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recA Protein-promoted DNA Strand Exchange

STABLE COMPLEXES OF recA PROTEIN AND SINGLE-STRANDED DNA FORMED IN THE PRESENCE OF ATP AND SINGLE-STRANDED DNA BINDING PROTEIN*

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The recA protein of *Escherichia coli* promotes the complete exchange of strands between full length linear duplex and single-stranded circular DNA molecules. An early step in this reaction consists of the binding of recA protein to single-stranded DNA. In the presence of ATP and the single-stranded DNA binding protein, recA protein and single-stranded DNA interact to form a complex whose stability depends upon the single-stranded DNA binding protein. Duplex DNA is not required for complex formation. Subsequent steps occur within this complex which contains up to 1 recA protein monomer per 2 nucleotides of single-stranded DNA. Although several hundred ATPs are hydrolyzed per recA protein monomer, recA protein is not released from the complex at any step during or subsequent to strand exchange. The complex is kinetically competent with respect to both its rate of formation and its rate of reaction with homologous duplex DNA. It is therefore a significant intermediate in the overall reaction pathway. These results have served as the basis for an expanded model for recA protein-promoted strand exchange.

recA protein can promote a variety of DNA strand exchanges that are analogous to those steps in homologous recombination *in vivo* in which recA protein probably participates (1–4). Of these, exchange of strands between circular single-stranded and linear duplex DNAs has been particularly informative (3, 5–8). recA protein-promoted strand exchange proceeds in two kinetically distinguishable phases (3, 7). The first, formation of a short heteroduplex region (a D-loop), is relatively fast, proceeds in the presence of the nonhydrolyzable ATP analog adenosine 5′-*O*-(thiotriphosphate), and can occur at either end of the duplex. The second phase, branch migration, is relatively slow, exhibits a continued requirement for ATP hydrolysis, and shows a unique polarity. Stoichiometric, rather than catalytic, amounts of recA protein are required and the reaction is strongly stimulated by SSB.[1]

These features of strand exchange appear to define a coupled vectorial process in which the chemical energy of ATP is utilized to bring about a unidirectional branch migration. In principle, the reaction should be subject to the same constraints as muscle contraction and ion transport (9). Thus, the mechanism may be of fundamental interest not only with respect to homologous recombination but to the mechanism of biochemical energy conversion, as well.

As a first step in the elucidation of the mechanism of recA protein-promoted strand exchange, we have investigated the need for stoichiometric amounts of recA protein and have attempted to define the role of SSB. We have found that in the presence of SSB and ATP, recA protein forms a tight complex with single-stranded DNA. Furthermore, the recA protein remains associated with the complex through the course of strand exchange and is not recycled even after the reaction is complete.

EXPERIMENTAL PROCEDURES

Materials

recA protein was purified to homogeneity as described (10). Its concentration was calculated from the extinction coefficient, $E^{1\%}_{280} = 5.9$ (11). SSB was the generous gift of J. Kaguni of this department. Its concentration was determined by the method of Bradford (12) using bovine serum albumin as a standard. Nuclease S1 was purchased from Sigma; the restriction endonucleases *Ava* I and *Hpa* II were purchased from New England Biolabs; phosphoenolpyruvate and pyruvate kinase were purchased from Sigma. To reduce the salt content in 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.

Preparation of linear duplex and labeled or unlabeled circular φX SS DNA has been described (3). Supercoiled SV40 DNA was a gift from Michael Fromm of this department. It was linearized by cleavage with the restriction endonuclease *Hpa* II, then extracted with phenol. ^{3}H-labeled φX SS DNA preparations had specific radioactivities ≥ 100,000 cpm/μg (nucleotide). DNA concentrations were determined by using an A_{260} of 1.0 as equivalent to 50 and 36 μg of DNA/ml for double- and single-stranded DNAs, respectively.

Methods

Assay of Heteroduplex Formation—This assay measures the conversion of ^{3}H-labeled circular φX SS DNA into an S1 nuclease-resistant form. Standard reaction mixtures contained: 25 mM Tris·HCl, 80% cation (final pH = 7.2), or 20 mM sodium maleate, 65% dianion (pH 6.0); 5% (v/v) glycerol; 10 mM $MgCl_2$; 1 mM dithiothreitol; 1.2 mM ATP; 5.6 μM linear duplex φX DNA; 3.3 μM circular φX SS DNA ((+) strands); 1.8 μM recA protein; and 0.15 μM SSB. All reactions were performed at 37 °C in 1.5-ml plastic Eppendorf tubes. Unless otherwise indicated, reactions were initiated by the addition of ATP and SSB together, following a 10-min preincubation of a mixture containing the other components. Unless otherwise indicated, reactions were performed at pH 7.2.

Where indicated, an ATP regenerating system containing 1 or 2 mM phosphoenolpyruvate and an amount of pyruvate kinase that could convert 0.8 mM ADP to ATP with a $t_{1/2}$ of 15 s was added. Modifications of standard reaction conditions are indicated in the

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[1] The abbreviations used are: SSB, *Escherichia coli* SS DNA-binding protein; φX, bacteriophage φX174; RFI (replicative form I), supercoiled double-stranded DNA; RFII (replicative form II), double-stranded DNA containing a nick in one strand; SS DNA, single-stranded DNA.

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legends to the figures and table.

Aliquots (50 μl) were removed from the reaction mixture at the times indicated and placed in 1.5-ml plastic Eppendorf tubes containing 5 μl of 10% sodium dodecyl sulfate. The samples were then mixed briefly and placed on ice until all samples had been collected. Subsequent steps were carried out in the same tubes. To each aliquot was added 620 μl of a solution containing 500 mM NaCl, 50 mM sodium acetate, 50% cation (pH 4.6), 1 mM zinc acetate, 32 μg/ml of heat-denatured calf thymus DNA, and S1 nuclease at 2.5 Sigma units/ml. (One unit is defined as the amount of S1 nuclease needed to digest 1 μg of SS DNA in 1 min at 37 °C.) After incubation at 37 °C for 30 min, carrier DNA (40 μl of heat-denatured calf thymus DNA at 1 mg/ml) was added to each tube, followed by 700 μl of cold 10% trichloroacetic acid. After at least 35 min on ice, the mixtures were filtered through Whatman GF/C filters which had been briefly presoaked in 10% trichloroacetic acid. Tubes and filters were rinsed with 3 ml of cold 10% trichloroacetic acid, followed by a final rinse of the filter with 1 ml of 95% ethanol. Filters were then dried and assayed for radioactivity.

To define the total acid-precipitable tritium, one aliquot of each reaction mixture was treated identically except that no S1 nuclease was added. For each ^{3}H-labeled DNA preparation, a standard value for this control was obtained by averaging 5–10 such determinations. The samples in which this value differed by more than 8% from the averaged standard were discarded. Under the conditions of the assay, approximately 2.5% of the maximum acid-precipitable radioactivity was S1 nuclease-resistant in the absence of recA protein. This background was subtracted from all values and from the control before the calculations described below were made.

Treatment of Data—Under standard reaction conditions, the SS DNA is in slight excess so that if all of the linear duplex molecules were converted to RFII, only 85% of the single-stranded circles would have been utilized, and thereby become S1 nuclease-resistant. The acid-precipitable maximum was thus corrected by a factor of 0.85. When higher concentrations of SS DNA were used, different correction factors were employed so that "% heteroduplex" represents the percentage of the potential heteroduplex that could be formed, and not the percentage of total SS DNA that had reacted.

To evaluate the efficiency of the assay, reactions were carried out under several conditions with labeled duplex or SS DNA, followed by agarose gel electrophoresis under conditions that have been described previously (3). Bands corresponding to reactants and products were visualized by staining with ethidium bromide and cut out; they were then solubilized and their radioactivity was determined (13). As demonstrated in Fig. 1, strand exchange can proceed essentially to completion. Under these conditions, 98% of the tritium from the linear duplex was found as RFII and linear single strands after 60 min. In a parallel experiment, the S1 assay indicated that 80% of the possible heteroduplex had been formed. Several such measurements demonstrated that the S1 assay in this case was 80–82% efficient relative to the more direct gel assay. This factor varies for different preparations of ^{3}H-labeled SS DNA, most likely due to the presence of labeled impurities or a slight degradation of the product RFII by the S1 nuclease under assay conditions. These results necessitated additional correction factors. In the case above, the acid-precipitable maximum was corrected by a factor of 0.81 × 0.85 = 0.69, and this value was taken as "100% heteroduplex formation." This example was chosen because it represents the largest correction necessary for any of the DNA preparations.

Where measurements of extent are reported, the values represent the average of at least three determinations taken between 40 and 60 min after the start of the reaction. Where both labeled and unlabeled φX SS DNA were present, % heteroduplex refers to the fraction of ^{3}H-labeled DNA incorporated into heteroduplex rather than the fraction of the total DNA that had reacted. In the absence of an ATP regenerating system, ATP concentrations reported for various reaction conditions were chosen to ensure that ATP was not the limiting component.

RESULTS

Characterization of recA Protein-promoted Strand Exchange in the Presence of SSB

Under standard reaction conditions, approximately 70% of the linear duplex DNA was converted to RFII-like molecules. Increasing the concentrations of SS DNA, SSB, and recA protein by 60% resulted in nearly complete conversion to RFII

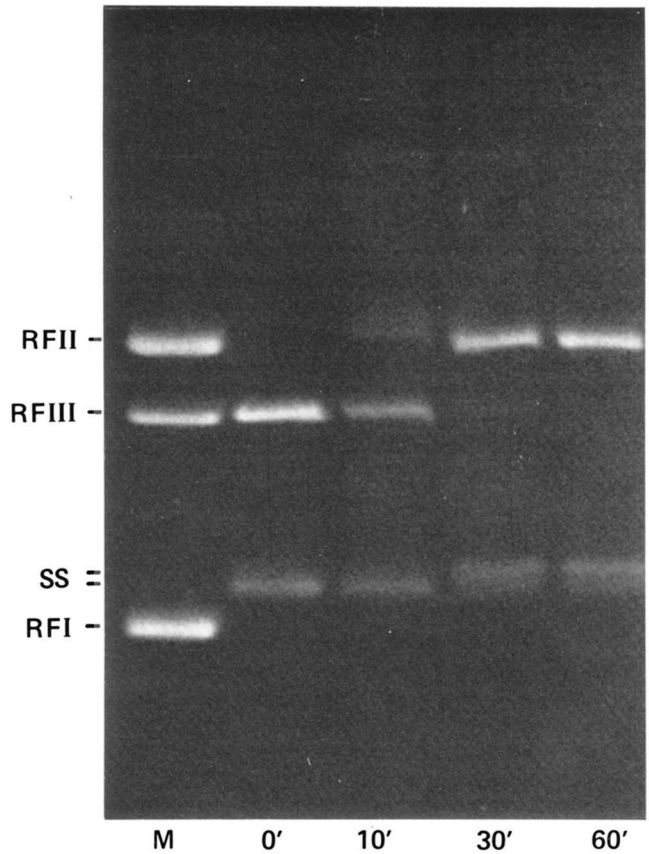


FIG. 1. **Measurement of DNA strand exchange by agarose gel electrophoresis.** Strand exchange was performed under standard reaction conditions, except that the concentrations of recA protein, SS DNA, SSB, and ATP were increased by 60%. Agarose gel electrophoresis was as described previously (3). The upper band in the single-stranded DNA doublet represents linear molecules. *M* = markers; *numbers* under the other lanes indicate the time of reaction in minutes.

(Figs. 1 and 2). Similar results were obtained at pH 6.0 where, in contrast to pH 7.2, duplex DNA is bound efficiently by recA protein even in the absence of SS DNA and efficient duplex DNA-dependent hydrolysis of ATP occurs (14).

The dependence of the extent of reaction on the concentration of recA protein is shown in Fig. 3. The apparent cooperativity is the same as that observed for the duplex DNA-dependent ATPase and duplex DNA binding activities of recA protein at pH 6.0 (14, 15). Saturation occurred at approximately 1 recA protein monomer per 4 nucleotides of SS DNA.

Effect of Divalent Cations—The effect of divalent cation concentration on strand exchange, shown in Fig. 4, is similar to that observed for other recA protein-associated activities (15, 16). The effect of Zn^{2+} is noteworthy in that not only did it fail to support strand exchange, but it blocked an ongoing reaction proceeding in the presence of 10 mM Mg^{2+} (see Fig. 6). Zn^{2+} also inhibits the SS DNA-dependent ATPase activity of recA protein.[2]

Effect of Monovalent Cations—As shown in Fig. 5, strand exchange was strongly inhibited by NaCl concentrations above 20 mM.[3] The inhibition by NaCl is identical with that observed for D-loop formation (17), suggesting that it represents an effect on the D-loop formation rather than on the branch migration phase of strand exchange. Thus, when 0.1 M NaCl was added to an ongoing reaction, heteroduplex

[2] M. M. Cox and I. R. Lehman, unpublished results.

[3] The ionic strength under standard reaction conditions is approximately equivalent to 32 mM NaCl.

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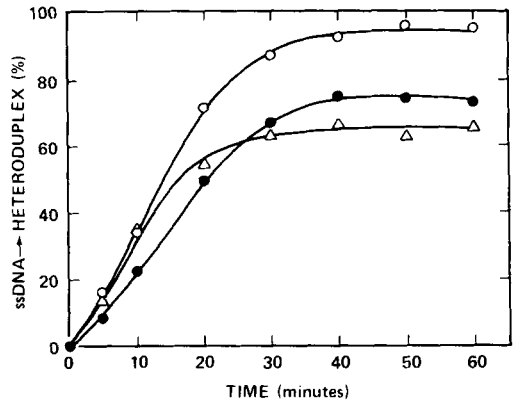


FIG. 2. **Measurement of DNA strand exchange by conversion of ^{3}H-labeled φX SS DNA to S1 nuclease resistance.** ●——●, time course of recA protein-promoted strand exchange under standard reaction conditions; ○——○, recA protein-promoted strand exchange under the conditions described for the reaction in Fig. 1; △——△, recA protein-promoted strand exchange at pH 6.0.

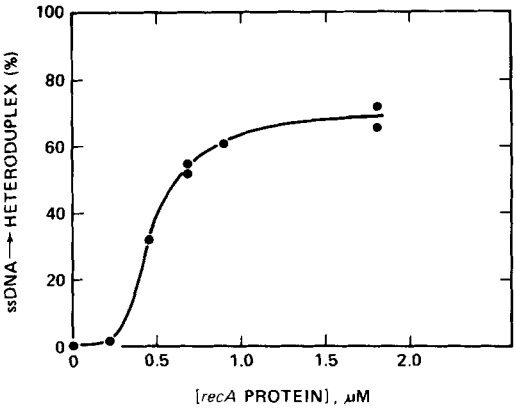


FIG. 3. **recA protein-promoted strand exchange: dependence on recA protein concentration.** Reactions were performed under standard reaction conditions. The *points* shown represent extents of reaction.

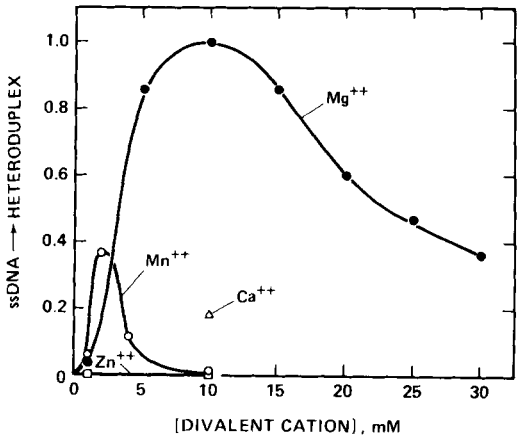


FIG. 4. **recA protein-promoted strand exchange: effects of divalent cations.** Extents of reaction are normalized to standard reaction conditions, which is set at 1.0.

extension continued unimpeded for another 10 min (Fig. 6). The duplex DNA-dependent ATPase and duplex DNA binding activities of recA protein show a similar response to added NaCl (14, 15), suggesting that the binding of duplex DNA may be the salt-sensitive step in D-loop formation.

Effects of SSB—The kinetics of the strand exchange reaction are affected not only by reaction conditions, but by the

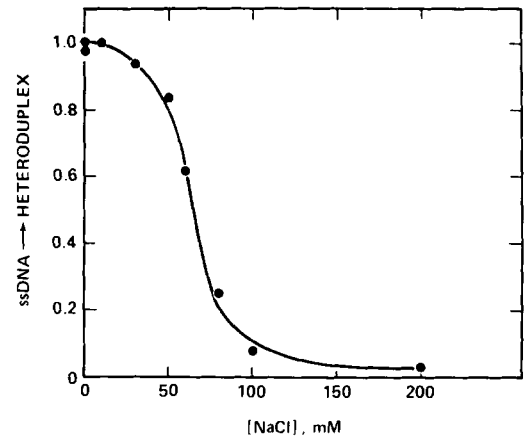


FIG. 5. **recA protein-promoted strand exchange: effects of ionic strength.** Extents of reaction are normalized to standard reaction conditions which is set at 1.0.

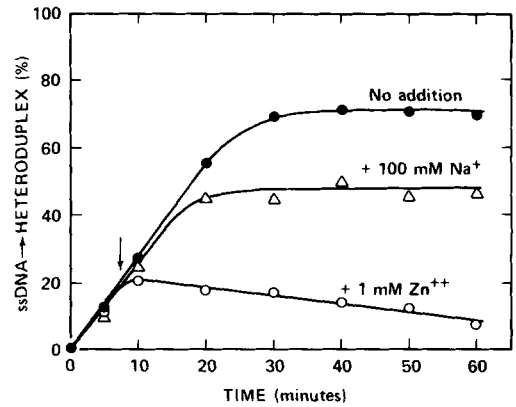


FIG. 6. **Effect of addition of NaCl and $ZnCl_2$ to ongoing strand exchange.** Reactions were performed under standard reaction conditions. Additions of NaCl and $ZnCl_2$ were made at 7.5 min (*arrow*).

order of addition of reaction components. The enhancement of the reaction by SSB is particularly sensitive to the point at which SSB is added. The most efficient reaction was observed when SSB was added together with or after the point at which ATP and recA protein were first mixed in the presence of the two DNA substrates (Fig. 7). The final extent of reaction in this case was the same using SSB concentrations that were either half-saturating (1 monomer per 20 nucleotides of SS DNA) or saturating (1 monomer per 10 nucleotides of SS DNA), although the rate was greater in the latter case.

When half-saturating SSB and ATP were added to the DNA before the recA protein, there was a lag of approximately 5 min, and the extent of the reaction was decreased by 30% (Fig. 7). A slow step is thus introduced into the pathway under these conditions. The lag was abolished by preincubating recA protein together with SSB for 5–10 min before addition of ATP. Inasmuch as binding of recA protein to SS DNA can occur in the absence of ATP (15), this is probably the affected step. These findings imply that SSB can inhibit the binding of recA protein to SS DNA, even when the amount of SSB is sufficient to saturate only about one-half of the SS DNA.

When levels of SSB sufficient to saturate the SS DNA and ATP were added before recA protein, a lag again resulted and there was a 50% inhibition in the rate and extent of reaction. These effects were not overcome by preincubation of recA protein with SSB before the addition of ATP. Moreover, the relative inhibition was independent of the order of addition of the two proteins during preincubation (not shown). These

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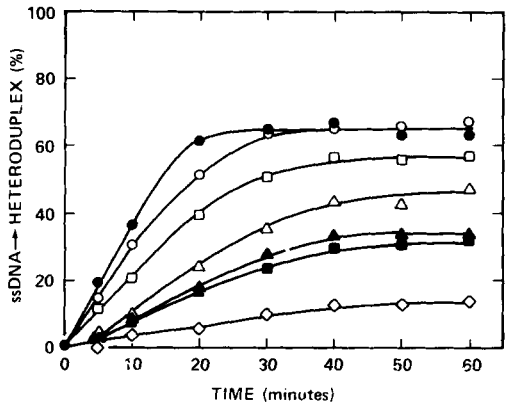


FIG. 7. **recA protein-promoted strand exchange: effect of the time of addition of SSB.** Reactions were performed under standard reaction conditions with changes as indicated. All preincubations included both SS and duplex DNA substrates and were carried out for at least 10 min at 37 °C. ○——○, recA protein in preincubation, reaction started with ATP and 0.15 μM SSB; □——□, recA protein and 0.15 μM SSB in preincubation, reaction started with ATP; △——△, 0.15 μM SSB and ATP in preincubation, reaction started with recA protein; ●——●, ■——■, ▲——▲, same order of addition as three preceding reactions, SSB at 0.3 μM; ◇——◇, no SSB.

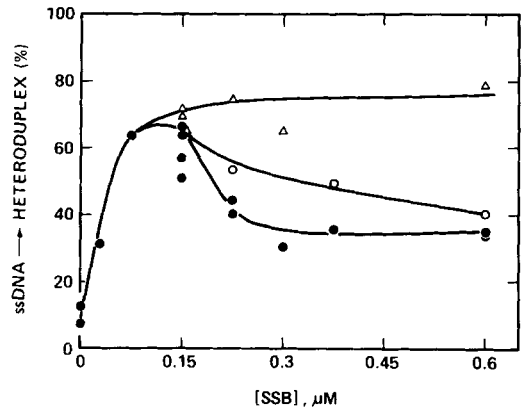


FIG. 8. **recA protein-promoted strand exchange: dependence on SSB concentration.** Values represent extents of reaction under standard reaction conditions with changes as indicated. All preincubations included both SS and duplex DNA substrates and proceeded for 10 min at 37 °C. ●——●, 1.8 μM recA protein in preincubation with indicated concentration of SSB, reaction started with ATP; ○——○, 4.5 μM recA protein, same order of addition; △——△, 1.8 μM recA protein in preincubation, reaction started with ATP and the indicated amount of SSB.

results may reflect a competition by recA protein and SSB at saturating levels of SSB for available binding sites on the SS DNA, consistent with the effects of SSB reported previously (18, 19). As noted above, however, this competition is eliminated if SSB is added to the DNA coincident with or subsequent to the point at which recA protein and ATP are mixed. The efficiency of the reaction in the absence of SSB was less than that in its presence, regardless of the order of addition.

The detailed effects of SSB on the extent of heteroduplex formation are summarized in Fig. 8. When SSB was added last, optimal reaction occurred even in the presence of a large excess of SSB. The relative inhibition associated with early addition of SSB occurred at SSB concentrations above 0.15 μM (~1 SSB monomer per 22 nucleotides of SS DNA). The inhibition never became absolute, even with a large excess of SSB. In addition, the inhibition could be partially overcome by increasing the concentration of recA protein. The lack of total inhibition at high SSB concentrations suggests that SSB does not compete efficiently for all sites on the SS DNA, perhaps because of secondary structure in the φX SS DNA. A more complete understanding of these effects awaits a more detailed analysis of the binding properties of the two proteins to SS DNA.

As indicated above, SSB affects the interaction of recA protein and SS DNA even when the concentration of SSB is not sufficient to saturate the DNA. The preference of recA protein for binding SS DNA not coated with SSB is demonstrated more directly in Fig. 9. When recA protein was added to a reaction mixture containing the DNA substrates and ATP 30 s before addition of sufficient SSB to half-saturate the SS DNA, efficient strand exchange took place. When the SSB was added prior to the recA protein, the lag and 30% inhibition described above resulted. In both cases, recA protein is present in 2-fold excess relative to the amount required for a maximal reaction. Nevertheless, if recA protein was added to equal concentrations of free SS DNA and DNA half-saturated with SSB, the free DNA participated almost exclusively in strand exchange while inhibition of the reaction of the SSB-coated DNA was greater than 75%.

recA Protein Is Not Recycled during DNA Strand Exchange

To determine whether the recA protein is recycled in the course of strand exchange, the reaction was initiated under conditions in which recA protein was the limiting component.

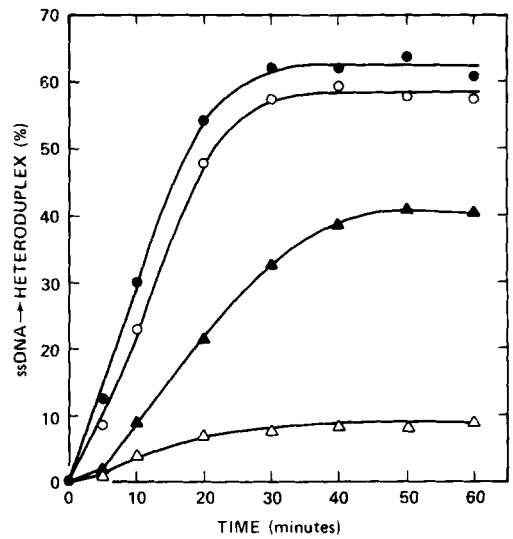


FIG. 9. **Preference of recA protein for free SS DNA.** Reactions were carried out as described under "Methods." The following experimental conditions were used.

Reaction	Designation	Time of addition (min)				
		−20	−15	−5	0	0.5
1	●——●	[^{3}H]SS	ATP		recA	SSB
2	○——○	SS	SSB ATP	[^{3}H]SS	recA	SSB
3	▲——▲	[^{3}H]SS	SSB ATP		recA	
4	△——△	[^{3}H]SS	SSB ATP	SS	recA	SSB

Duplex DNA was added to a final concentration of 5.6 μM before the indicated additions. Final concentrations following additions were: SSB, 0.15 μM (where this component appears twice, final concentration is doubled); recA protein, 1.8 μM; ^{3}H-labeled circular φX SS DNA, ([^{3}H]SS), 3.3 μM; unlabeled circular φX SS DNA (SS), 3.3 μM; ATP, 1.2 μM. Based on the dissociation constant for SSB (27), it is assumed that SSB binds SS DNA upon addition and is not significantly redistributed during the preincubation.

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The reaction was then challenged at various times with additional DNAs. Any reaction with the "challenging" DNA is taken as evidence that recA protein involved in the initial reaction has been recycled.

In the first set of experiments, both ϕX SS and duplex DNA were included in a preincubation with recA protein. After initiation of the reaction, the recA protein was challenged at various times with another equivalent each of duplex DNA and SS DNA. Heteroduplex formation involving the substrates in the preincubation is termed reaction 1. Heteroduplex formation involving the substrates in the challenge constitutes reaction 2. Reactions 1 or 2 can be followed independently by using ^{3}H-labeled SS DNA in one or the other. When the SS DNA in reaction 1 is labeled, it is not in reaction 2, and vice versa. The point at which reaction 2 was initiated defines $t = 0$, unless stated otherwise. The final extent of incorporation of ^{3}H-labeled SS DNA into heteroduplex in reactions 1 or 2 was measured by averaging values obtained at 40, 50, and 60 min after $t = 0$. In every case there was no significant increase in heteroduplex formation between 40 and 60 min. There is an approximate 2-fold excess of recA protein under standard reaction conditions (see Fig. 3) so that the concentration of recA protein was decreased 50% for these experiments (to 0.9 μM). With the final DNA concentration doubled as well, the amount of recA protein was sufficient to promote only about half the usual amount of heteroduplex formation in reaction 1 or reaction 2 assuming both sets of substrates had an equal opportunity to interact with recA protein. Appropriate amounts of SSB were always included after addition of the DNA substrates in reaction 1 and reaction 2, either at the same time or subsequent to the addition of ATP. This protocol provides optimal conditions for heteroduplex formation in either reaction 1 or reaction 2 if recA protein is available. Inclusion of duplex DNA in both cases ensured that this component was not limiting. An ATP regenerating system was provided in each experiment and assays of ATP hydrolysis showed that there was no net hydrolysis of ATP for at least 80 min.

When reactions 1 and 2 were preincubated together with recA protein and the reaction was initiated with ATP and SSB ($t = 0$ here is defined by the addition of ATP and SSB), about 28% of the SS DNA in each reaction was converted to heteroduplex (Fig. 10). When the substrates of reaction 1 were preincubated with recA protein and ATP was added with reaction 2 substrates, a competition for the available recA protein again occurred. Reaction 2, however, was somewhat more efficient than reaction 1 in this case (Table I, experiments 1A and 2A, and Fig. 10). The inhibition of reaction 1 relative to reaction 2 when the former was preincubated with recA protein may be due to transient and unproductive interactions between reaction components in the absence of ATP. When reaction 1 was initiated by adding ATP and SSB at various times before $t = 0$, a different result was obtained (Fig. 10). Thus, when ATP and SSB were introduced only 2 min before the substrates of reaction 2, the final extent of heteroduplex formation in reaction 1 was doubled. When added 5 min before the reaction 2 substrates, the extent of heteroduplex formation was equivalent to that observed in the absence of the DNA of reaction 2. This was paralleled by a reciprocal decrease in the final extent of heteroduplex formation in reaction 2. When the reaction 2 substrates were added 5 min after the initiation of reaction 1, the final extent of heteroduplex formation in reaction 2 was decreased by 90%. The DNA of reaction 2 was effectively excluded from the reaction, even 60 min after $t = 0$. If, 30 min after initiation of reaction 1, additional recA protein was added together with the reaction 2 substrates, reaction 2 proceeded with optimal efficiency (Fig. 10). Thus, inhibition of reaction 2 by the preinitiation reaction 1 is a direct result of the sequestering of recA protein in reaction 1. More than 80% of the optimal reaction was restored even when the reaction 2 substrates were added 5 min before the additional recA protein and SSB (not shown). This finding eliminates the possibility that the second reaction is simply being inhibited by the SSB added for reaction 1. Taken together, these results demonstrate that a tight complex of recA protein is formed early in strand exchange. This complex involves at least the SS DNA of reaction 1, and its formation is largely irreversible.

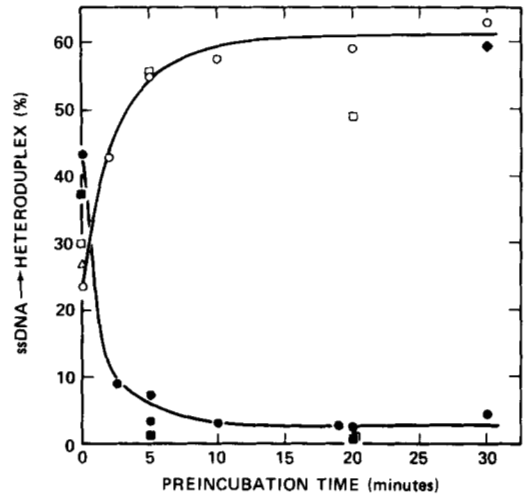


FIG. 10. **Time course of complex formation.** Reactions were performed as described under "Methods." The following conditions were used:

Designation	Time of addition (min)					
	−40	−35	−x	0	0.5	1.0
●—●	DS SS	recA	ATP SSB	[^{3}H]SS DS	SSB	
○—○	DS [^{3}H]SS	recA	ATP SSB	SS DS	SSB	
■—■	SS	recA	ATP 2× SSB	[^{3}H]SS DS	2× SSB	
□—□	[^{3}H]SS	recA	ATP 2× SSB	SS DS	2× SSB	
△	[^{3}H]SS 2× DS, SS	recA	ATP 2× SSB			
●	DS, SS	recA	ATP SSB	[^{3}H]SS, DS	recA	SSB

Conditions and final concentration of components resulting from the indicated addition are as described in Table I. Preincubation time refers to x in minutes. Where $x = 0$, SSB additions were made at $t = 0.5$ min rather than $t = 0$; DS, duplex DNA.

Requirements for Complex Formation—The requirements for complex formation are presented in Table I. When ATP was added at $t = 0$ with reaction 2, after preincubation of recA protein with reaction 1, reaction 2 was somewhat more efficient than reaction 1 (experiments 1A and 2A). In contrast, when reaction 1 was preinitiated, it became more than 10-fold more efficient than reaction 2 (experiments 1B and 2B). (Note that experiments 1 and 2 are identical except for the time of introduction of ^{3}H-labeled SS DNA.)

When reaction 1 was initiated without SSB, the subsequent inhibition of reaction 2 was minimal (experiment 3B *versus* 3A) and the final extent of heteroduplex formation in reaction 1 did not increase (experiment 4B *versus* 4A). Thus, SSB strongly stimulates complex formation. Increasing the concentration of SSB used to initiate reaction 1 resulted in a somewhat greater inhibition of reaction 2 (experiment 5B *versus* 5A). On the other hand, SSB is not sufficient for complex

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TABLE I

Requirements for complex formation

Reactions were carried out as described under "Methods." Buffer components and an ATP regenerating system are present in all cases before the first addition listed. The extent of heteroduplex formation is the average of three measurements carried out at 40, 50, and 60 min after $t = 0$. For each pair of experiments, experiment A is the control to which experiment B should be compared. Final concentrations resulting from the indicated additions are: unlabeled circular ϕX SS DNA (SS), 3.3 μM; ^{3}H-labeled ϕX circular SS DNA ([^{3}H]SS), 3.3 μM; full length linear ϕX duplex DNA (DS), 5.6 μM; full length linear duplex SV40 DNA (SV40 DS), 5.6 μM; recA protein (recA) 0.9 μM; SSB, 0.15 μM; ATP, 1.2 mM. Where a number precedes the indicated addition (*i.e.* 2X SSB), the final concentration resulting from that addition should be increased by that factor (to 0.3 μM in the case above). Components added at the same time point were generally added as a mixture. Where an addition appears more than once in the same experiment, the full amount indicated was added each time. Reaction mixtures were kept at 37 °C for 5 min before the first indicated addition.

Experiment	Additions								Extent of heteroduplex formation		Relative reaction (B/A)	
	−40[a]	−30	−24.5	−20	−5	−4.5	0	+0.5	1	2[b]	1	2
									%			
1 A	SS DS	recA					[^{3}H]SS DS/ATP	2× SSB	45.7	43.6		
B	SS DS	recA		ATP SSB			[^{3}H]SS DS	SSB	4.7	4.1	0.1	0.09
2 A	[^{3}H]SS DS	recA					SS DS/ATP	2× SSB	23.5			
B	[^{3}H]SS DS	recA		ATP SSB			SS DS	SSB	58.8		2.5	
3 A	SS DS	recA					[^{3}H]SS DS/ATP	2× SSB	50.2	46.2		
B	SS DS	recA		ATP			[^{3}H]SS DS	2× SSB	38.4	39.6	0.77	0.86
4 A	[^{3}H]SS DS	recA					SS DS/ATP	2× SSB	26.3			
B	[^{3}H]SS DS	recA		ATP			SS DS	2× SSB	26.2		0.99	
5 A	SS DS	recA					[^{3}H]SS DS/ATP	4× SSB	44.7			
B	SS DS			ATP 2× SSB			[^{3}H]SS DS	2× SSB	3.5		0.08	
6 A	SS	recA					[^{3}H]SS DS/ATP	4× SSB	37.5			
B	SS	recA		2× SSB			[^{3}H]SS DS/ATP	2× SSB	56.7		1.51	
7 A	[^{3}H]SS	recA					SS DS/ATP	4× SSB	26.1			
B	[^{3}H]SS	recA		2× SSB			SS DS/ATP	2× SSB	0.9		0.03	
8 A	SS	recA					[^{3}H]SS DS/ATP	2× SSB	43.7	44.7		
B	SS	recA		ATP SSB			[^{3}H]SS DS	SSB	7.9	6.0	0.18	0.13
9 A	SS	recA					[^{3}H]SS DS	4× SSB	37.4	36.0		
B	SS	recA		ATP 2× SSB			[^{3}H]SS DS	2× SSB	1.7	1.4	0.05	0.04
10 A	[^{3}H]SS	recA					SS DS/ATP	4× SSB	30.3			
B	[^{3}H]SS	recA		ATP 2× SSB			SS DS	2× SSB	49.1		1.62	
11 A	SS	recA					[^{3}H]SS DS/ATP	2× SSB	44.7			
B	SS	recA	ATP SSB		[^{3}H]SS	SSB	DS		4.9		0.11	
12 A		recA		SSB			[^{3}H]SS DS/ATP		40.2			
B		recA		ATP SSB			[^{3}H]SS DS		46.6		1.16	
13 A	[^{3}H]SS	recA		ATP SSB			DS		52.1			
B	[^{3}H]SS SV40 DS	recA		ATP SSB			DS		51.5		0.99	

[a] Time (t) is given in minutes.
[b] Duplicate experiment.

formation, as shown in experiments 6 and 7. When SSB was added without ATP at $t = -20$ min, reaction 2 was strongly stimulated while reaction 1 was strongly inhibited. These findings are consistent with those presented in Fig. 9. Thus, both ATP and SSB are required for complex formation.

In contrast, duplex DNA is not required for complex formation. This point is illustrated in experiments in which the reaction 1 and reaction 2 substrates did not include duplex

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DNA. One equivalent of duplex DNA was added at $t = 0$ with the SS DNA of reaction 2. When ATP was added at $t = 0$ as well, after preincubation of recA protein and reaction 1 SS DNA, the SS DNA of reaction 2 competed efficiently with reaction 1 SS DNA for both the recA protein and the duplex DNA (experiment 8A). When ATP and SSB were added to reaction 1 SS DNA prior to the addition of the reaction 2 SS DNA, formation of heteroduplex with the latter was inhibited by more than 80% (experiment 8B). This inhibition occurred even though duplex DNA was not added until introduction of the reaction 2 SS DNA. Increasing the concentration of SSB added to the reaction 1 SS DNA resulted in a greater than 95% inhibition of reaction 2 (experiment 9B *versus* 9A). As expected, there was a reciprocal increase in the formation of heteroduplex involving reaction 1 SS DNA (experiment 10B *versus* 10A). Thus, in the presence of ATP and SSB, recA protein forms a complex with SS DNA from which it is not recycled. However, the complexes react efficiently with homologous duplex DNA added subsequently.

It is possible that some recycling from the complex occurred in the absence of duplex DNA, but that reaction of the complex with duplex DNA is faster than the recycling process. The duplex DNA might thus become sequestered by reaction 1 before sufficient recA protein had been recycled to allow the reaction 2 SS DNA to participate. This possibility was examined by forming the complex with reaction 1 SS DNA, recA protein, SSB, and ATP as before, then adding the ^{3}H-labeled reaction 2 SS DNA 5 min before the duplex DNA (experiment 11B *versus* 11A). Under these conditions, the extent of heteroduplex formation involving reaction 2 SS DNA did not increase and in fact appeared to decrease (the levels of SSB used are such that experiment 11 should be compared with experiment 8). Consequently, once the complex is formed, recA protein is not recycled even in the absence of duplex DNA.

When recA protein was preincubated with SSB and ATP in the absence of DNA, its subsequent reaction with SS DNA and duplex DNA in the strand exchange reaction was not impaired (experiment 12B *versus* 12A). Thus, SSB does not simply inhibit interaction of recA protein with SS DNA added after the addition of SSB.

Time Course of Complex Formation—The results presented thus far indicate that a tight complex of recA protein and SS DNA is formed in the presence of ATP and SSB. It should be noted that a 50% inhibition of reaction 2 implies that the amount of the recA protein that would normally react in reaction 1 has entered the complex together with 50% of the protein that would normally react in reaction 2, or approximately 75% of the total. It is therefore likely that the time course shown in Fig. 10 underestimates the rate of complex formation. In fact, we estimate that the half-time for complex formation is less than 1 min. The less complete data for the reaction with higher SSB concentrations suggest an even greater rate of complex formation.

Dependence of Complex Formation on the Concentration of SS DNA—The concentration of reaction 1 substrates was varied to determine the amount of SS DNA required for complex formation (Fig. 11). Concentrations of SSB in the preincubation were decreased proportionately, while the concentration of recA protein was maintained at 0.9 μM. In the absence of duplex DNA, the final extent of heteroduplex formation in reaction 2 was inhibited by 58% in the presence of 60% of the standard concentration of reaction 1 SS DNA and enough SSB to half-saturate the single strands. If we assume that this inhibition reflects complex formation of 58% of the 0.9 μM recA protein (0.52 μM) with 2.0 μM SS DNA (0.6 × 3.3), then one recA protein monomer is complexed per 3.8 nucleotides of SS DNA. For the reasons given above, this value may be an overestimate of the amount of SS DNA required. Although duplex DNA is not required for complex formation, its presence in the preincubation increases the apparent complex formation almost 2-fold, to 1 recA protein monomer per 2 nucleotides of SS DNA. Increasing the concentration of SSB in the preincubation by 2-fold (to 1 SSB per 10 nucleotides of SS DNA) had essentially the same effect as addition of duplex DNA. Most activities of recA protein show saturation in the presence of 1.5–4 nucleotides of SS DNA per recA protein monomer (15, 17). Thus, association of sufficient recA protein with the complex to saturate the SS DNA occurs in the presence of SSB concentrations that are also sufficient to saturate the SS DNA.

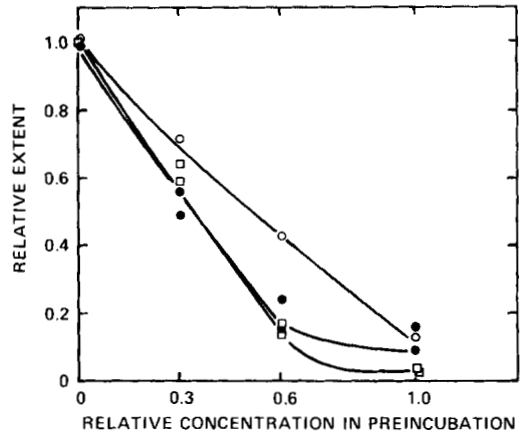


FIG. 11. **Effect of SS DNA concentration on complex formation.** Reactions were carried out as described under "Methods." The following conditions were used:

Designation	Time of addition (min)				
	−40	−30	−20	0	0.5
○—○	SS	recA	ATP SSB	[^{3}H]SS DS	SSB
●—●	SS DS	recA	ATP SSB	[^{3}H]SS DS	SSB
□—□	SS	recA	ATP 2× SSB	[^{3}H]SS DS	2× SSB

Conditions and final concentrations of components resulting from the indicated additions are as described in Table I. Where the "Relative Concentration in the Preincubation" is less than 1.0, the final concentration of all additions at $t = -40$, as well as the SSB added at $t = -20$, should be multiplied by the indicated factor. For each point a separate control was performed (with DNA and SSB added to the same final concentrations) in which the ATP was added at $t = 0$ and SSB was added at $t = 0.5$.

Binding of the Complex to Duplex DNA Is Reversible—The complex was preformed by incubation of the required components, then duplex DNA was added at $t = 0$ (Table I, experiment 13). When an amount of linear duplex SV40 DNA was present in the preincubation equivalent to that of the φX duplex DNA added subsequently, the extent of heteroduplex formation remained unaffected. When complex was preformed by incubation of the necessary components for 20 min in the presence and absence of a concentration of SV40 DNA 2-fold greater than that of the homologous duplex DNA, there was an approximately 40% inhibition of the subsequent rate of heteroduplex formation, although the reaction appeared to approach the same extent (Fig. 12). This inhibition represents an effect on initiation of strand exchange since addition of the SV40 DNA 11 min after addition of the homologous duplex had no effect on the reaction. These results strongly suggest that there is a simple competitive inhibition by the SV40 DNA for the binding of the homologous duplex. The inhibition

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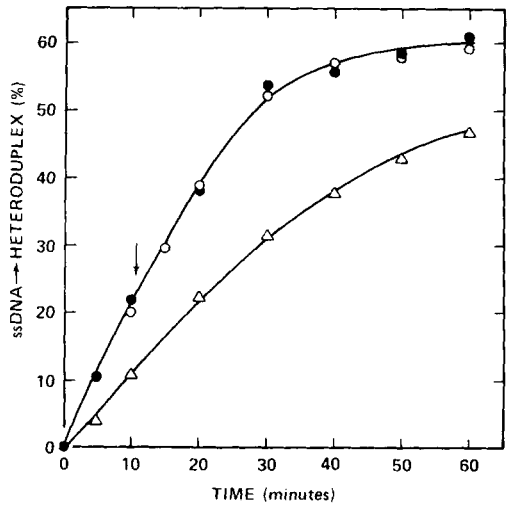


FIG. 12. **Lack of inhibition of strand exchange by heterologous duplex DNA.** Reactions were carried out as described under "Methods." The following conditions were used:

Designation	Time of addition (min)				
	−40	−30	−20	0	11
●——●	[^{3}H]SS	recA	ATP SSB	DS	
△——△	[^{3}H]SS 2× SV40 DS	recA	ATP SSB	DS	
○——○	[^{3}H]SS	recA	ATP SSB	DS	2× SV40 DS[a]

[a] Addition indicated by *arrow*.

Conditions and final concentrations of each component resulting from the indicated additions are as described in Table I.

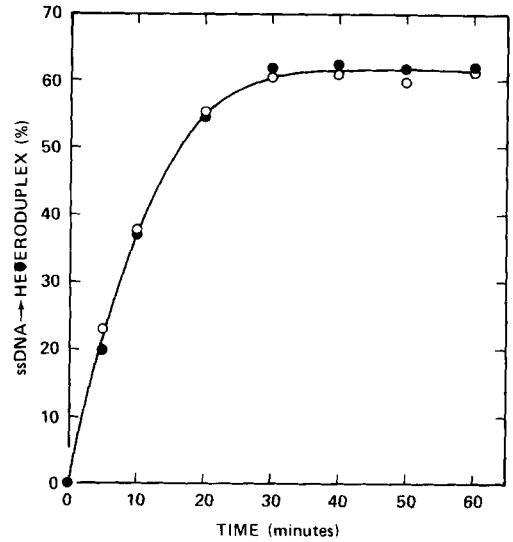


FIG. 13. **Kinetic competence of complex in strand exchange.** Reactions were carried out as described under "Methods." The following conditions were used:

Designation	Time of addition (min)			
	−30	−20	−5	0
●——●	[^{3}H]SS	recA		DS, ATP 2× SSB
○——○	[^{3}H]SS	recA	ATP 2× SSB	DS

Conditions and final concentrations of components resulting from indicated additions are as described in Table I.

implies that the complex binds heterologous duplex DNA, as would be expected from previous results (20, 21). The absence of total inhibition, despite the fact that the SV40 DNA was present in 70% excess over the complex, indicates the rapid reversibility of the interaction of heterologous duplex DNA with the complex.

Kinetic Competence of the Stable Complex—The demonstration that a complex or intermediate exists does not identify it as an obligatory step in a reaction pathway. Thus, the complex may represent an unproductive side path having little to do with the reaction. To establish that an intermediate lies on the normal reaction pathway, three conditions must be met (22): (i) it must be isolated and characterized, (ii) it must be *formed* fast enough to be on the reaction pathway, and (iii) it must *react* fast enough to be on the pathway. With a half-time of formation of a minute or less, the complex is clearly formed fast enough to be on the reaction pathway. For example, half-time for formation of stable D-loops under standard reaction conditions is approximately 4 min (3). The rate of heteroduplex formation is the same whether the complex is preformed or not before addition of duplex DNA (Fig. 13). This result tentatively fulfills the third condition. Identification of the components necessary for formation of the complex represents the initial step in satisfying the first condition. Further kinetic and physical studies are, however, required for a detailed understanding of its structure.

DISCUSSION

The effects of various reaction conditions on recA protein-promoted strand exchange are similar to those observed previously for other activities associated with the recA protein. The inhibition by high ionic strength, as well as the cooperative dependence on recA protein concentration, closely parallel the effects observed in the binding of duplex DNA by recA protein at pH 6.0. The binding of duplex DNA would thus appear to be the most likely candidate for the salt-sensitive step in DNA strand exchange, although an effect on protein-protein interactions is also possible.

The two phases of strand exchange, *i.e.* D-loop formation and branch migration, have previously been distinguished by differences in rate, in their requirements for ATP hydrolysis, and in their polarity (3, 7). The characterization of strand exchange presented here provides an additional feature that distinguishes D-loop formation from branch migration. Thus, D-loop formation is more sensitive to inhibition by both salt and heterologous duplex DNA than is branch migration.

SSB greatly enhances the rate and extent of branch migration even when there is more than enough recA protein to saturate the SS DNA. SSB also prevents dissociation of recA protein from SS DNA which normally occurs in the presence of ATP. These findings suggest that there is a direct interaction between SSB and recA protein which requires ATP, and that SSB is an important and integral component in DNA strand exchange. Correspondingly, there is evidence that SSB plays a role in homologous recombination *in vivo* (23, 24).

In the presence of ATP and SSB, recA protein is rapidly incorporated into a complex with single-stranded DNA and does not dissociate measurably from this complex for as long as 90 min. All subsequent steps in strand exchange, including D-loop formation and branch migration, occur within this complex. We previously suggested that recA protein might drive branch migration by an association of the crossover junction with the end of a growing recA protein filament (3). The results presented here render that hypothesis unlikely. recA protein is clearly incorporated into a stable complex long before heteroduplex formation is complete. We assume for the present that the complex is related to the filaments of recA protein described previously (15, 25, 26).

The inability to form a stable complex may explain the relative inefficiency of strand exchange in the absence of SSB.

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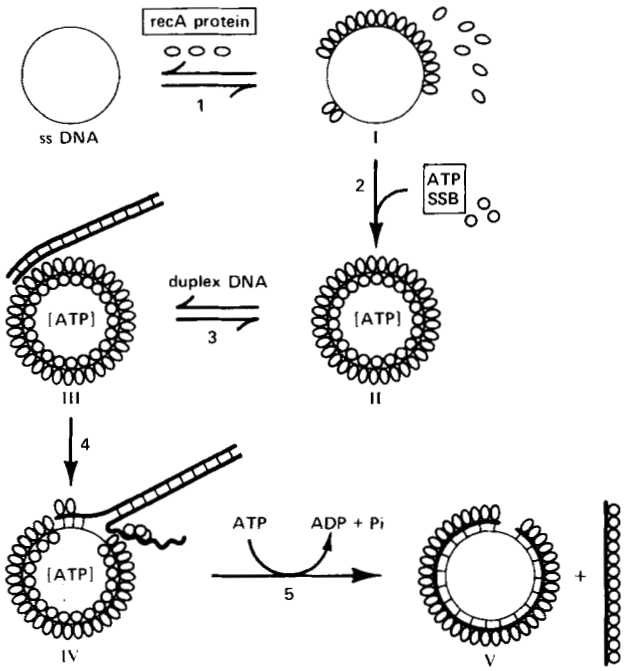


FIG. 14. **Model for recA protein-promoted DNA strand exchange.** See text for details.

We cannot, however, exclude the possibility that some stable complex formation occurs in the absence of SSB which is proportional to the efficiency of the reaction under those conditions.

A model which incorporates most of our findings is presented in Fig. 14. Inasmuch as we do not yet possess a detailed knowledge of the structure of these complexes, the location of recA protein and SSB at various stages in the reaction is speculative. Step 1 is shown to be reversible because recent experiments have indicated that recA protein bound to SS DNA is rapidly redistributed among different DNA molecules.[2] We have shown in the present work that step 2 leading to complex formation is largely irreversible. The large amounts of both recA protein and SSB that can be incorporated into the complex make it unlikely that both recA protein and SSB are bound only to the SS DNA; a direct interaction between recA protein and SSB is therefore likely. It is also plausible that much of the recA protein is bound within the complex only through protein-protein interactions, and is thus free to bind duplex DNA. The subsequent reaction of the complex in step 3 is reversible as shown by the reversibility of the interaction of this complex with heterologous SV40 duplex DNA. Step 4 is rendered irreversible by the polarity of the branch migration which occurs in step 5 (5–8). There is one qualification to the irreversibility of step 4; we have demonstrated that D-loops are at least occasionally formed at the "wrong" end of the duplex DNA (the 5′ end of the (−) strand) (7). The 3′ → 5′ polarity of branch migration relative to the (−) strand should, however, result in the elimination of these D-loops.

The pairing that occurs in step 4 might come about by a processive movement of the DNA substrates relative to each other or by a distributive mechanism. Alternatively, recA protein could promote pairing by simply holding unwound duplex DNA and SS DNA together; a facilitated diffusion mechanism which could take a variety of forms. There is, as yet, no decisive evidence to support either view, although the observation that D-loops can be formed in the presence of ATP hydrolysis (3, 18) would tend to argue against the processive mechanism. A detailed analysis of the rate of step 4 relative to the rate of the reverse reaction should help answer this question by establishing the number of times duplex DNA is bound and released before pairing occurs.

Step 5 is of particular interest. recA protein-promoted branch migration has a constant requirement for ATP hydrolysis and proceeds with a unique polarity. The demonstration that it occurs within a recA protein complex adds a new dimension to the strand exchange reaction. The existence of such a complex explains the requirement for stoichiometric amounts of recA protein and provides a role for SSB. The mechanism by which branch migration occurs within the complex must await further study. The state of the complex at the end of the reaction is not known; however it is clear that recA protein is not in a form in which it can interact with added DNA. Structure V is, therefore, only one of several possibilities.

One would expect that the recA protein is recycled. Such recycling is probably not directly coupled to ATP hydrolysis, since hundreds of ATPs are hydrolyzed per recA protein monomer during the lifetime of the complex. Furthermore, there is no reason to suspect that the recA protein has been inactivated during the reaction. Thus, the specific activity of recA protein-catalyzed ATP hydrolysis is unchanged following strand exchange, and no degradation of recA protein occurs.[2] It is possible that recycling *in vivo* requires additional factors. In fact, we have observed that under certain conditions recA protein can be recovered from the reaction in an active form.[2]

It is important to note that a slow recycling may occur that would not have been detected by the methods we have employed in this study. For example, a "treadmilling" reaction could take place in which monomers released from a recA protein filament were incorporated into other filaments in preference to binding free DNA. The effect of SSB in stabilizing complexes of SS DNA and recA protein, however, is clear.

A stable complex of recA protein might serve several purposes in homologous recombination. By not dissociating from the DNA, recA protein ensures that heteroduplexes formed are not reversed by spontaneous branch migration. The complex may also serve to protect recombination intermediates from unproductive and possibly deleterious nuclease action.

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