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DNA Strand Exchange Promoted by *recA* Protein and Single-stranded DNA-binding Protein of *Escherichia coli*

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The *recA* gene plays a central role in homologous genetic recombination in *Escherichia coli* (Radding 1978). It is also a key element in a regulatory pathway mediating cellular responses to DNA damage (Gottesman 1981). The product of the *recA* gene has been isolated (Ogawa et al. 1979; Roberts et al. 1979; Shibata et al. 1979; Weinstock et al. 1979), and its primary structure has been determined (Horii et al. 1980; Sancar et al. 1980). Activities relevant to both of its roles in vivo have been identified (Roberts et al. 1979; Radding 1981; McEntee and Weinstock 1981), and a variety of assays have been developed with which they can be measured (Roberts et al. 1979; Shibata et al. 1979; Weinstock et al. 1979; Cox and Lehman 1981a; West et al. 1981a).

Our aim is to determine the mechanism of action of *recA* protein in homologous recombination. Our recent efforts have concentrated on one of the *recA*-protein-promoted reactions, the exchange of strands between circular (plus) single-stranded ϕX174 DNA (SS DNA) and homologous linear duplex ϕX174 DNA coupled to the hydrolysis of ATP to ADP and P_i (Cox and Lehman 1981a,b, 1982; Cox et al. 1982a,b) (Fig. 1). This reaction mimics, in vitro, steps in homologous recombination in which *recA* protein very likely participates in vivo. It has several important advantages: (1) both substrates and products are well characterized and easily distinguishable; (2) a variety of assays are available, including one that permits the direct measurement of heteroduplex formation; and (3) the reaction is efficient, thereby simplifying kinetic analysis.

The efficiency of strand exchange is dependent on the presence of the single-stranded DNA-binding protein (SSB) of *E. coli*. There is, in fact, genetic evidence that SSB may play a role in recombination and recombinational repair (Johnson 1977; Glassberg et al. 1979; Whittier and Chase 1981).

In addition to the three-strand exchange described above, *recA* protein will promote a reciprocal exchange involving four DNA strands (West et al. 1981a; DasGupta et al. 1981). West et al. (1982) have recently shown that SSB, although stimulating three-strand exchanges, either has no effect on or slightly inhibits a four-strand exchange. More work is required to understand the relationship of the two types of exchange to homologous recombination in vivo.

Our initial efforts to determine the mechanism by which *recA* protein and SSB interact to bring about DNA strand exchange are described in this paper.

RESULTS

DNA Strand-exchange Reaction

recA-protein-promoted DNA strand exchange is diagramed in Figure 1 as a two-phase reaction. An initial pairing event yields an intermediate structure for which we use the term D loop regardless of its topology. This part of the reaction has been variously designated D-loop formation, strand assimilation, homologous pairing, and synapsis (McEntee et al. 1979; Shibata et al. 1979; Cox and Lehman 1981a; DasGupta and Radding 1982). The second phase consists of extension of the short heteroduplex within the D loop by branch migration to yield a circular nicked duplex DNA (RFII) and the displaced linear (plus) single strand.[1] Each of these phases very likely represents a complex series of chemical steps. However, it is useful to distinguish the two phases as an initial approach to dissecting the entire reaction pathway.

The strand-exchange reaction in the presence of SSB is illustrated in Figures 2 and 3. In agarose gel electrophoresis, a band corresponding to the product RFII can be detected early in the reaction. The substrates, and, at later times, the reaction intermediates and products, are readily identified by electron microscopy (Cox and Lehman 1981b). All of the D loops originate from an end of the linear duplex, as expected from topological considerations (DasGupta et al. 1980), and heteroduplex formation is largely concerted with the displacement of the plus strand of the linear duplex.

As shown in Figure 4, most of the linear duplex molecules are incorporated into D loops within 5 minutes, whereas net formation of heteroduplex DNA is considerably slower (see Appendix for a description of the assays for D-loop formation and heteroduplex extension). The most straightforward interpretation of these findings is that they reflect the two phases of strand exchange illustrated above and that D-loop formation is fast, whereas branch migration is slow. Other kinetic interpretations are possible, however, and additional experiments were carried out to distinguish between them (Cox and Lehman 1981a,b). These have confirmed that

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[1]The term "strand exchange" is utilized by Radding and colleagues (DasGupta and Radding 1982) to describe this second phase while we use it to describe the entire reaction.

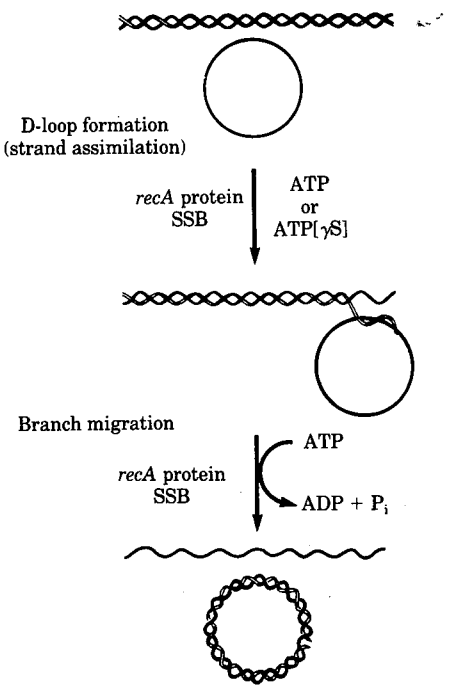

Figure 1. Two kinetically distinguishable phases of *recA*-protein-promoted DNA strand exchange. See text for details (Cox and Lehman 1981a).

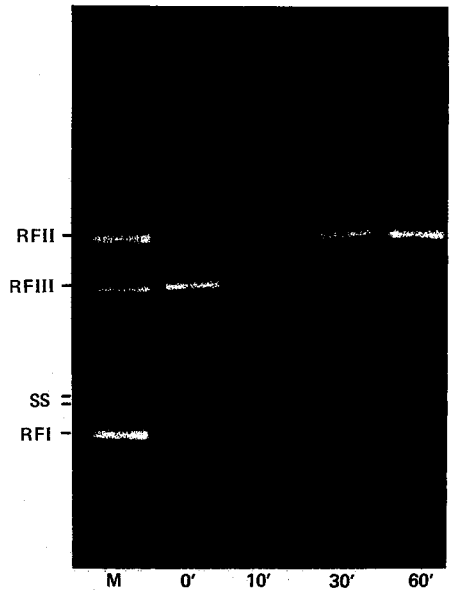

Figure 2. Agarose gel electrophoretic analysis of *recA*-protein-promoted DNA strand exchange. Reactions were carried out as described previously (Cox and Lehman 1982). Reaction mixtures contained 25 mM Tris-HCl, 80% cation (final pH 7.2), 5% (v/v) glycerol, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM ATP, 5.6 μM linear duplex φX DNA, 5.6 μM circular φX SS DNA (plus strands), 1.8 μM *recA* protein, and 0.24 μM SSB. The upper band in the single-stranded DNA doublet represents linear molecules. M indicates markers; numbers indicate the time of reaction in minutes.

in the presence of SSB, the straightforward interpretation is correct. This is most simply demonstrated by examining populations of molecules at various times by electron microscopy (Fig. 5). Structures containing D loops accumulate early in the reaction in a pattern typical of chemical intermediates formed prior to a slow step in a pathway, followed by the appearance of products.

With this information we could use the assays for D-loop formation and heteroduplex extension to characterize the two phases of the reaction separately. The first phase requires ATP but not its hydrolysis, since significant numbers of D loops can be formed in the presence of adenosine-5′-*O*-(3-thiotriphosphate) (ATPγS), which is not hydrolyzed at a significant rate by *recA* protein (Cox and Lehman 1981a; Weinstock et al. 1981c). The branch migration phase of the reaction, in contrast, has a continuous requirement for ATP hydrolysis (Cox and Lehman 1981a); thus, it does not proceed spontaneously but is a process directed by *recA* protein. As described below, the branch migration phase of the reaction shows polarity, further implicating *recA* protein in this process.

In addition to ATP, the reaction requires stoichiometric amounts of *recA* protein (Cox and Lehman 1981a, 1982) and is saturated only when its concentration is equivalent to 1 monomer per 2–4 nucleotides of SS DNA.

Branch migration directed by* recA *protein is polar. *recA*-protein-promoted strand exchange is polar (Cox and Lehman 1981b; Kahn et al. 1981; West et al. 1981b; DasGupta and Radding 1982). The polarity is a property of branch migration rather than pairing (Cox and Lehman 1981b). Thus, early in the reaction a significant number of double D loops are observed in which two circular single strands have reacted with both ends of a linear duplex or two linear duplexes have interacted with one circular single strand. Since all the single-stranded circles are plus strands and must therefore pair with the minus strand of the duplex, these structures provide a direct demonstration that the initial pairing resulting in D-loop formation has no absolute polarity.

The polarity of the entire strand-exchange reaction can be followed by measuring the incorporation of ^3H from ^3H-labeled, circular SS DNA into fragments obtained after *Hha*I restriction endonuclease cleavage of the products formed as the reaction with linear duplex DNA proceeds. As shown in Figure 6, ^3H appears in fragment 4, located near the 3′ end of the minus strand, at least 12 minutes before detectable label is found in fragment 1, located near the 5′ end of the minus strand. This 3′→5′ polarity relative to the minus strand must be a property of the branch migration phase of the reaction. Presumably, D loops formed at the "wrong" end of the linear duplex are quickly eliminated by the polarity of the subsequent branch migration. The possibility that some preference for one end over the other in D-loop formation might have remained undetected. This possibility has recently been

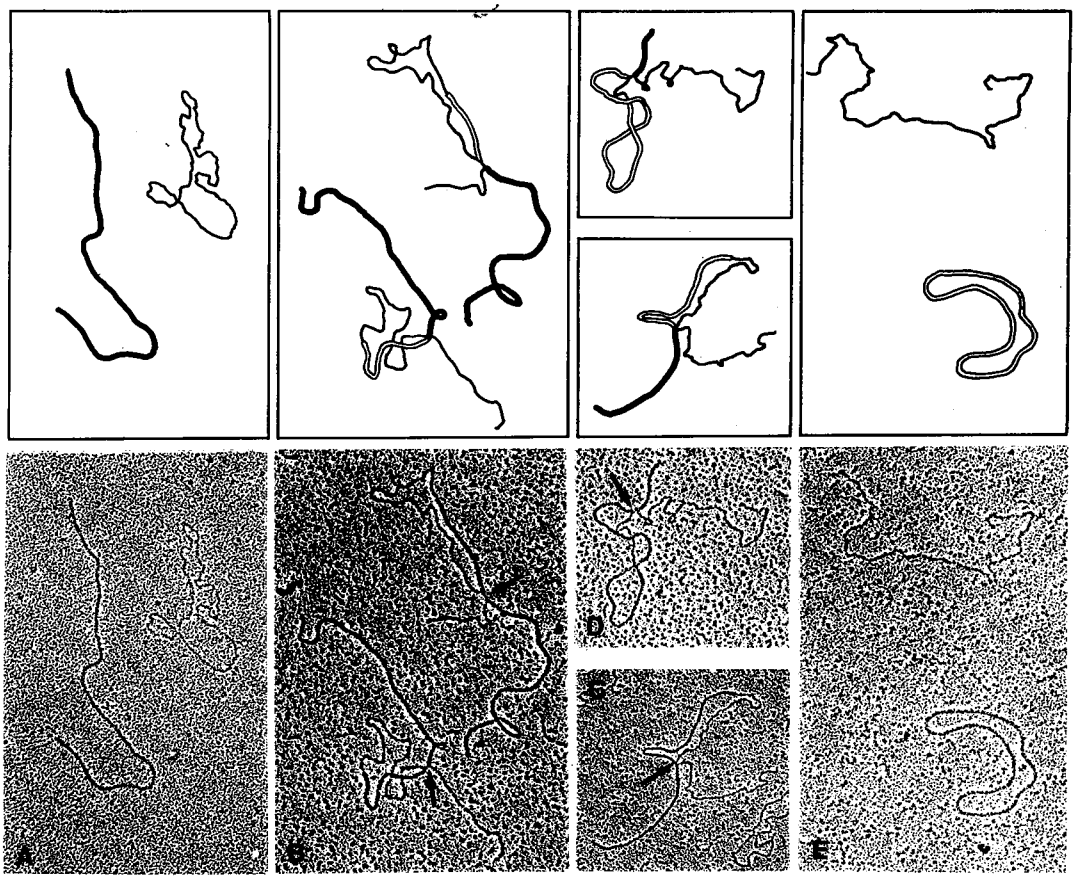

Figure 3. Electron microscopy of *recA*-protein-promoted strand exchange. In the interpretive drawings, heavy lines indicate duplex DNA, thin lines indicate SS DNA, and parallel lines indicate heteroduplex. (*A*) Substrates: linear duplex DNA and circular ϕX SS DNA. (*B–D*) D loops in various stages of reaction. (→) Branch points. (*E*) Products: linear SS DNA and RFII. Reactions were carried out as described previously (Cox and Lehman 1981b).

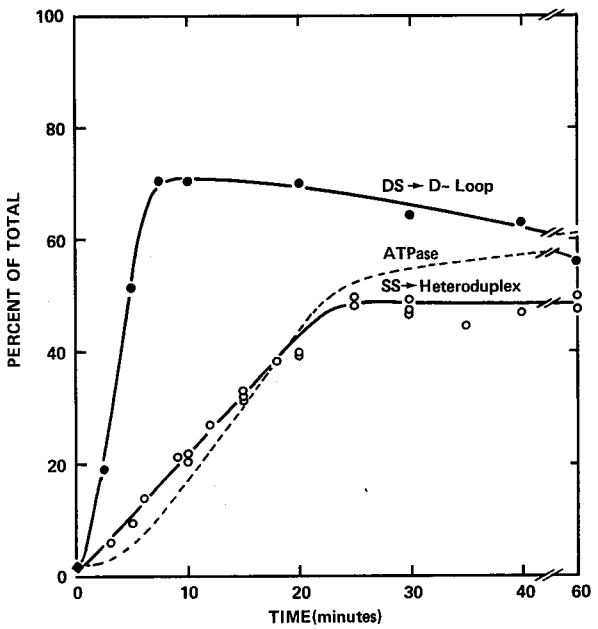

Figure 4. D-loop formation and heteroduplex formation during *recA*-protein-promoted DNA strand exchange. Reactions were carried out as described previously (Cox and Lehman 1981a). Assays are described in the Appendix.

eliminated by Wu et al. (1982), who demonstrated directly that D-loop formation proceeds at equivalent rates at both ends of the linear duplex.

recA-*protein-promoted strand exchange is stimulated by SSB.* Strand exchange is stimulated 5-fold to 20-fold by SSB (Cox and Lehman 1981a, 1982; Wu et al. 1982). This stimulation is depicted in Figure 7, using the nuclease-S1 assay for heteroduplex formation. Stimulation by SSB is also observed by agarose gel electrophoresis (Cox and Lehman 1981a; Cox et al. 1982a). The extent of stimulation is strongly dependent on the order of the addition of reaction components. The greatest stimulation is observed when SSB is added last (Cox and Lehman 1982; West et al. 1982). Stimulation of D-loop formation by SSB was observed previously (McEntee et al. 1980; Shibata et al. 1980). These earlier findings suggested that the stimulation was due to the ability of SSB to titrate excess SS DNA, thereby preventing it from sequestering *recA* protein. Inasmuch as stimulation of strand exchange occurs in the presence of more than enough *recA* protein to saturate the SS DNA, it is unlikely that SSB is simply serving in such a sparing role. Recent results, in fact, point to a much more complex interaction between SSB and *recA* protein.

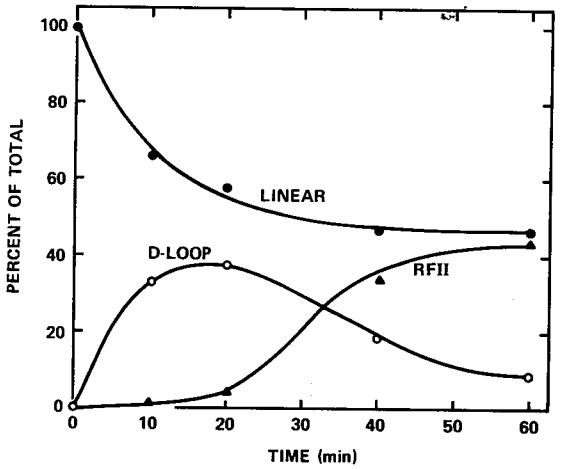

Figure 5. Fate of DNA structures observed during *recA*-protein-promoted DNA strand exchange. (Data from Cox and Lehman 1981b.)

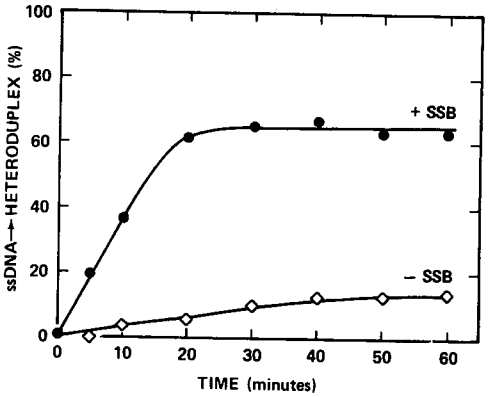

Figure 7. Stimulation of heteroduplex formation by SSB. Reactions were carried out as described previously (Cox and Lehman 1982). Reaction mixtures were carried out as described in Fig. 1, except that the concentrations of ATP, SS DNA, and SSB (where present) were decreased by 41%. Assay is described in Appendix.

SSB-dependent Formation of a Stable Complex between *recA* Protein and SS DNA

An early step in DNA strand exchange involves binding of *recA* protein to SS DNA. In the presence of SSB and ATP, this interaction is strongly stabilized. Under these conditions, *recA* protein remains associated with the SS DNA with which it had first interacted and does not dissociate measurably for up to 90 minutes (Cox and Lehman 1982). Formation of this stable complex, which occurs with $t_{½}$ of less than 1 minute, is completely dependent on both ATP and SSB but does not require duplex DNA. If sufficient SSB is added (~1 monomer/8–10 nucleotides SS DNA), up to 1 *recA* protein monomer per 2 nucleotides of SS DNA can be incorporated into the complex. The amount of both proteins effectively sequestered in the complexes makes it unlikely that all of the protein is bound directly to the DNA. We would therefore infer that much of it is bound through protein-protein interactions. The complex is kinetically competent, with respect to both its rate of formation and the rate of its subsequent reaction with homologous duplex DNA, strengthening the conclusion that it is an intermediate in the normal reaction pathway (Cox and Lehman 1982).

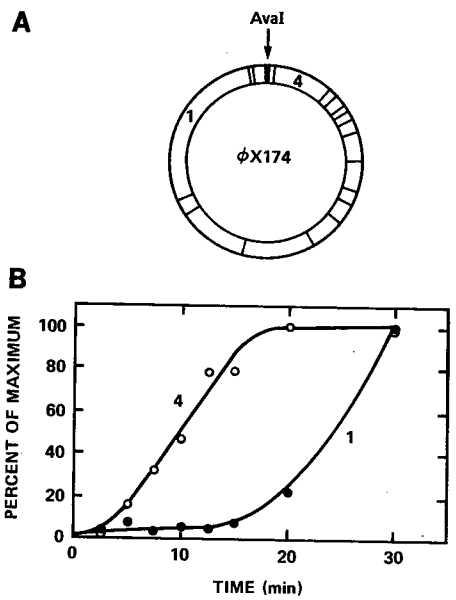

Figure 6. Restriction endonuclease analysis of polarity of *recA*-protein-promoted branch migration. (*A*) *Hha*I restriction map of ϕX RFI with the *Ava*I site (defining the ends of the linear duplex substrate) and the two fragments of interest indicated. (*B*) Rate of appearance of radioactivity in *Hha*I restriction fragments 1 and 4. Reactions were carried out as described previously (Cox and Lehman 1981b).

Although *recA* protein in the *recA* protein–SS DNA complex will not react with SS DNA added after complex formation, the complexes will react readily with duplex DNA to carry out strand exchange. Strand exchange is inhibited approximately 40% by a twofold excess of heterologous duplex DNA added prior to the addition of homologous duplex. However, the reaction appears to approach the same extent whether or not the heterologous DNA is present. These results indicate that the stable complexes will bind heterologous duplex DNA but that this binding is reversible. If the heterologous DNA is added to an ongoing reaction (after D-loop formation), it has no effect on extension of the heteroduplex. Heterologous duplex DNA thus acts as a competitive inhibitor of the binding of homologous duplex DNA to the complex (Cox and Lehman 1982).

Under the conditions in which complex is formed, as defined kinetically, a structure is generated that migrates at approximately 75S in a sucrose density gradient. Labeled SS DNA and *recA* protein cosediment in stoichiometries consistent with the findings described above. A detailed analysis of these complexes will be reported elsewhere (D.A. Soltis and I.R. Lehman, in prep.).

An SSB-dependent Change in the Rate-determining Phase of DNA Strand Exchange

As described above, stable complexes are formed between *recA* protein and SS DNA in the presence of SSB and ATP. In contrast, *recA* protein moves rapidly from one molecule of SS DNA to another when SSB is not present. *recA* protein in this case will completely equilibrate between two populations of SS DNA in less than 1 minute. Rapid equilibration occurs whether or not ATP is present. In addition, movement involves a rapid equilibrium between free and bound forms of *recA* protein, rather than a direct transfer of *recA* protein from one molecule of DNA to another (Cox et al. 1982).

The instability of *recA* protein–SS DNA complexes in the absence of SSB has an important kinetic consequence. As shown in Figure 8, SSB stimulates the initial pairing reaction as measured by nitrocellulose-filter binding (see Appendix). The extent of stimulation, ≥12-fold if the linear portions of each curve are compared, is equivalent to that observed for strand exchange as measured with the nuclease-S1 assay. Inasmuch as stimulation must involve the rate-limiting step of a reaction, the rate-limiting step during strand exchange in the absence of SSB must be the initial formation of D loops or one of the steps leading up to it. This is in contrast to the reaction in the presence of SSB, where the extension of heteroduplex regions following D-loop formation is clearly rate-limiting.

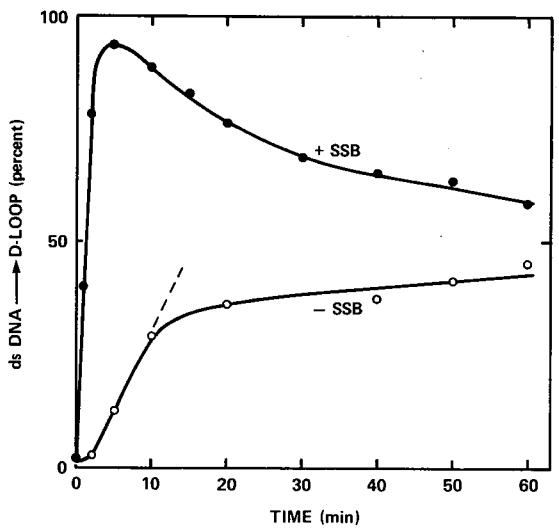

Figure 8. Stimulation of D-loop formation by SSB. Reaction mixtures were carried out as described in Fig. 7, except that an ATP-regeneration system was included (phosphoenolpyruvate and pyruvate kinase). The assay is described in the Appendix. Note that in the D-loop form, all of the ^3H from the linear duplex DNA will be retained on the nitrocellulose filters, whereas in the products, half of the label will be in a completely duplex form (RFII) and thus will not be retained by the filters. The decrease seen with time in the "+SSB" reaction thus reflects the formation of products rather than a reversal of D-loop formation. The greater efficiency of D-loop formation observed here relative to that in Fig. 4 reflects a difference in the order of addition of reaction components. In this case, ATP and SSB were added as a mixture to start the "+SSB" reaction.

Heteroduplex extension occurs at rates up to 20 bp per second (Cox et al. 1982a). It is not known whether SSB has a significant effect on this phase of the reaction. Given a sufficiently long stretch of heteroduplex DNA to be formed, this process will require at least several minutes, and thus will always be kinetically significant. As indicated above, however, the events leading up to the initial pairing in the absence of SSB are considerably slower than branch migration. SSB accelerates the reaction by stimulating one of the steps in this first phase of the reaction so that D-loop formation becomes much faster than branch migration. The rate-limiting phase of strand exchange is thus determined by the presence or absence of SSB. Additional evidence supporting this conclusion is described elsewhere (Cox et al. 1982a).

The observation that SSB affects an early phase of strand exchange correlates well with the known effect of SSB in stabilizing *recA* protein–SS DNA complexes. It is likely, therefore, that stabilization of the complex represents the primary effect of SSB. Thus, the steps leading to D-loop formation in the absence of SSB are not intrinsically slow. This phase is apparently rate-limiting because dissociation of *recA* protein from the DNA is faster than one or more of the steps in this part of the reaction. Under these conditions, *recA* protein does not remain associated with the DNA long enough for the reaction to proceed to completion.

Role of ATP Hydrolysis in DNA Strand Exchange Promoted by *recA* Protein

The DNA-dependent ATPase activity of *recA* protein is sensitive to changes in salt concentration, the DNA used as effector, DNA concentration, and pH (Weinstock et al. 1981a,b). The activity is characterized by a high Hill coefficient under most conditions (Weinstock et al. 1981b). ATP is required for all of the DNA-pairing reactions promoted by *recA* protein; however, its precise role is not understood.

One of the more perplexing characteristics of *recA*-protein-promoted ATP hydrolysis during strand exchange is its inefficiency. More than 1000 ATPs are hydrolyzed per base pair of heteroduplex formed when the reaction is carried out in the absence of SSB. This efficiency can be improved by one order of magnitude by adding SSB. We have recently found that in the presence of the appropriate amounts of ADP, only 10–15 ATPs are hydrolyzed per base pair of heteroduplex formed (Cox et al. 1982b), indicating that at least 90% of the ATP hydrolyzed is unrelated to strand exchange even in the presence of SSB.

Another characteristic of the ATPase activity noted in earlier work is relevant to the structure of the stable *recA* protein–SS DNA complex. At pH 6.0, ATP hydrolysis with a duplex-DNA effector does not proceed to completion but ceases when about 60% of the ATP present has reacted (Weinstock et al. 1981a). This limit is observed regardless of the initial concentration of ATP greater than 200 mM. The same effect occurs during strand exchange at pH 7.2, in the presence of SSB.

The cessation of ATP hydrolysis well before the reaction is complete is a consequence of the dissociation of *recA* protein from the stable complexes. *recA* protein can be recycled to begin a new round of strand exchange if an ATP-regenerating system is provided. The structure of the stable *recA* protein–SS DNA complexes is thus sensitive to the ratio of ADP to ATP in the reaction (Cox et al. 1982b).

DISCUSSION

We have presented here a summary of our efforts to date to understand the mechanism of *recA*-protein-promoted DNA strand exchange and, in particular, the role of SSB in the reaction. A number of important questions remain to be resolved by the kinetic and structural studies now in progress. A minimal scheme that describes these results and that can serve as a framework for further investigation is presented in Figure 9. *recA* protein is in rapid equilibrium between free and SS DNA-bound forms until addition of ATP and SSB promotes formation of a stable complex in the second step. Formation of this complex is irreversible as long as ADP is not permitted to build up significantly. However, binding of the complex to duplex DNA is reversible. Formation of D loops (at the 3′ end of the minus strand) is rendered irreversible by the polarity of the subsequent heteroduplex extension via branch migration.

In stabilizing the *recA* protein–SS DNA complexes, SSB increases the rate of the overall reaction and alters the rate-limiting step. Under these conditions, SSB converts strand exchange from an inefficient reaction to an irreversible reaction that proceeds rapidly to completion (Cox and Lehman 1982).

The positions of *recA* protein and SSB at various stages of the reaction are, for the moment, speculative, and structures indicated in Figure 9 represent only one of many possibilities. Efforts are under way to examine these structures directly. It is known that *recA* protein will form highly structured filaments under a variety of conditions (McEntee et al. 1981; DiCapua et al. 1982; Dunn et al. 1982; Flory and Radding 1982). SSB has also been shown to have an effect on the growth of these filaments (Flory and Radding 1982). Although the experiments demonstrating the effect of SSB were carried out in the absence of ATP, and thus cannot be compared directly with the stable complexes we have observed kinetically, it is reasonable to assume that the complexes will prove to be closely related to these filamentous structures.

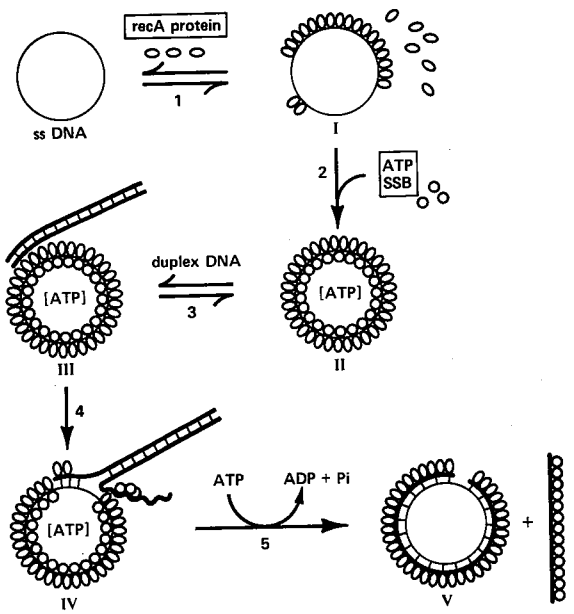

Figure 9. Model for *recA*-protein-promoted DNA strand exchange in the presence of SSB. See text and Cox and Lehman (1982) for details.

recA-protein-promoted strand-exchange reactions have been ultilized in the study of postreplication repair as well as recombination (West et al. 1981c). Thus, these reactions have been shown to bypass short mismatches (DasGupta and Radding 1982) and thymine dimers (Livneh and Lehman 1982).

The system described here possesses an additional feature of interest. The two most important characteristics that distinguish the second phase of strand exchange (i.e., branch migration) from the initial pairing phase are its continual requirement for ATP hydrolysis and its polarity. To the extent that ATP hydrolysis is coupled to unidirectional branch migration, this system exemplifies the classic biochemical problem of how chemical energy is coupled to vectorial processes. A detailed understanding of this process may well have importance beyond our understanding of homologous recombination and repair.

APPENDIX

Measurement of DNA Strand Exchange

Five assays have been used routinely for the measurement of DNA strand exchange; together they permit a quantitative evaluation of the fate of each of the substrates in the course of conversion to products:

1. Hydrolysis of ATP to ADP and P_i.
2. Electron microscopic analysis to identify all DNA species and intermediate structures formed. In addition to permitting visualization of unusual intermediates, electron microscopy provides a means to confirm the general characteristics of the reaction inferred from other assays.
3. Agarose gel electrophoresis to observe formation of the circular, nicked duplex DNA (RFII) product.
4. Nitrocellulose filter binding. In this assay, based on a procedure described by Beattie et al. (1977), the fate of ^{3}H-labeled, linear duplex DNA can be determined by passing aliquots of the reaction through nitrocellulose filters at high ionic strength (Shibata et al. 1979; Cox and Lehman 1981a). Under these conditions, duplex DNA is retained only if it is associated with SS DNA. Thus, the only duplex DNA

retained is that which has been incorporated into a D loop (Fig. 1) and as a result has some single-stranded character. The assay is therefore an indirect measure of the initial pairing reaction, or the fraction of duplex molecules involved in structures containing D loops. There are several limitations. Its efficiency is adversely affected by detergents and by treatments that permit spontaneous branch migration in deproteinized D loops. A second difficulty is the uncertainty regarding the amount of heteroduplex that must be formed before the structure will be retained by the filter. Measurements carried out in the presence of ATPγS indicate that about 300 bp of heteroduplex are sufficient (Cox and Lehman 1981a); however, it is not clear whether shorter base-paired regions can suffice. Advantages of the assay are its ease and rapidity. It also permits a reliable evaluation of relative rates of reaction even though the absolute rates may be slightly underestimated because of the above-mentioned considerations.

5. Incorporation of ^{3}H-labeled, SS DNA into nuclease-S1-resistant material. This assay provides a quantitative measure of the formation of heteroduplex DNA (Cox and Lehman 1981a, 1982), and the results correlate well with those obtained by electron microscopy and agarose gel electrophoresis (Cox and Lehman 1981a,b, 1982). This assay can be used to provide information about all stages of strand exchange but is especially useful for obtaining kinetic information about heteroduplex extension following initial pairing. It also complements the nitrocellulose assay in that it follows the fate of the SS DNA rather than the linear duplex substrate.

ACKNOWLEDGMENTS

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