. 9. **RecA protein-promoted heteroduplex formation in the absence of SSB: inhibition by ssDNA added at 10 min.** Reactions were carried out as described under "Methods." Concentrations of reaction components were as described in the legend to Fig. 8 except that no SSB was used in the reaction except after a challenge. The *solid line* represents an unchallenged reaction in the presence of SSB, data from Fig. 8. ○——○, 0.35 μM SSB added at t = 12 min (*arrow 2*); △——△, ^{3}H-labeled φX ssDNA in reaction challenged with 6.6 μM unlabeled φX ssDNA at t = 10 min (*arrow 1*) and 0.7 μM SSB at t = 12 min; ▲——▲, unlabeled φX ssDNA in reaction challenged with 6.6 μM ^{3}H-labeled φX ssDNA at t = 10 min and 0.7 μM SSB at t = 12 min; ●——●, no challenge.

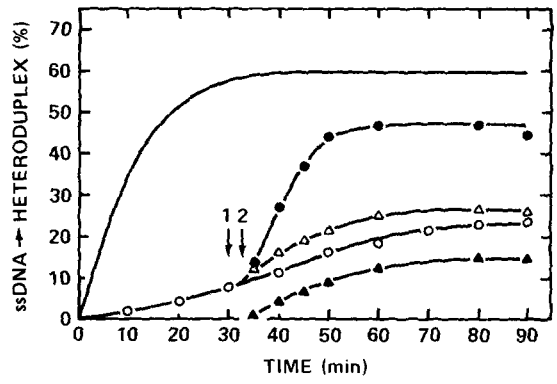


FIG. 10. **RecA protein-promoted heteroduplex formation in the absence of SSB: inhibition by ssDNA added at 30 min.** Reactions were carried out as described under "Methods" and in the legend to Fig. 9, except that challenges with φX ssDNA and SSB were carried out at 30 and 32 min, respectively. Experiments represented by *open* and *closed triangles* parallel experiments with the same designations in Fig. 9. *Open circles* represent an unchallenged reaction, while *closed circles* represent a reaction challenged only with 0.35 μM SSB at $t = 32$ min.

ated with SSB and ATP. When the challenging DNA was added at 10 min, both the rate and extent of reaction upon addition of SSB were decreased by about 50% (Fig. 9). Moreover, the challenging DNA participated in the reaction with a rate and to an extent comparable to that observed with the DNA in the initial reaction. This result is plausibly explained by movement of recA protein to the challenging DNA during the 2-min period between the challenge and the addition of SSB. At 10 min after the beginning of a reaction in the absence of SSB, 30% of the duplex DNA was incorporated into D-loops (Fig. 2).[4] The efficiency of the reaction with the challenging DNA implies that little or no recA protein had been sequestered as a result of D-loop formation. The extent of the reaction with the challenging DNA further implies that the D-loops themselves are unstable and that some of the duplex DNA already incorporated into D-loops is still available to react with the challenging DNA. A similar experiment was carried out 30 min after the beginning of the reaction (Fig. 10). At this point, more than 40% of the duplex DNA had been incorporated into D-loops and some RFII had been formed.[5] Nevertheless, the challenging DNA was still as reactive as the original DNA. After 32 min, 9.5% of the potential heteroduplex DNA had been formed in the reaction lacking SSB. If allowance is made for this amount of reaction, the rate and extent of heteroduplex formation with both the challenging and the original DNA were equivalent (Fig. 10 and Table I). Thus, rapid movement of recA protein takes place between ssDNA molecules in the absence of SSB even in an ongoing strand exchange reaction.

Mechanisms of Transfer of recA Protein between DNA Molecules—In the presence of SSB, recA protein is stably bound in a complex (4). Thus, it is to be expected that the rate of reaction in the presence of SSB should reach saturation at a fixed ratio of recA protein to ssDNA. In the absence of SSB, recA protein is rapidly transferred between DNA molecules. There are two possible mechanisms involved in such a transfer. (i) recA protein might dissociate from the first DNA molecule to join the pool of free recA protein. Free recA protein would then bind the second DNA molecule. (ii) recA protein could be transferred directly between DNA molecules. A direct transfer would imply a small pool of free recA protein, and the rate of reaction should reach saturation at a ratio of recA protein to ssDNA similar to that observed in a reaction in the presence of SSB. Transfer of recA protein involving a rapid equilibrium between free and bound forms would imply a relatively large pool of free recA protein. In this case, recA protein in excess of that required to saturate the ssDNA might

TABLE I

Movement of recA protein after 30 min of reaction in the absence of SSB

Experiments were carried out as described under "Methods" under conditions described in the legend to Fig. 10. In Experiment 2, additional linear duplex DNA, to a total final concentration of 22.4 μM, was added with the ssDNA at the time of the challenge.

Experiment	Labeled DNA	Final extent of heteroduplex formation[a]	Corrected extent[b]
1 Challenge with ssDNA only	Reaction DNA	27.3	17.8
	Challenge DNA	17.4	17.4
2 Challenge with ssDNA and dsDNA[c]	Reaction DNA	34.3	24.8
	Challenge DNA	23.2	23.2

[a] Measurements of extent represent the average of three values obtained between 80 and 90 min.

[b] Corrected extents are listed for the Reaction DNA only. The heteroduplex present at the time of challenge (9.5%) has been subtracted so that these values reflect the amount of heteroduplex DNA formed after the challenge.

[c] *Double-stranded* DNA.

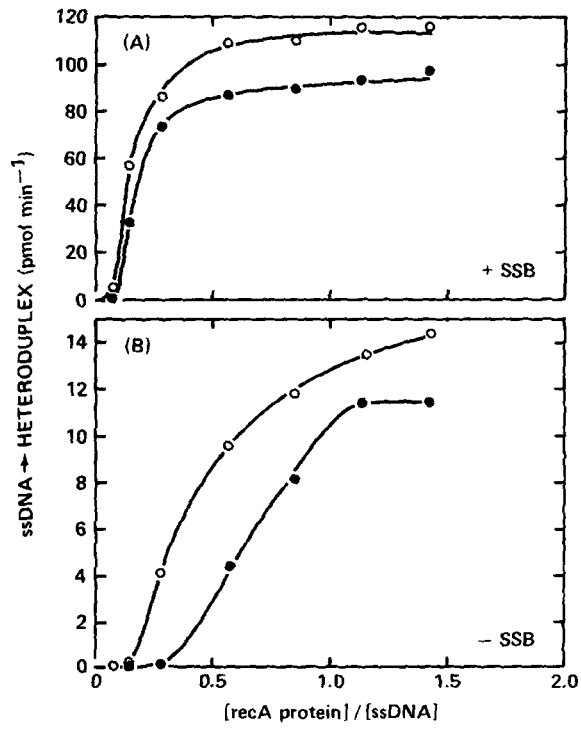


FIG. 11. **RecA protein-promoted heteroduplex formation: dependence on recA protein concentration.** Reactions were carried out as described under "Methods." Reactions indicated with *closed circles* contained 3.3 μM ^{3}H-labeled φX ssDNA, 5.6 μM linear duplex φX DNA, and 3.0 mM ATP. In reactions indicated with *open circles*, the concentration of both DNA substrates was doubled. *A*, reactions included SSB (1 monomer/10 nucleotides of ssDNA); *B*, reactions without SSB. RecA protein was added as indicated. Reaction volumes were 157 μl. For reactions containing SSB, initial rates were calculated from at least four points taken in the linear portion of the time course between 1 and 5 min after start of the reaction. For reactions in the absence of SSB, points were taken in the linear region of the time course between 10 and 25 min.

[4] M. M. Cox, D. A. Soltis, Z. Livneh, and I. R. Lehman, unpublished data.

[5] M. M. Cox, D. A. Soltis, and I. R. Lehman, unpublished results.

promote the reaction by driving the equilibrium toward the bound form. Results presented in Figs. 11 through 13 support the idea of transfer via a pool of free recA protein. The rate of heteroduplex formation in the presence of SSB reached saturation at a ratio of 1 recA protein monomer/2 nucleotides of ssDNA at two different DNA concentrations (Fig. 11*A*). This stoichiometry is consistent with the amount of recA protein that can be incorporated into stable complexes (4). In contrast, in the absence of SSB, rates of D-loop and heteroduplex formation were enhanced by increasing the concentration of recA protein even beyond a stoichiometry of 1 recA protein monomer to 1 nucleotide of ssDNA (Figs. 11*B* and 12). This result is contrary to the findings of Shibata *et al.* (25, 26), in which D-loop formation in the absence of SSB reached saturation at 1 recA protein monomer/2–4 nucleotides of ssDNA. The reason for this discrepancy is unclear, although it may be due to a difference in the DNA substrates or the methods of analysis employed. Some of the experiments from which the data of Fig. 12 were derived are shown in Fig. 13. D-loop formation is reliably linear only between 2 and 10 min into the reaction. Rate measurements were therefore taken from points in this region. All of the reactions leveled off when 30–40% of the duplex DNA had been incorporated into D-loops. This extent or steady state level of D-loop formation showed saturation at lower concentrations of recA protein than the initial rate. In controls in which bacteriophage M13 ssDNA was substituted for ϕX ssDNA, less than 1% of the duplex DNA was retained on nitrocellulose filters even at the highest concentration of recA protein employed. Thus, the continuing increase in rate observed at very high recA protein concentrations requires the presence of complementary DNA and is therefore not an artifact associated with the addition of high concentrations of recA protein.

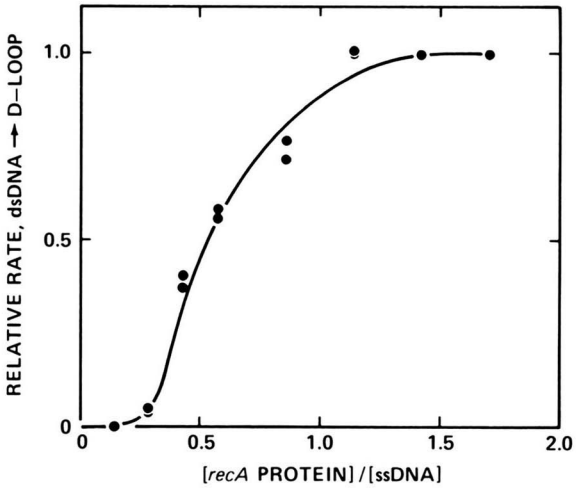


FIG. 12. **RecA protein-promoted D-loop formation in the absence of SSB: dependence on recA protein concentration.** Reactions were carried out as described under "Methods." Reaction mixtures contained 3.3 μM ϕX ssDNA, 5.6 μM ϕX linear duplex DNA, 3.0 μM ATP, and recA protein as indicated. Initial rates of D-loop formation were taken from at least five points in the linear region of the time course between 2 and 10 min. Rates are reported relative to the highest rate observed. dsDNA, double-stranded DNA.

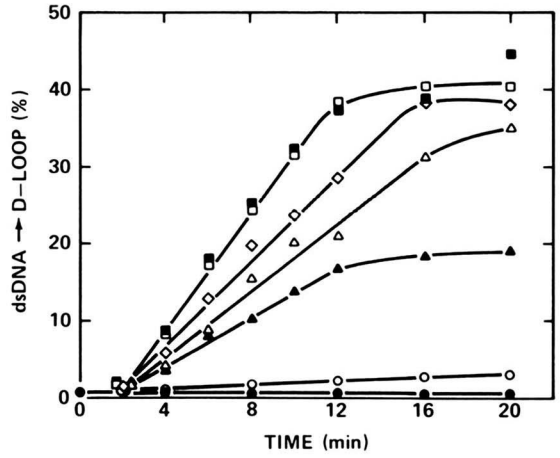


FIG. 13. **Dependence of the initial rate of D-loop formation in the absence of SSB on the concentration of recA protein.** Reactions were carried out as described under "Methods" and the legend to Fig. 12. Concentrations of recA protein are for ●——●, 0.47 μM; ○——○, 0.93 μM; ▲——▲, 1.4 μM; △——△, 1.86 μM; ◇——◇, 2.8 μM; □——□, 3.7 μM; and ■——■, 4.67 μM. *dsDNA*, double-stranded DNA.

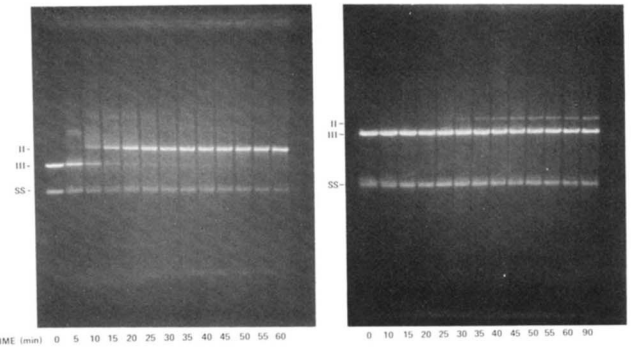


FIG. 14. **RecA protein-promoted DNA strand exchange: the effect of SSB.** Reactions were carried out as described under "Methods." The reaction with SSB contained 6.0 μM linear duplex ϕX DNA, 4.2 μM circular ϕX ssDNA, 1.7 μM recA protein, 0.53 μM SSB, 2.3 mM ATP, and an ATP-regenerating system. The reaction without SSB contained 11.9 μM linear duplex ϕX DNA, 8.3 μM circular ϕX ssDNA, 3.4 μM recA protein, 2.3 mM ATP, and an ATP-regenerating system. Aliquots were removed as indicated, and the reaction was stopped by the addition of sodium dodecyl sulfate to a final concentration of 1% and subjected to agarose-gel electrophoresis as described under "Methods." *II*, circular duplex DNA containing a nick (RFII); *III*, linear duplex DNA (RFIII); *SS*, single-stranded DNA.

The Effect of SSB on Branch Migration—The rate of strand exchange can be examined by observing formation of the product RFII by agarose-gel electrophoresis. The course of the reaction in the presence and absence of SSB is shown in Fig.14. In the presence of SSB, a small amount of RFII was formed even at 5 min. This corresponds to a rate of branch migration on at least some molecules of up to 20 base pairs·s^{-1}. In the absence of SSB, RFII was detectable at 20 min so that branch migration in some molecules must proceed at a rate of at least 4.5 base pairs·s^{-1}. As shown above, D-loops formed early in a reaction in the absence of SSB are probably unstable. Furthermore, there is a 5–10-min lag in heteroduplex formation under these conditions. Thus, the RFIIs observed at 20 min may have resulted from D-loops formed at 10–15 min or later. The branch migration phase of the reaction may therefore require 5 min or less even in the absence of SSB. Thus, while the rate of branch migration on individual D-loops may be minimally affected by the presence of SSB, the stability of the D-loops, hence, the probability that they will be converted to products, is strongly influenced by SSB.

DISCUSSION

Our findings are consistent with the minimal reaction pathway for strand exchange that we presented previously, based on the finding that SSB and ATP are required for the for-

mation of stable complexes of recA protein and ssDNA (4). We have found that SSB affects an early step or steps in strand exchange. This finding correlates well with the known effect of SSB in stabilizing recA protein·ssDNA complexes and supports the idea that such stabilization is the primary effect of SSB. SSB determines which of the two phases of strand exchange is rate-limiting. In the absence of SSB, D-loop formation is slow, whereas in the presence of SSB, the branch migration phase of the reaction is clearly rate-limiting (1, 2). Our results suggest, however, that the steps within the D-loop formation phase of the reaction in the absence of SSB are not intrinsically slow. SSB does not increase the rate of D-loop formation by accelerating an early step of the reaction, rather it exerts its effect by rendering these early steps irreversible. This is accomplished by preventing dissociation of recA protein from ssDNA. The recA protein complexes do not remain intact long enough to promote a complete strand exchange reaction unless SSB is present.

The stabilization of recA protein·ssDNA complexes is dramatic. In the presence of SSB, recA protein is not recycled from the complexes for at least 90 min and strand exchange proceeds easily to completion (4). In the absence of SSB, recA protein equilibrates between two populations of ssDNA in less than 1 min.

Addition of sufficient SSB to bind excess ssDNA has little or no effect on either phase of strand exchange. Stimulation by SSB occurs only when the concentration of SSB exceeds that required to bind the excess ssDNA. This finding, combined with the fact that maximal stimulation occurs even when more than enough recA protein is present to saturate the ssDNA, suggests strongly that SSB acts *only* through the stabilization of recA protein·ssDNA complexes. While earlier work demonstrating a stimulatory effect of SSB on D-loop formation suggested that SSB acts by preventing the sequestering of recA protein by excess ssDNA, *i.e.* in a sparing role (5, 12), all of these effects are as easily explained by the complex stabilization mechanism. We would point out that this mechanism is also compatible with experiments in which SSB has been shown to stimulate the proteolytic activity of recA protein (27, 28). It is thus possible that SSB never acts simply in a sparing role.

RecA protein forms highly structured filaments under a variety of conditions (14–17). Presumably, it is present in the form of filaments during strand exchange whether or not SSB is present. In the absence of SSB, however, recA protein moves readily from one DNA molecule to another. Thus, if filaments are indeed present, either they are highly unstable, or the filaments are themselves more mobile.

Flory and Radding (17) reported an effect of SSB on the rate of formation of recA protein filaments in the absence of a nucleotide cofactor. Since both ATP and SSB are required for formation of the stable complexes that we have defined kinetically (4), it is not clear whether the two sets of results can be compared directly. However, both sets of observations, taken together with our earlier findings (1, 5, 13), again suggest that recA protein and SSB interact directly.

A model that accommodates our findings on strand exchange in the absence of SSB is presented in Fig. 15. For the most part, the steps parallel those presented earlier (4). However, in this case, recA protein dissociates from the DNA at a rapid rate at every stage of the reaction. If dissociation occurs early, D-loops are not formed and the DNA and protein are recycled. If dissociation occurs after D-loop formation, they are rendered unstable. Random branch migration will convert the D-loops to either products or substrates depending on the extent to which directed branch migration has progressed, the rate of recA protein reassociation, and the probability factors

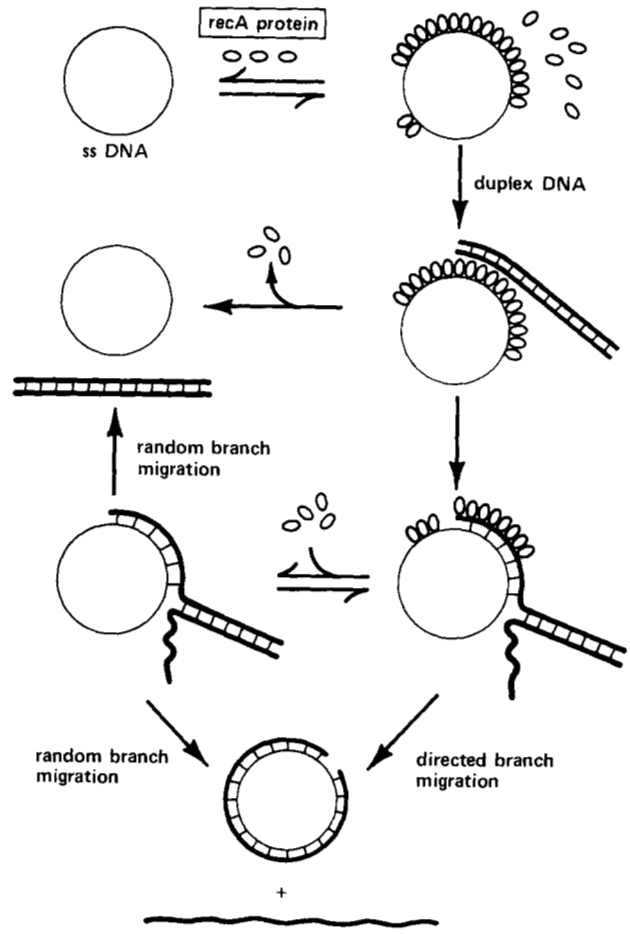


FIG. 15. **Model for recA protein-promoted strand exchange in the absence of SSB.** See text for details.

related to a random walk (29). The increase in the percentage of duplex DNA molecules involved in D-loops observed in the early part of a reaction in the absence of SSB reflects an approach to a steady state concentration of D-loops. However, under these conditions, few of the D-loops present at any point are converted to products; instead, most are recycled to the substrate pool. Since every D-loop will have a finite probability of being converted to products, the slow accumulation of heteroduplex DNA observed in Fig. 2 is not the result of a synchronous but slow branch migration involving all of the D-loops detected early in the reaction. Rather, it results from a relatively fast branch migration involving a small percentage of these D-loops to form products. Thus, the amount of heteroduplex DNA under these conditions more closely reflects the accumulation of product molecules rather than the extent of branch migration in individual molecules. A point may be reached even in the reaction without SSB at which strand exchange becomes irreversible. If this does occur, it is beyond the point at which a D-loop is formed that is detectable by the nitrocellulose filter binding assay. It is clear that the bulk of the recA protein is readily redistributed in the absence of SSB even 30 min after the reaction is started. If a small amount of recA protein were sequestered in product RFIIs in this system, it might not be detected by the methods we have employed.

RecA protein can promote reciprocal strand exchange reactions involving four strands of DNA (23, 24). West *et al.* (3, 30) have recently found that, while SSB strongly stimulates three-strand exchanges such as the one considered here, it is not required for four-strand exchanges and in some cases can

be inhibitory. In reactions involving a reciprocal exchange of thousands of base pairs, initiated at a single-stranded gap, SSB appears to stimulate the initial pairing but not the four-strand branch migration that follows (30). In this work, we have documented an effect of SSB on the early stages of strand exchange, but we have not yet found any evidence to suggest an effect of SSB on branch migration. SSB may thus be of primary importance in stabilizing recA protein complexes during three-strand exchanges such as may occur during postreplication repair or recombination following conjugation in *E. coli* (31). In four-strand exchanges, SSB may be important for stabilizing recA protein complexes in the single-stranded gap where such exchanges are presumably initiated.

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