THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 262, No. 3, Issue of January 25, pp. 1337–1343, 1987
Printed in U.S.A.

recA Protein Binding to the Heteroduplex Product of DNA Strand Exchange*

(Received for publication, August 8, 1986)

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Following DNA strand exchange, recA protein remains associated with the heteroduplex DNA product of the reaction. This association exists without significant changes (as measured by nuclease protection, topological state of the heteroduplex DNA, and rates of ATP hydrolysis) for at least 30 min after strand exchange is complete. The heteroduplex DNA is unwound as a result of this association to an extent at least as great as the unwinding observed when recA protein is bound to duplex DNA at pH 6.35. The extensive unwinding, in combination with the rates of ATP hydrolysis reported elsewhere, provide evidence that recA protein binding is contiguous throughout the heteroduplex DNA. This binding is disrupted upon challenge with heterologous single-stranded DNA, with rapid migration of recA protein to the challenging DNA. These results are discussed in relation to the mechanism of recA protein-promoted branch migration.

The recA protein of *Escherichia coli* promotes *in vitro* a set of DNA strand exchange reactions which mimic its *in vivo* function in homologous genetic recombination (1–4). For convenience, the *in vitro* systems generally employ DNA substrates derived from small bacteriophages. The best characterized system is the exchange of strands between circular single strands and homologous linear duplex DNA molecules (Fig. 1). The reaction can be divided into three experimentally distinguishable phases. The first phase is the cooperative assembly of a stoichiometric complex of recA protein and ssDNA[1] (2, 3, 5). About one recA monomer is present/four nucleotides in this complex, which exhibits a distinctly filamentous structure in the electron microscope (6, 7). This complex is the active species in subsequent stages of the reaction. The second phase involves a search for homology which results in the pairing of sequences within the ssDNA with homologous sequences in the duplex. This phase requires ATP but not ATP hydrolysis (1, 8). In the third and final phase, recA protein promotes a unidirectional branch migration reaction coupled to ATP hydrolysis.

* This work was supported in part by National Institutes of Health Grant GM32335. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by National Institutes of Health Research Career Development Award AI00599.

[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); FI, supercoiled closed circular form of a DNA molecule as isolated from *E. coli* cells; FII, nicked circular form of the same DNA molecule; SSB, *E. coli* single-stranded DNA binding protein.

The branch migration phase of recA protein-promoted DNA strand exchange is the least understood part of the reaction. We are interested in the mechanism by which ATP hydrolysis is coupled to the unidirectional branch movement of this phase. This reaction represents a coupled vectorial system analogous in a sense to muscle contraction and ATP-driven ion pumps. The active species in this reaction is a large filamentous nucleoprotein complex of recA protein on DNA formed in the first phase. The present challenge is to outline a model for this reaction which provides a role for this complex and also explains and reconciles a wide range of experimental observations.

A number of models for this reaction have been discussed (9–11) and are outlined in Fig. 1 with respect to the possibilities for the disposition of recA protein during strand exchange. Movement of the branch could involve some activity of the entire nucleoprotein complex, or it could be coupled to association or dissociation of recA protein at filament ends. ATP hydrolysis is required for the reaction and should therefore be coupled to the important chemical steps in any model.

One of the central factors in this problem is the interaction of recA protein with single- and double-stranded DNA. At pH values above 7, and under conditions optimal for DNA strand exchange, recA protein binds to ssDNA rapidly while binding to dsDNA is almost undetectable (unless ATPγS is used) (12–14). Binding to ssDNA stimulates binding to dsDNA (15, 16), which is one of the facts upon which the sequence of events described above is based. As the branch point passes a given recA monomer in the nucleoprotein complex, it must move from an association with ssDNA ahead of the branch to an association with duplex DNA behind the branch. The apparent inability of previous studies to detect recA protein binding to dsDNA under these conditions might lead to a conclusion that recA protein must dissociate as DNA strand exchange proceeds. A detailed model in which branch migration is accompanied by dissociation of recA protein from the end of a nucleoprotein filament has been proposed by Howard-Flanders and colleagues (9). However, an alternative interpretation of this apparent week binding to dsDNA is provided in the accompanying paper (18). The lack of binding observed at pH 7.5 is not the result of an unfavorable binding equilibrium, but instead reflects a kinetically slow step in the association pathway for recA protein. This slow step could potentially be circumvented by DNA strand exchange.

Recent studies have demonstrated that recA nucleoprotein filaments do not exhibit some of the properties which might be expected in a system where the important events occur at filament ends. In particular, it has been shown that ATP hydrolysis is not tightly coupled to association and dissociation of recA protein with and from these complexes (17), and that ATP hydrolysis is not restricted to recA monomers at

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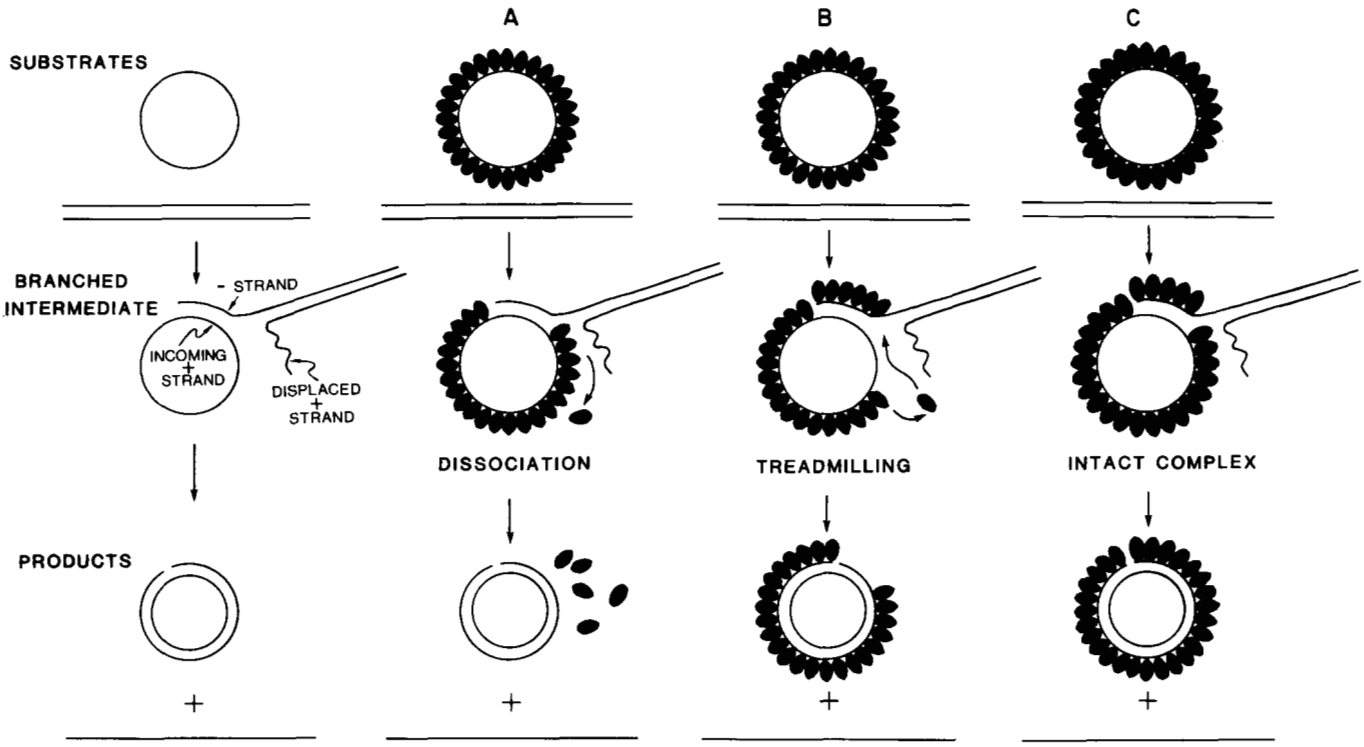


FIG. 1. **Alternative possibilities for the disposition of recA protein during DNA strand exchange.** See text for details.

filament ends.[2] Furthermore, the demonstration in the accompanying paper (18) that the apparent weak binding of recA protein to dsDNA reflects a slow step in the association pathway rather than an unfavorable binding equilibrium indicates that the apparent requirement implied above for dissociation of recA protein at the branch point during strand exchange does not exist.

To fully evaluate models for DNA strand exchange, it is clearly necessary to obtain more detailed information about the location of recA protein at every stage of the reaction. Especially important is the location of recA protein after the reaction is complete. Several studies have addressed this question. Soltis and Lehman (19) obtained results for strand exchange in the presence of SSB which indicated that recA protein was bound to the heteroduplex DNA product and SSB was bound to the displaced ssDNA. The use of ATPγS to stabilize recA·DNA complexes in this study left open the possibility that recA protein binding to the heteroduplex DNA was enhanced by ATPγS. Since ATPγS should stabilize binding of recA to either ssDNA or dsDNA, however, these results provide strong evidence that SSB (when it is included in the reaction) is bound to the displaced single strand and recA protein is not. Observations employing electron microscopy after formaldehyde fixation have indicated either little (20) or partial (21) binding of recA protein to heteroduplex DNA. A more recent study by Chow *et al.* (22) employed nuclease protection in experiments designed to circumvent the interpretative problems imposed by the use of ATPγS or sample fixation. This study again provided evidence that at least some recA protein remained bound to heteroduplex DNA and that this binding afforded greater protection to the "+" strand (this is the incoming strand which is complexed with recA protein before the reaction begins) than to the "−" strand. Protection of the displaced "+" strand by recA protein was also observed. No SSB was included in this study, however. Strand exchange reactions are severalfold more efficient in the presence of SSB than under the conditions employed (23). The possible presence of a significant population of unreacted substrates could lead to an overestimation of the degree of protection of the "+" strand of the heteroduplex product and/or an underestimate of the degree of protection of the "−" strand. SSB would also block the observed association of recA protein with the displaced "+" strand.

To provide additional information about the disposition of recA protein during and after strand exchange, we have examined reactions carried out in the presence of SSB. It has been noted elsewhere[2] that recA protein-promoted ATP hydrolysis continues without a detectable decrease during and at least 30 min after the completion of DNA strand exchange, since SSB should block recA protein binding to the displaced single strand, this ATP hydrolysis suggests continued binding to the heteroduplex DNA product of the reaction. The experiments reported here, employing nuclease protection and observed changes in DNA topology, provide unambiguous evidence that recA protein remains bound to the heteroduplex DNA well after DNA strand exchange is complete. In addition, the results indicate that the heteroduplex DNA is completely and uniformly bound by recA protein. The heteroduplex DNA within this complex is highly unwound.

EXPERIMENTAL PROCEDURES

Enzymes and DNA—*E. coli* recA protein and single- and double-stranded DNA substrates in various topological forms were prepared and characterized as described in the accompanying paper (18). *E. coli* SSB was prepared, characterized, and stored as described elsewhere (24). SSB stock concentrations were determined by the absorbance at 280 nm, using an extinction coefficient of $\epsilon_{280} = 15.1$ mg^{-1} ml (25). Type II DNase I was purchased from Sigma.

Methods—The coupled ATPase assay, nuclease protection assay, the trapping of topologically unwound duplex DNA by ligation, and

[2] Brenner, S. L., Mitchell, R. S., Morrical, S. W., Neuendorf, S. K., Schutte, B. C., and Cox, M. M. (1987) *J. Biol. Chem.*, submitted.

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agarose gel electrophoresis are described in the accompanying paper (18). The S_1 assay for formation of heteroduplex DNA is described in detail elsewhere (2). This assay is based on the incorporation of ^{3}H-labeled ssDNA into an S_1 nuclease-resistant form. Product formation in DNA strand exchange reactions was also followed by a previously described agarose gel electrophoresis assay (1).

Reaction Conditions—Reactions were carried out as described by Cox and Lehman (2). Briefly, reactions contained 25 mM Tris-HCl (80% cation, pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 5% glycerol (buffer B). Unless noted, reactions included 1 mM ATP, an ATP-regenerating system (2.24 units/ml pyruvate kinase, 2.34 mM phosphoenolpyruvate, and 0.44 mM KCl final), 3.3 μM circular M13mp8 ssDNA (+-strand, ^{3}H-labeled where appropriate), 2 μM recA protein, 0.3 μM SSB, and 5 μM linear M13mp8 dsDNA (^{3}H-labeled where appropriate). Linear φX174 dsDNA was employed for heterologous reactions. RecA protein was preincubated with ssDNA and the other buffer components for 5 min followed by ATP and SSB for 5 min. Reactions were started by the addition of dsDNA. All reactions were carried out at 37 °C.

RESULTS

As described in the accompanying paper (18), recA protein binding to duplex DNA is characterized by a binding density of one recA monomer/4 base pairs, extensive unwinding of the bound DNA, and a partial protection from nuclease digestion. In the present study, the location of recA protein during DNA strand exchange was followed by two methods. Nuclease protection permitted some resolution of binding to different substrates and products and provided information about recA protein binding throughout the reaction. Binding of recA protein to heteroduplex DNA was followed by sealing the nick in the FII heteroduplex products of strand exchange and trapping any topological changes in this DNA brought about by recA protein binding. ATP hydrolysis during DNA strand exchange is examined elsewhere.

Nuclease Protection—The state of the DNAs participating in strand exchange was examined by nuclease protection. The level of protection from DNase I degradation afforded to DNA by recA protein binding varies depending on the type of DNA (ss or ds), conditions, level of DNase I employed, etc. To provide meaningful information on the time scale of DNA strand exchange (10–30 min) it was necessary to keep DNase I incubations as short as possible. In these assays the incubation time was 30 s. Binding of saturating amounts of recA protein to ssDNA partially protects the DNA from nuclease digestion, affording approximately 80% protection under standard strand exchange conditions when high levels of DNase I are employed (Fig. 2*A*). In this experiment SSB was omitted. By itself, saturating levels of SSB also provide approximately 80% protection (Fig. 2*B*) at the highest levels of DNase I employed. When SSB was added to recA·ssDNA complexes, 100% protection was observed, even over a full range of DNase I concentrations (Fig. 2*C*). When recA protein was incubated with duplex DNA for 10 min under these conditions (pH 7.5) no protection of the DNA was observed (Fig. 2*D*). As described in the accompanying paper, recA protein binding to dsDNA at pH 7.5 is very slow and will not produce significant nuclease protection over a 10-min time course. Incubation of duplex DNA with heterologous ssDNA complexes containing recA protein and SSB in the presence of ATP also produced no detectable protection (Fig. 2*D*). This experiment served to mimic the situation in which the presynaptic filament must bind heterologous duplex DNA as a first step in the search for homology (26). This assay is not sensitive enough to detect this interaction, but to a first approximation the binding of heterologous duplex DNA by recA·ssDNA complexes does not result in significant protection of that duplex DNA from nuclease digestion.

Direct binding of recA protein to duplex DNA (at pH 6.35)

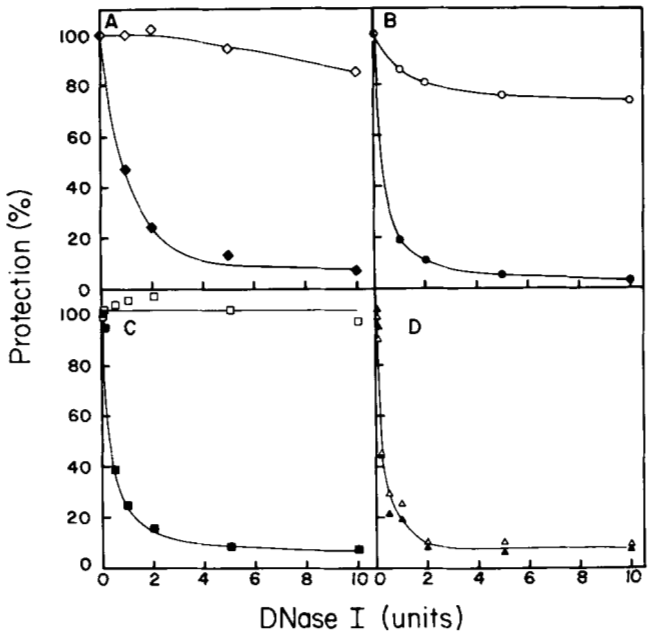


FIG. 2. **Modes of nuclease protection of DNA during various stages of strand exchange.** All reactions were performed in buffer B as described under "Experimental Procedures." *Panel A*, recA·ssDNA complexes: ◇, protection of 5 μM ssDNA (^{3}H-M13mp8(+)) after incubation with 3 μM recA protein and 1 mM ATP for 30 min; ◆, recA protein omitted. *Panel B*, SSB·ssDNA complexes: ○, protection of 5 μM ssDNA (^{3}H-M13mp8(+)) after incubation with 5 μM SSB for 15 min; ●, SSB omitted. *Panel C*, recA·SSB·ssDNA complexes: □, 2 μM recA protein was preincubated with 5 μM ssDNA (^{3}H-M13mp8(+)) for 5 min, then 0.3 μM SSB and 1 mM ATP was added and incubation continued for 20 min before being assayed for protection; ■, recA protein and SSB omitted. *Panel D*, heterologous dsDNA in recA·SSB·ssDNA complexes: △, reaction was as described in *panel C* except that 3.3 μM ssDNA (φX174(+)) was used. Linear dsDNA (^{3}H-M13mp8, 10 μM) was added 5 min after the addition of SSB and ATP and incubation continued for 10 min before assaying for protection; ▲, ssDNA omitted.

results in only a partial protection of the DNA (see accompanying paper (18)) which is highly dependent on the amount of DNase I present. This partial protection could be due to at least three factors: (*a*) incomplete binding by recA protein, (*b*) a dynamic association and dissociation of recA protein which would permit higher levels of DNase I to compete for binding sites, or (*c*) incomplete protection of one or both strands of DNA in the duplex due to the prevalent structure of the complex. At pH 6.35, the level of nuclease protection observed is largely independent of recA protein concentration above saturation. In the accompanying papers (18) we show that partial nuclease protection is characteristic of recA·dsDNA complexes in which the recA binding density is one recA monomer/4 base pairs. These and other results presented below provide strong evidence that the DNA is bound uniformly by recA protein. The partial protection therefore is a function of the complex structure or its dynamic state. We have not yet distinguished between these two possibilities. At present we note only that partial protection is characteristic of the recA protein binding to duplex DNA we wish to investigate here. The level of DNase I used in these experiments (2 units) provides the optimal compromise between high protection observed when recA protein is bound and low backgrounds observed when it is not bound (maximum signal to noise ratio). A series of experiments have shown that the error observed, in measurements carried out with this assay and which involve recA protein binding to duplex DNA, is on the order of 10% in experiments carried out on different days.

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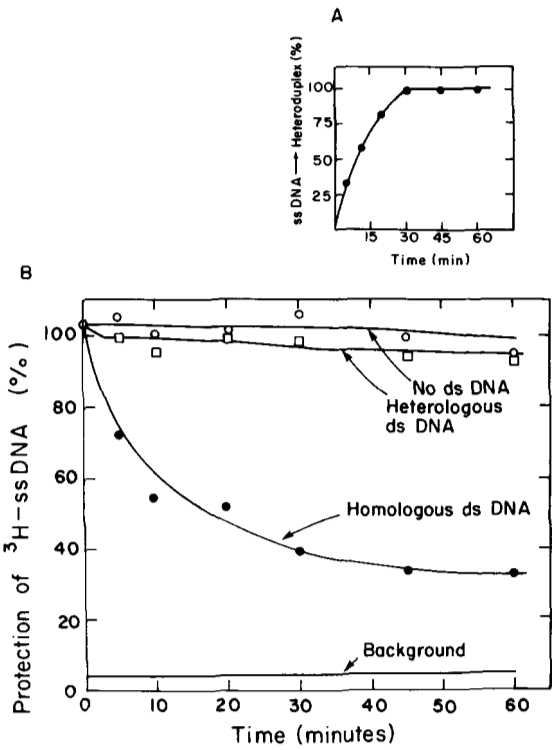


FIG. 3. **Interaction of recA protein with ssDNA during DNA strand exchange.** *A*, standard DNA strand exchange as measured by S_1 nuclease assay. *B*: ●, same reaction shown in *panel A* except samples were assayed for nuclease protection at the indicated time points. □, nuclease protection when heterologous dsDNA (ϕX174) is substituted for M13mp8 dsDNA; ○, dsDNA omitted. Background represents the percent of total radioactivity that remained acid precipitable after DNase I digestion of linear dsDNA (^{3}H-M13mp8) in the absence of added proteins.

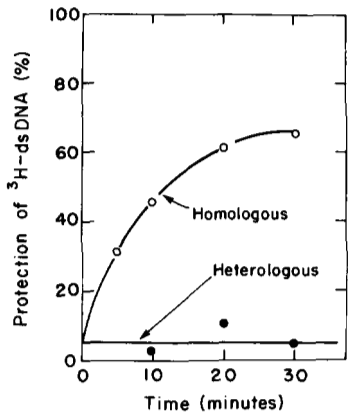


FIG. 4. **Interaction of recA protein with dsDNA during DNA strand exchange.** Reactions were carried out as described under "Experimental Procedures" and contained 3.5 μM recA protein, 5 μM either homologous (M13mp8(+)) or heterologous (ϕX174(+)) ssDNA, 0.5 μM SSB, and 5 μM linear dsDNA (M13mp8). Strand exchange proceeded to greater than 90% as measured by the agarose gel assay.

Nuclease Protection and Strand Exchange—Possibilities for the disposition of recA protein during DNA strand exchange are illustrated in Fig 1. Since SSB is included in these reactions, we assume that recA protein is not bound to the displaced "+" strand during the reaction. The results of Soltis and Lehman (19) provide evidence that this strand should be bound by the SSB present and recA protein binding should therefore be blocked. If recA protein remains bound to the heteroduplex DNA after strand exchange and does not dissociate during the reaction, we predict that protection of the incoming "+" strand should decrease as strand exchange progresses to the level characteristic of the recA·dsDNA complex. Protection of the linear duplex DNA should increase to a level consistent with binding of the displaced "+" strand by SSB and partial protection of the "−" strand within the recA·heteroduplex complex. If recA protein dissociates as strand exchange proceeds, then the protection of the incoming "+" strand would fall to 0 as the reaction progressed and protection of the linear duplex should reflect only SSB binding to the displaced "+" strand. Independent measurements of the efficiency of the overall DNA strand exchange reaction are critical in these experiments to distinguish between partial protection of heteroduplex DNA by recA protein and recA protein binding to unreacted circular ssDNA.

The first of the two possibilities described above is found experimentally and typical results are presented in Figs. 3 and 4. When the incoming circular "+" strand was labeled with ^{3}H and subjected to nuclease digestion at various stages, the level of protection was observed to drop from 100% at the beginning of the reaction to approximately 33% at the end (Fig. 3). In control reactions, identical except that heterologous duplex had been added or duplex DNA omitted, the ssDNA remained completely resistant to DNase I over the entire time course of the experiment. The drop in protection therefore was dependent upon the addition of homologous duplex DNA. Separate measurements of the strand exchange reaction by the S_1 assay and the agarose gel assay confirmed that virtually all of the circular ssDNA was incorporated into heteroduplex in the experiment. The partial protection observed at the end of the experiment therefore represents recA protein binding to heteroduplex DNA rather than binding in leftover unreacted recA·ssDNA complexes. Since virtually all of the ssDNA became resistant to S_1 nuclease as the reaction progressed, and no decrease in acid precipitable counts was observed when DNase I was omitted from the procedure (not shown), the drop in protection cannot be attributed to the introduction of a nonspecific nuclease in the reactions containing homologous duplex DNA.

In Fig. 4, the fate of the linear duplex substrate during strand exchange is examined. Duplex DNA uniformly labeled with ^{3}H was employed in this experiment. As strand exchange proceeds, the level of protection rises from zero to 65% after 30 min. This rise in protection parallels the rate at which strand exchange proceeds (see Figs. 3 and 7). This suggests that only the fraction of the incoming duplex which has undergone strand exchange is protected. The overall strand exchange reaction resulted in conversion of over 90% of the duplex DNA into product in this experiment as measured by the gel electrophoresis assay (not shown). Since the label is equally partitioned into the displaced "+" strand which should have SSB bound and the "−" strand which has recA protein bound, the level of protection observed for individual strands cannot be easily assessed here. Since much of the observed protection of the duplex substrate presumably involves SSB binding, the estimates of protection of the "−" strand alone are subject to considerable error. Whereas we believe that the observed levels of protection are significant, the quantitation is insufficient to determine if the protection of the "+" strand in the heteroduplex is greater than protection of the "−" strand, as indicated elsewhere (22).

As with recA protein bound to dsDNA at pH 6.35, partial protection of the heteroduplex varies with DNase I concentration as shown in Fig. 5. If ATPγS is added just before DNase I treatment then the heteroduplex DNA remains fully

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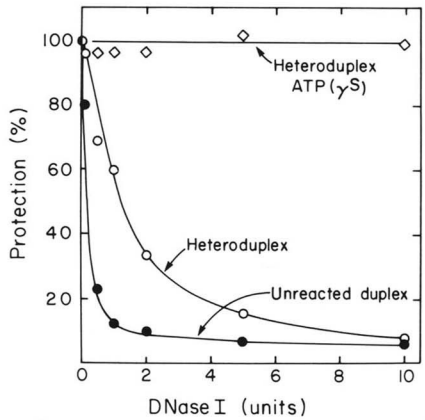


FIG. 5. **Nuclease protection of heteroduplex DNA.** ○, standard strand exchange reactions (see "Experimental Procedures") containing ^{3}H-labeled ssDNA (M13mp8(+)) were allowed to proceed for 60 min, then assayed for protection at various DNase I concentrations. ●, reactions were identical to those above except linear ^{3}H-labeled dsDNA (M13mp8) was incubated in the absence of ssDNA. ◇, strand exchange reactions were as described above except 0.5 mM ATPγS was added to the reaction 5 min before being assayed for protection. Strand exchange reactions proceeded to 75% completion as measured by the S_1 assay (not shown). Data has been corrected for the contribution of the unreacted ssDNA, based on the assumption that unreacted ssDNA is 100% protected from nuclease digestion (see Fig. 2*C*).

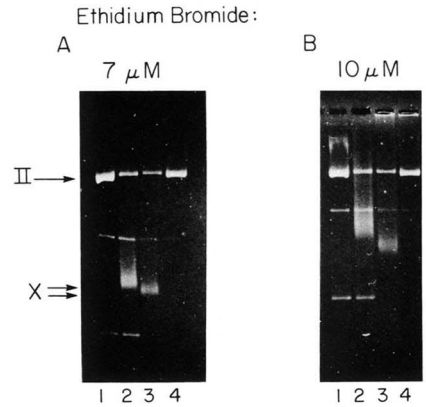


FIG. 6. **RecA protein unwinds heteroduplex DNA.** Reactions were carried out as described in the legend to Fig. 4. Lane identification for both gels: *lane 1*, M13mp8 FII; *lane 2*, M13mp8 form X (made at pH 6.35 from M13mp8 FII with recA protein and DNA ligase, see accompanying paper for details); *lane 3*, strand exchange reaction of *lane 4* but with 1 mg/ml DNA ligase added for 5 min; *lane 4*, product of DNA strand exchange without ligase treatment. Strand exchange had proceeded for 60 min in the experiments illustrated in *lanes 3* and *4*. Aliquots (25 μl) were adjusted to 10% (v/v) glycerol, 12 mM EDTA, 1% sodium dodecyl sulfate, and 0.01% (wt/v) bromphenol blue. Electrophoresis was carried out in the presence of (*A*) 7 μM or (*B*) 10 μM ethidium bromide in 0.8% agarose gels using a Tris acetate buffer system. Samples in both gels are from the same reaction mixtures.

protected over the range of DNase I concentrations tested. Independent measurements (18) indicate that the length of the incubation employed here is insufficient to significantly increase the level of recA protein binding to dsDNA under these conditions. We interpret the effect of ATPγS as trapping a recA nucleoprotein complex conformation which provides greater protection throughout the heteroduplex. This data is consistent with contiguous binding of recA protein to the heteroduplex DNA under these conditions. If instead of adding ATPγS to the completed strand exchange reaction we added glucose and hexokinase which rapidly converts all the ATP into ADP, then there is a rapid loss of protection (data not shown). Nuclease protection provides evidence here that recA protein is bound to DNA throughout the strand exchange reaction and remains associated with the heteroduplex DNA after the reaction is over. The partial protection afforded the heteroduplex by recA protein binding does not permit quantitation of this binding. The nature of the apparent decrease in this partial protection relative to that observed at pH 6.35 is unclear. If DNase I competes for binding with recA protein this may reflect different rates of dissociation at the two pH values. The level of protection observed in the presence of ATPγS, however, is sufficient to be consistent with the idea that the heteroduplex DNA is saturated with recA protein.

The Heteroduplex DNA Product of Strand Exchange Is Extensively Unwound—The topological state of duplex DNA provides an independent measure of recA protein binding. Duplex DNA is extensively unwound whenever recA protein is bound to it (see accompanying paper (18)), and this unwound state can be trapped by the addition of large concentrations of DNA ligase to recA·dsDNA complexes. This is particularly effective when the duplex DNA is FII (as is the product of strand exchange in this system), with up to 60–80% of the DNA molecules covalently sealed even in the presence of large excesses of recA protein (18). The topological form obtained after ligating FII DNA completely bound with recA protein at pH 6.35 is an extensively unwound isomer called form X (18, 26). The degree of unwinding form X DNA produced by recA protein binding to dsDNA at pH 6.35 is much greater than that in FI DNA. Form X DNA migrates faster than FI DNA in an agarose gel. Significant relaxation (and slower migration) of the form X DNA is not observed unless ethidium bromide is included in the gel at concentrations of 1–10 μM (18). This is 10–100-fold more ethidium bromide than is required to relax FI DNA. Electron microscopy has been employed to establish that dsDNA saturated with recA protein in the presence of ATP is unwound 28–30% relative to relaxed B form DNA.[3]

If recA protein is bound uniformly to the heteroduplex after strand exchange, it is expected that the DNA would be as highly unwound as form X described above. A strand exchange reaction was run as described under "Experimental Procedures" in which over 90% of the linear duplex substrate was converted to FII heteroduplex product. When treated with DNA ligase 30 min after completion of the reaction (60 min after the initiation of the reaction), most of the FII DNA was converted to a form which migrated faster than FI in an agarose gel containing no ethidium bromide (11). This reflects an unwinding of the DNA which is characteristic of recA protein binding (18). The reaction was undisturbed except for the addition of ligase and the topological state of the heteroduplex DNA should therefore reflect the presence or absence of recA protein. To determine the extent of unwinding of this fast-migrating species, samples of this DNA before and after ligation were electrophoresed in agarose gels containing 7 and 10 μM ethidium bromide (Fig. 6). Migration was compared to the migration of form X DNA produced by recA·dsDNA complexes at pH 6.35. The results indicate that the fast-migrating species is at least as unwound as form X DNA. The form X resulting from strand exchange actually migrates somewhat faster than the form X prepared at pH 6.35. We have shown in the accompanying paper (18) that the degree of unwinding manifested by form X and partial nuclease protection of duplex DNA is characteristic of a recA protein binding density of one recA monomer/4 base pairs. We have noted elsewhere[2] that the rate of recA protein-mediated ATP

[3] B. F. Pugh, M. M. Cox, and R. B. Inman, unpublished results.

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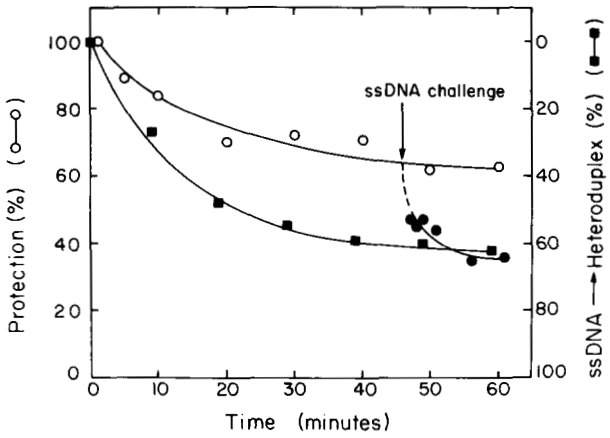


FIG. 7. **recA protein migrates rapidly from heteroduplex DNA to challenging ssDNA.** A strand exchange reaction was carried out as described under "Experimental Procedures" under conditions identical to those described in legend to Fig. 4. At 45 min after the initiation of strand exchange an aliquot of the reaction was challenged with an 8-fold excess (25 μM) of heterologous ssDNA (ϕX174(+)) (*arrow*). ■, formation of heteroduplex DNA as monitored by the S_1 nuclease assay. Data is inverted to reflect fraction of ssDNA remaining unreacted; ○, nuclease protection of the ^{3}H-labeled ssDNA during strand exchange. Data is not corrected for protection contributed by unreacted ssDNA. ●, protection observed after ssDNA challenge. Background levels of protection of ^{3}H-dsDNA in the absence of recA protein and SSB were unaltered by the addition of a similar amount of ssDNA (not shown).

hydrolysis does not decrease during or following DNA strand exchange, providing additional evidence that significant dissociation of recA protein does not occur during the reaction.[4]

recA Protein Migrates Rapidly from Heteroduplex DNA to Challenging ssDNA—As part of an effort to evaluate the stability of the recA·heteroduplex DNA complex revealed in the studies described above, excess (25 μM) ssDNA was added to a reaction after strand exchange had been completed. The results are presented in Fig. 7. The partial protection of the heteroduplex DNA by recA protein rapidly deteriorates after the addition of the challenging ssDNA. As shown in Fig. 7, independent measurements by the S_1 assay showed that 62% of the labeled ssDNA was converted to heteroduplex DNA in this experiment. After correction for unreacted ssDNA remaining in the reaction, the protection of the heteroduplex is effectively reduced to 0 within approximately 5 min. The nuclease protection remaining after the challenge reflects unreacted ssDNA in complexes with recA protein and SSB. In a parallel experiment to which no dsDNA was added, no drop in protection of the ssDNA in the recA·SSB·ssDNA complexes was observed after challenging with an equivalent amount of ssDNA (not shown). This experiment demonstrates that if free ssDNA is available, recA protein will be transferred rapidly to it from dsDNA. It is unclear whether this transfer is direct or involves a free intermediate.

DISCUSSION

Our primary conclusion is that recA protein remains bound to the heteroduplex product of DNA strand exchange well after the reaction is complete. This is supported qualitatively by nuclease protection patterns and more quantitatively by the topological state of the heteroduplex DNA. In the form X DNA derived from the heteroduplex DNA product of strand exchange, the extent of unwinding is characteristic of a recA protein binding density of at least one recA monomer/4 base pairs (18). We have reported elsewhere that once strand exchange is initiated under these conditions, the rate of recA protein-promoted ATP hydrolysis remains constant during and at least 30 min after DNA strand exchange.[2] The rate of this ATP hydrolytic reaction is again consistent with recA protein saturation of the heteroduplex DNA. This also serves as evidence that significant net dissociation of recA protein does not occur during strand exchange. A net dissociation of recA protein is therefore not part of the mechanism by which recA protein promotes DNA strand exchange, these results substantiate the results and conclusions of Soltis and Lehman (19).

This result presents a paradox which has been resolved in the accompanying paper (18). Under the conditions employed in this study, recA protein binds rapidly to ssDNA but binding to dsDNA is nearly undetectable (12–14). As branch migration proceeds in the 3 strand exchange reaction (Fig. 1), recA protein must be associated with ssDNA ahead of the branch but with dsDNA after the branch has passed. The previously reported weak binding of recA protein to dsDNA could be used to argue that recA should dissociate as the branch passes. The results presented in the accompanying paper (18), however, demonstrate that the apparent weak binding to dsDNA reflects a slow step in the association pathway rather than an unfavorable binding equilibrium. The slow step can be circumvented by binding at low pH (12–14, 18), adding homologous ssDNA fragments (15, 16, 27), unwinding the DNA before binding (18), or by strand exchange (this paper; Ref. 22). Once bound to unwound dsDNA, the binding is stable and constant for periods of at least 1 h. The apparent requirement for recA protein dissociation at the branch point does not exist. In fact, as we have shown here, no net dissociation occurs.

The disposition of recA protein and SSB during strand exchange suggested by this study is essentially equivalent to a model presented previously (2), although it was originally proposed at a time when many features were unsubstantiated. Evidence now exists that recA protein is bound to heteroduplex DNA at the end of the strand exchange reaction (19, 22; this study) and that SSB, if present, is bound to the displaced single strand (19).

The fate of recA protein described above applies strictly only to experiments in which SSB is included. SSB is transferred to the displaced "+" strand during strand exchange (19)[5] where it should block binding by recA protein (2). Our challenge experiments indicate that recA protein will migrate rapidly from dsDNA to available free ssDNA. This indicates that although binding to dsDNA at pH 7.5 is stable, binding to ssDNA is still preferred. The results also indicate that recA protein should bind to the displaced "+" single strand when SSB is not present. This binding has been observed in experiments carried out in the absence of SSB by Chow *et al.* (22). We argue at present that binding of recA protein to the displaced "+" strand, where it occurs, is fortuitous rather than an important part of the reaction mechanism. We note that strand exchange is severalfold more efficient in the presence of SSB than in its absence, regardless of the protocol employed (23).

Our results conflict with the results of Chow *et al.* (22) in one respect. These workers did not observe a change in the pattern of nuclease protection of the parental "+" strand

[4] These results would also be obtained if strand exchange and branch migration involved treadmilling within a complex which had a short gap in the recA protein filament. Most of the DNA would still remain bound with recA protein throughout the reaction. Other evidence, however, indicates that treadmilling in the classical sense does not occur in recA filaments (17) (see Footnote 2). See Refs. 9–11 for a description of possible models for the reaction.

[5] B. C. Schutte and M. M. Cox, unpublished results.

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during strand exchange in reactions carried out in the absence of SSB. We observe a large decline in the protection of this incoming "+" strand during strand exchange, as we expected from previous studies of the nuclease protection of duplex DNA bound by recA protein (18). Our result is not an artifact resulting from the unintentional introduction of a nonspecific nuclease to the reaction, since the same ssDNA becomes resistant to S_1 nuclease as the reaction proceeds and the decrease in acid precipitable counts is entirely dependent on the addition of DNase I. Chow *et al.* (22) reported the efficiency of the pairing phase of strand exchange in their experiments, but did not provide data about the overall reaction. Recent experiments have shown that the efficiency of strand exchange is reduced in the absence of SSB, regardless of the protocol employed. If an efficient strand exchange occurred, we would expect some changes in the pattern of protection of the incoming "+" strand and cannot explain the discrepancy. If strand exchange was inefficient, the continuous protection of the incoming "+" strand could simply reflect a large population of unreacted DNA molecules.

We note that the rate at which the incoming duplex DNA acquires protection from nuclease digestion in a strand exchange reaction coincides with the rate of strand exchange. This implies that recA protein binds only the heteroduplex DNA in the three-stranded D-loop complex; *i.e.* only that part of the incoming dsDNA which has undergone strand exchange is protein bound. This suggests that any given segment of the incoming dsDNA remains outside of the recA nucleoprotein complex until the migrating branch passes that point. Qualitatively this is inconsistent with a strict interpretation of the model proposed by Howard-Flanders (9) which describes a recA protein filament forming around all three strands before strand exchange proceeds.

The results reported here are also relevant to the mechanism by which recA protein promotes the unidirectional branch migration coupled to ATP hydrolysis as the last phase in strand exchange. While we cannot eliminate the possibility that association and dissociation of recA protein takes place during branch migration and strand exchange, these and other results (18, 22) provide good evidence that no net dissociation occurs. Any models considered for this process must account for the continued presence of recA protein on the heteroduplex DNA after strand exchange is complete. When combined with results presented elsewhere[2] (17), we argue that ample evidence now exists to merit more serious consideration of models for DNA strand exchange in which the recA nucleoprotein filament remains intact throughout the reaction.

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