THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1986 by The American Society of Biological Chemists, Inc.

Vol. 261, No. 18, Issue of June 25, pp. 8276–8282, 1986
Printed in U.S.A.

Exchange of recA Protein between Adjacent recA Protein-Single-stranded DNA Complexes*

(Received for publication, September 13, 1985)

Sandra K. Neuendorf‡ and Michael M. Cox§

From the Department of Biochemistry, College of Agriculture and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706

We have examined the exchange of recA protein between stable complexes formed with single-stranded DNA (ssDNA) and (*a*) other complexes and (*b*) a pool of free recA protein. We have also examined the relationship of ATP hydrolysis to these exchange reactions. Exchange was observed between two different recA·ssDNA complexes in the presence of ATP. Complete equilibration between two sets of complexes occurred with a $t_{1/2}$ of 3–7 min under a set of conditions previously found to be optimal for recA protein-promoted DNA strand exchange. Approximately 200 ATPs were hydrolyzed for every detected migration of a recA monomer from one complex to another. This exchange occurred primarily between adjacent complexes, however. Little or no exchange was observed between recA·ssDNA complexes and the free recA protein pool, even after several hundred molecules of ATP had been hydrolyzed for every recA monomer present. ATP hydrolysis is not coupled to complete dissociation or association of recA protein from or with recA·ssDNA complexes under these conditions.

The recA protein of *Escherichia coli* promotes several steps of homologous genetic recombination *in vivo*. These steps have been mimicked with a variety of *in vitro* systems employing purified recA protein. In one of the most informative of these systems, recA protein carries out a three-stranded exchange reaction between circular single-stranded and linear duplex DNA substrates derived from bacteriophages. This exchange results in the production of nicked circular duplex and linear single-stranded DNAs. Three major phases of this reaction have been defined (1–4). First, recA protein polymerizes on single-stranded DNA (ssDNA[1]). The resulting complex contains stoichiometric amounts of recA protein organized in a highly structured filament which coats the entire DNA molecule. This filamentous complex represents the active form of recA protein in subsequent reactions. The second phase involves a search for homology between the single-stranded and duplex DNAs and pairing of homologous regions. The final phase is a polar branch migration reaction which is promoted by recA protein and requires ATP hydrolysis.

The strand exchange reaction is stimulated by the single-stranded DNA binding protein of *E. coli* (SSB). Evidence described elsewhere indicates that this stimulation reflects an effect of SSB on the formation and stability of the filamentous recA complex formed in the first phase (4–6). SSB may act in part by binding to single-stranded DNA to remove secondary structure (7), which could inhibit complex formation, or it may play a more direct role in the formation of the complex (8). Recent evidence indicates that SSB is continuously associated with the complex after it is formed for periods up to 1 h (5). recA protein·ssDNA complexes formed in the presence of SSB differ markedly from those formed in its absence. This difference is reflected in experiments in which recA/ssDNA complexes are challenged with ssDNA which has no recA protein bound to it (1). recA protein in complexes formed in the presence of SSB and ATP will not detectably migrate to the challenging ssDNA. If SSB is omitted, approximately half of the recA protein rapidly becomes associated with the challenging DNA. Additionally, SSB-stabilized complexes enhance the rate of strand exchange 5–10-fold relative to reactions carried out in the absence of SSB (4). The efficiency of strand exchange is similarly improved, as the ratio of ATP hydrolyzed per base pair of heteroduplex DNA formed decreases by an order of magnitude when SSB is present (9). These *in vitro* effects of SSB complement a growing body of evidence showing that SSB exerts a profound effect on a variety of reactions carried out by recA protein *in vivo* (10–12).

We are interested in the mechanism by which these filamentous complexes of recA protein promote a unidirectional branch migration reaction coupled to ATP hydrolysis. To this end, we have recently considered several general types of models for this reaction (13). One of these involves a process referred to as treadmilling. Treadmilling occurs in a protein filament when repeated association and dissociation of monomers at the ends of the filament lead to a net gain at one end. This process is coupled to the hydrolysis of ATP or GTP and has been best characterized in the cases of actin and tubulin filaments (14). recA protein could promote branch migration by a similar mechanism if movement of the branch point is coupled to association of recA monomers to the head of a filament and/or dissociation of recA monomers from the tail. A detailed description of a model in which branch migration is coupled to dissociation of recA protein from these filaments has recently been provided by Howard-Flanders *et*

* This work was funded in part by National Institutes of Health Grant GM32335. Supplementary research support was provided by a Shaw Research Fellowship (Milwaukee Foundation) (to M. M. C.) and by Steenbock Career Development funds administered by the Department of Biochemistry, University of Wisconsin-Madison. 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.

‡ Present address: Incell Corp., 1600 W. Cornell, Milwaukee, WI 53209.

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

[1] The abbreviations used are: ssDNA, single-stranded DNA; SSB, *E. coli* single-stranded DNA binding protein; ATPγS, adenosine 5′-*O*-(thiotriphosphate); SDS, sodium dodecyl sulfate; φX174am3(+), single-stranded DNA from phage φX174am3; M13oriC26(+), single-stranded DNA from M13oriC26 phage.

al. (15). In all models of this type, the assumption must be made that the filamentous complexes of recA protein on ssDNA have a distinct head and tail or that these endpoints are formed before branch migration begins. Register and Griffith (16) have shown that recA protein monomers bind unidirectionally on ssDNA in the presence of SSB and ATP, thus providing evidence that recA protein filaments have an intrinsic structural polarity.

recA·ssDNA complexes formed in the presence of SSB and ATP promote a highly efficient DNA strand exchange reaction. As stated above, recA monomers do not exchange between these complexes and uncomplexed ssDNA molecules added as a challenge. Since association and dissociation of recA monomers must occur during treadmilling, this fact would seem to provide an argument against treadmilling or related models for recA protein-promoted branch migration. However, binding of recA protein to DNA is a highly cooperative process (17–19). It has been pointed out that cooperativity can greatly amplify the probability of a protein monomer binding adjacent to a protein cluster already formed on DNA at the expense of binding to a DNA molecule where no such cluster exists (20). This would occur even if the intrinsic affinity of the protein for the two sites differs only slightly. It is therefore possible that this cooperativity may preclude binding of transiently free recA monomers to uncomplexed ssDNA, while exchange of monomers between filaments (which would not have been detected in the earlier experiments (1)) could occur readily. Therefore, it is important to determine whether recA protein can migrate from one filamentous recA·ssDNA complex to another. An exchange of recA protein between complexes and a pool of free recA protein might also be detectable.

The possibility of coupling between ATP hydrolysis and association or dissociation of recA protein with or from a filamentous recA·DNA complex is a central issue in the consideration of models for DNA strand exchange. During strand exchange, the rate of ATP hydrolysis exceeds the rate of heteroduplex formation even under the most favorable conditions (9). A treadmilling model for branch migration provides one mechanism by which this excess hydrolysis might be rationalized. If treadmilling of the filament is similar to the treadmilling of actin filaments, then the efficiency of unidirectional elongation would depend on the number of protein monomer associations and dissociations at each end of the filament that results in a net increase of one monomer at the growing end. If treadmilling is relatively inefficient, and if each association/dissociation is accompanied by hydrolysis of one ATP molecule, then multiple ATPs might be hydrolyzed per base pair of heteroduplex formed. A treadmilling reaction would also provide an enzymatic purpose to the cycles of association and dissociation of recA monomers coupled to ATP hydrolysis proposed elsewhere (18).

We have initiated studies to test basic predictions derived from this and other models for recA protein-promoted DNA strand exchange. In this report we present the results of experiments which indicate that exchange of recA monomers does occur between recA·ssDNA complexes formed in the presence of SSB. This exchange, however, does not appear to reflect an actin or tubulin-like treadmilling reaction. A free recA monomer is not an intermediate in this reaction. Little or no exchange occurs between complexes and a pool of free recA protein. Instead, virtually all of the exchange occurs between adjacent complexes. The relationship of ATP hydrolysis to these exchange reactions is examined. The experimental system developed for this work has also been employed to re-examine a number of previously studied properties of recA protein-promoted DNA strand exchange reactions. A portion of this work has been described in a preliminary report (13).

MATERIALS AND METHODS

$H_2[^{35}S]O_4$ was purchased from New England Nuclear and [^{3}H]ATP from Amersham. ATPγS was obtained from Boehringer Mannheim and was further purified by DEAE-Sephadex A25 (Pharmacia) chromatography. Proteinase K was obtained from Beckman. All other biochemicals were purchased from Sigma. *E. coli* K37 (Hfr sup D r_k+ m_k+) and M13oriC26 phage (21) were kindly provided by Jon Kaguni (Michigan State University).

recA protein and SSB were prepared as described (22, 23). Labeling of recA protein with ^{35}S was carried out using a published procedure (3). The specific activity of labeled recA protein preparations used in this study ranged between 4.6×10^7 and 6.4×10^8 cpm/μmol protein. The labeled protein was tested for activity in DNA strand exchange reactions before it was employed in the sucrose density gradient experiments described below. No labeled recA protein was used in these experiments unless its activity was comparable to that of active, unlabeled recA protein as determined by a gel electrophoresis assay (24). φX174(+) was prepared by a modification of the method of Pagano and Hutchison (25). Extinction coefficients of $E^{1\%}_{280} = 5.9$ (26) and $E^{1\%}_{280} = 15.1$ (27) for recA protein and SSB protein, respectively, were used to determine protein concentrations. The concentration of ssDNA was determined by direct quantitation of the total phosphate concentration in the DNA solution (28) or by assuming a concentration of 36 μg/ml when $A_{260} = 1.0$. These values differed by less than 10%. Concentrations of DNA are reported as total nucleotides.

M13oriC26(+) was prepared by infecting *E. coli* K37, grown in LB (volumes ranged from 40 ml to 6 liters) to an $A_{550} = 0.45$, with M13oriC26 phage at a multiplicity of infection of 1000. This was then incubated with aeration at 37 °C overnight. The cells were removed by pelleting. To the cleared supernatant was added ⅕ volume of a solution containing 20% PEG 8000 and 2.5 M NaCl (final 4% PEG 8000, 0.5 M NaCl) and held at 0°C for 4 h to precipitate the phage. After centrifugation at 10,000 × *g* for 5 min, the precipitated phage was resuspended in 10 mM Tris (80% cation, pH 7.5), 1 mM EDTA (TE buffer) at 1/500 the original volume. The phage was then banded in CsCl density gradients (1.3 g CsCl/ml, 5 ml total volume) in a Beckman SW50.1 rotor at 40,000 rpm, 24 h, 5 °C. Phage bands were collected with a syringe, pooled (several gradients produced 2–3 ml), and dialyzed twice against 1 liter of TE buffer for at least 4 h. To extract the DNA, Proteinase K and sodium dodecyl sulfate were added to the phage preparations to final concentrations of 100 μg/ml and 0.1%, respectively, followed by incubation for 60 min at 37 °C. The resulting preparation was heated at 55 °C for 10 min, then diluted 2.5-fold with TE buffer and cooled to room temperature. It was then extracted twice with an equal volume of phenol (previously equilibrated to TE buffer) and then three times with an equal volume of ether. The phage DNA was then precipitated with ethanol and resuspended in a volume of TE buffer sufficient to yield a final A_{260} of 5.0 to 20.0.

ATPase Assay—The hydrolysis of ATP by recA protein was monitored by a previously described assay employing ^{3}H-labeled ATP (29) or by a spectrophotometric coupled assay (5). Results obtained with either assay were equivalent.

recA Protein Exchange Reactions—To form a recA·ssDNA complex, 8.8 μM ssDNA and 2.2 μM recA protein were incubated with an ATP-regenerating system for 5 min at 37 °C in 20 mM Tris (80% cation, pH 7.5), 10 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 5% glycerol. A mixture of ATP and SSB were added to final concentrations of 4 mM and 0.88 μM, respectively, and incubation was continued for 10 min. This is the preincubation mixture referred to in the text. The ATP-regenerating system contained 2 mM phosphoenolpyruvate and 2 units of pyruvate kinase/ml of preincubation mixture. This maintained the ADP/ATP ratio <0.05 for at least 60 min.

Experiments designed to measure exchange of recA protein between two recA·ssDNA complexes were carried out according to the following protocol. In one preincubation mixture, ^{35}S-labeled recA protein was used to form complexes with φX174(+), and in another preincubation mixture unlabeled recA protein was used to form complexes with M13oriC26(+), or vice versa. Although the two DNA molecules differ in size, the concentration of ssDNA in nucleotides and the concentration of recA protein were kept constant. For exchange experiments, equal volumes (which varied according to the number of samples to be taken in the course of the experiment) of

each of the preincubation mixtures were combined (t(min) = 0) and incubated at 37 °C. Aliquots (160 μl) were removed at the times indicated and reactions were stopped by adding ATPγS to a final concentration of 0.2 mM. After all samples had been collected, 150 μl of each was layered onto a separate sucrose density gradient. To determine the starting point for each experiment at t = 0, control experiments were carried out in which ATPγS was added prior to combining the two preincubation mixtures. The absence of exchange in these controls additionally served to confirm that the ATPγS solution employed in a given experiment effectively halted the reaction. These controls proved to be a sensitive indicator for the presence of contaminating ATP or ADP in an ATPγS preparation. Such contamination occasionally resulted in a significant apparent exchange reaction at t = 0, and this often coincided with an unusual distribution of labeled recA protein throughout the gradient. In every case where the apparent exchange at t = 0 involved more than 10% of the labeled recA protein, all data from the experiment was discarded.

A similar protocol was used to measure exchange of recA protein between recA·ssDNA complexes and pools of free recA protein. In this case, the ssDNA and SSB were eliminated from one preincubation mixture. The rest of the procedure was carried out as described above. Free recA protein (^{35}S-labeled or unlabeled) was therefore preincubated with ATP and then combined at t = 0 with recA (or ^{35}S-labeled recA)·ssDNA complexes. Concentrations of recA protein in the two preincubation mixtures were identical and equal volumes were combined at t = 0. Total reaction volumes were again adjusted according to the number of samples (in this case, 125 μl each) to be taken in the experiment. ATPγS was added to each timed aliquot to halt the reaction as described above, and 100 μl of this material was layered onto a sucrose density gradient. As described above, control reactions were carried out in which ATPγS was added before combining the preincubation mixtures. Experiments were again discarded if this control revealed an apparent exchange involving more than 10% of the labeled recA protein.

Sucrose Gradient Sedimentation—Preformed sucrose gradients (10–40%, 11 ml total), containing 25 mM Tris (80% cation, pH 7.5), 1 mM dithiothreitol, 1 mM $MgCl_2$, and 0.2 mM ATPγS, were used to separate the recA·ssDNA complexes. These were centrifuged in an SW40Ti rotor at 38K rpm, 5 °C for 3.5 h. recA·ssDNA complexes were separated from free recA protein on preformed 5–15% sucrose gradients (5 ml total, composition otherwise identical) by centrifuging for 1 h at 40,000 rpm, 5 °C, in an SW50.1 rotor. Three-drop fractions (~140μl) were collected from the bottom of each gradient onto Whatman GF/A filters. These were then dried and assayed for radioactivity by liquid scintillation. In some cases the fractions were collected directly into scintillation vials and dissolved in Aquasol (New England Nuclear).

The fraction of ^{35}S cpm in each of two partially separated gradient peaks representing the two different sized recA·ssDNA complexes was determined by peak height analysis (30). Quantitation by this method results in a 3% error when the peaks are clearly visible. When one of the peaks is less than 20% of the total, the error increases.

Separation of the recA·ssDNA complexes from free recA protein was always complete and the total radioactivity in each peak was determined by adding the counts in all peak fractions after subtraction of background. Background radioactivity was determined by averaging 10–15 fractions which were at least 4 fractions distant from a detectable peak. Peak fractions were taken as all fractions with a total radioactivity greater than 2-fold above background. Background radioactivity was approximately equivalent to that observed in measurements employing 140 μl of reaction buffer to which no radioactivity had been added.

RESULTS

Formation and Stoichiometry of recA·ssDNA Complexes—recA protein forms stable, stoichiometric complexes with ssDNA in the presence of SSB and ATP. The composition and properties of these complexes have been previously studied by Soltis and Lehman (3) employing sucrose density gradients. The integrity of the complexes during centrifugation, both in the earlier study and in the present work, depends on the prior addition of ATPγS. We determined that complex stability is further improved if ATPγS is present in the gradient. ATPγS halts most reactions of recA protein and promotes very tight binding to ssDNA (18, 24, 31).

It is possible that the addition of ATPγS affects the composition of the complexes, and several experiments were done in order to compare the complexes formed in these experiments with the recA·ssDNA complexes studied by other methods. Again as previously observed (3), formation of a recA·ssDNA complex which could be detected in a sucrose density gradient was almost completely dependent on the presence of SSB (Fig. 1). When recA protein, ssDNA, and ATP were incubated together for any period of time, at concentrations at which 1 recA monomer was present for every 4 nucleotides of ssDNA, no peak corresponding to a recA·ssDNA complex was observed after centrifugation. Complexes formed in the absence of SSB were not stable enough to survive sedimentation in these gradients, even though ATPγS was added before centrifugation. Instead the ^{35}S-labeled recA protein was found as a slowly migrating peak at the top of the gradient, in a position corresponding to free recA protein (Fig. 1*C*). In contrast, when SSB was added prior to the addition of ATPγS, virtually all of the recA protein was found in a single peak which migrated much faster in an identical gradient (Fig. 1*A*). Stoichiometric concentrations of ^{3}H-labeled ssDNA (4 nucleotides/recA monomer) co-migrated with this peak (data not shown). SSB-stabilized complexes are therefore efficiently detected in these experiments. If greater concentrations of recA protein were used in the experiment with SSB, two peaks corresponding to free and bound recA protein were observed (Fig. 1*B*).

Several additional experiments (data not shown) were carried out to determine the amounts of recA protein which could be incorporated into these complexes and the amount of SSB required to stabilize them. These experiments revealed that the complexes contained 1 recA monomer/4 nucleotides of ssDNA, in good agreement with other results (5, 18, 32, 41). Any recA protein in excess of this stoichiometry migrated in the position of free recA protein. In another series of experiments, the stoichiometry was set at 1 recA monomer/4 nucleotides and the amount of SSB was varied. The amount of

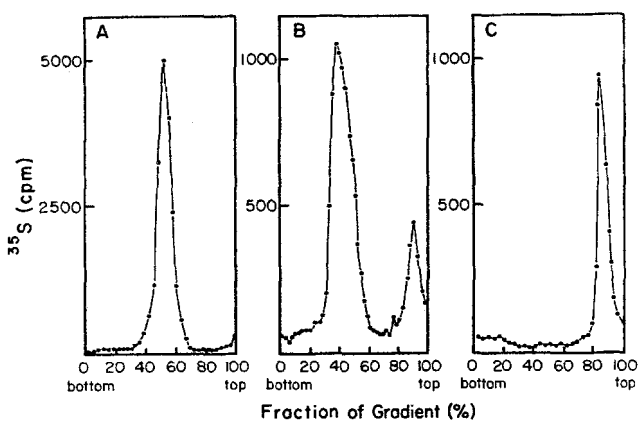

FIG. 1. **Effect of SSB on recA·ssDNA complexes.** Complexes of ^{35}S-labeled recA protein and ssDNA were formed in the presence of ATP at the indicated protein-to-nucleotide ratios, and the complexes were sedimented in 5–15% sucrose gradients as described under "Materials and Methods." Free recA protein migrated at the top of the gradients. recA protein·ssDNA complexes migrated in a peak at approximately the middle of the gradient under these conditions. Conditions employed in these experiments were: *A*, recA protein monomers:nucleotides = 1:4, SSB protein monomers:nucleotides = 1:10; *B*, recA protein monomers:nucleotides = 1:3, SSB protein monomers:nucleotides = 1:10; *C*, recA protein monomers:nucleotides = 1:4, no SSB present. The ssDNA concentration in each case was 8.8 μM.

recA protein complexed to ssDNA was found to be linearly dependent on the concentration of SSB (Fig. 2). One SSB monomer was required for the binding of 5 recA monomers, until a concentration was reached which corresponded to 1 SSB monomer/20 nucleotides of ssDNA. At this concentration, all of the recA protein was found in the rapidly migrating recA·ssDNA complex. Increasing the concentration of SSB by as much as 8-fold had no effect on this result. At subsaturating levels of SSB, the recA protein was divided between the peak corresponding to the saturated complex and the position corresponding to free recA protein, with none at intermediate positions. These results confirm that the complexes observed in this study exhibit a composition and properties consistent with those observed for recA·ssDNA complexes employing other methods.

Exchange of recA Protein between Stable Complexes—The following experiments were designed to detect transfer of recA protein between stable complexes.

SSB-stabilized complexes were formed on two different circular ssDNA molecules: ϕX174 (5,386 base pairs) and M13oriC26 (12,173 base pairs). The two complexes, which differ in size by more than 2-fold, were separable in sucrose density gradients under conditions described under "Materials and Methods." A diagram of the experiment designed to detect exchange of recA protein between complexes is shown in Fig. 3. Results are presented in Fig. 4. When stable complexes were formed on a mixture of these two DNA molecules, both present in equal concentrations in terms of total nucleotides, two peaks were distinguished on the gradients and the labeled recA protein was distributed nearly equally between them (Fig. 4*A*).

In these experiments, ^{35}S-labeled recA protein was used to form stable complexes on M13oriC26 ssDNA and unlabeled recA protein was used to form stable complexes on ϕX174 ssDNA. The two sets of complexes were then mixed together, and ATPγS was added at the appropriate time to halt any reaction. When ATPγS was added prior to mixing, no exchange of labeled recA protein from the M13oriC26 complexes to the ϕX174 complexes was observed up to 60 min after mixing (Fig. 4*B*). This control demonstrates that no exchange

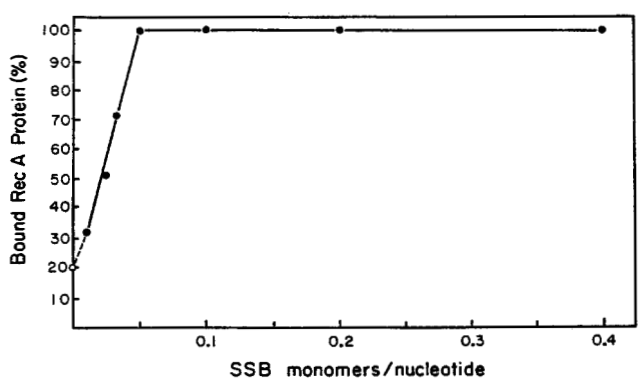

FIG. 2. **Titration of ^{35}S-labeled recA protein·ssDNA complexes with SSB protein.** Complexes of ^{35}S-labeled recA protein and ssDNA at a protein monomer-to-nucleotide ratio of 1:4 were formed in the presence of ATP and the concentrations of SSB indicated on the graph. The complexes were sedimented in the 5–15% sucrose gradients. Details are described under "Materials and Methods." Bound recA protein was calculated as the per cent of the total ^{35}S cpm present in the rapidly migrating peak. The ssDNA concentration in each experiment was 8.8 μM. In the reaction lacking SSB (*open circle*), the indicated level of rapidly migrating recA protein was found as a broad band extending between the gradient positions for recA·ssDNA complexes and free recA protein. In the points denoted by *closed circles*, the indicated level of recA protein was entirely included in a sharp, rapidly migrating peak.

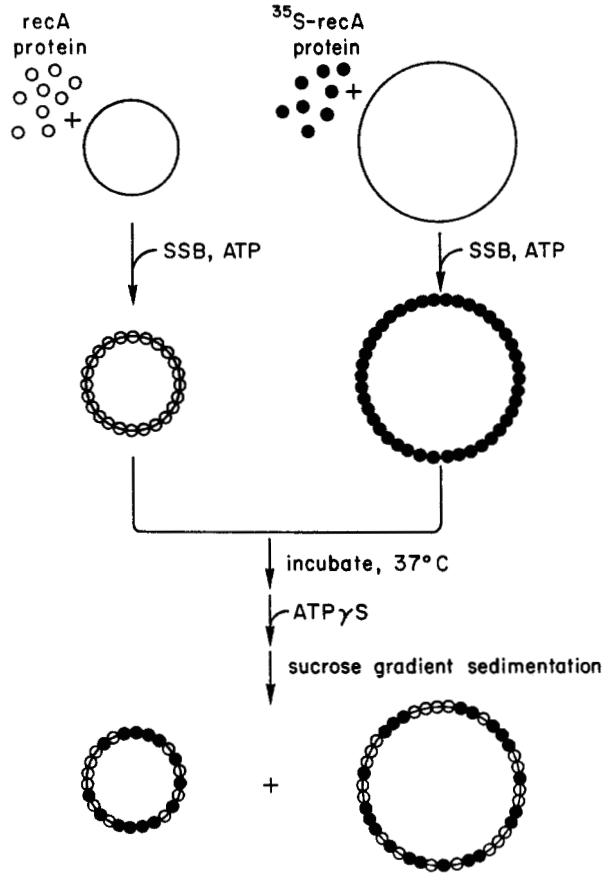

FIG. 3. **Outline of experimental protocol used to detect exchange between recA·ssDNA complexes.** See text for details.

of recA protein occurs after ATPγS addition. When complexes were mixed prior to addition of ATPγS, however, transfer of labeled recA protein from one set of complexes to the other was observed. Results obtained after incubation of the mixture for 15 and 30 min prior to the addition of ATPγS are shown in Fig. 4, *C* and *D*. At the apparent reaction endpoint, approximately 50% of the labeled recA protein was present in each complex. If unlabeled recA protein is omitted from the experiment so that uncomplexed ϕX174 ssDNA is mixed with the M13oriC26 stable complexes, no transfer of the labeled recA protein is observed up to 30 min after mixing (Fig. 4*E*). This is consistent with results obtained in earlier work (1) which demonstrated that recA protein does not migrate from complexes made in the presence of ATP and SSB to uncomplexed ssDNA added as a challenge. A slight shoulder present in Fig. 4, *B* and *E* was also observed in gradients within the same set of experiments in which recA·ssDNA complexes formed on M13oriC26(+) DNA were sedimented without the addition of ϕX174(+) DNA. Each of these experiments was repeated 2–4 times. Equivalent results were obtained when ^{35}S-labeled recA protein was used in the ϕX174 complexes and unlabeled recA protein was used in the M13oriC26 complexes.

Using results from many experiments, an approximate time course for exchange between complexes is presented in Fig. 5. An inability to achieve complete separation of the peaks corresponding to the two sets of complexes in these gradients precluded a quantitative examination of this exchange of recA monomers between complexes. The greatest error occurred at early times in the reaction. While the reaction clearly approaches an equilibrium in which approximately 50% of the labeled recA protein is present in each set of complexes, we

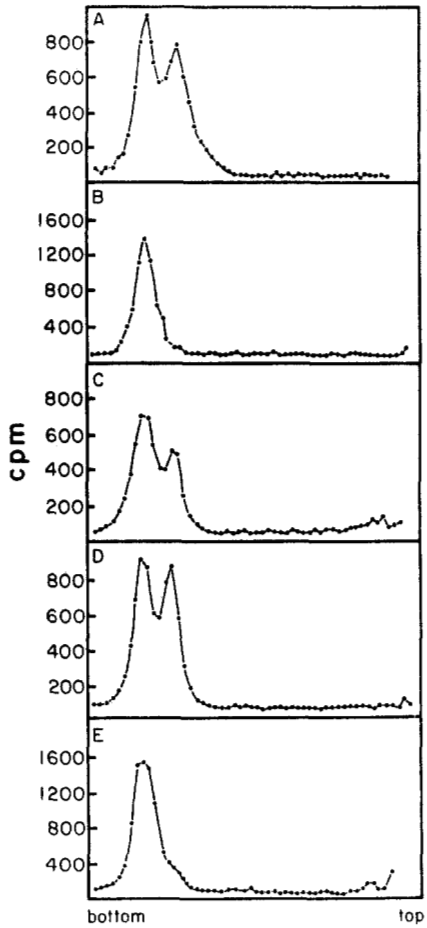

FIG. 4. **Exchange of recA protein monomers between complexes.** Mixtures of complexes of ^{35}S-labeled recA protein·M13oriC26(+) and unlabeled recA protein·ϕX174(+) were sedimented in 10–40% sucrose gradients. Experimental conditions and procedural details are described under "Materials and Methods." Experiments illustrated are: *A*, ^{35}S-labeled recA protein, unlabeled recA protein, M13oriC26(+), ϕX174(+), ATP, and SSB were incubated in the same preincubation mixture before addition of ATPγS. Peaks from *left* to *right* correspond to complexes on M13oriC26(+) and ϕX174(+), respectively. *B–D*, ^{35}S-labeled recA protein·M13oriC26(+) complexes and recA protein·ϕX174(+) complexes were preincubated separately and then mixed. The reactions were stopped with ATPγS at: *B*, $t = 0$ min (ATPγS added before mixing); *C*, $t = 15$ min after mixing; *D*, $t = 30$ min after mixing. *E*, ^{35}S-labeled recA protein·M13oriC26(+) complexes were mixed with ϕX174(+) preincubated in the absence of SSB and recA proteins. ATPγS was added 30 min after mixing.

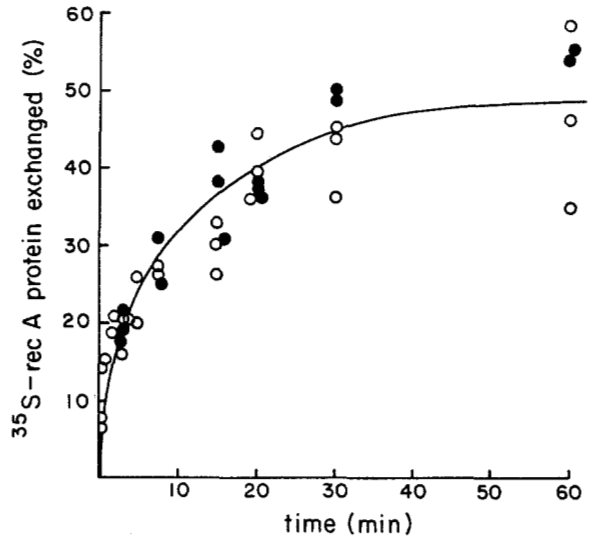

FIG. 5. **Time courses of recA protein exchange reactions.** Points indicate the per cent of total ^{35}S-labeled recA protein which has migrated from one state to another at the time indicated. Each point represents a separate experiment. Experimental procedures and conditions are described under "Materials and Methods." O——O, migration of recA protein from ϕX174 complexes to M13oriC26 complexes; ●——●, migration of recA protein from M13oriC26 complexes to ϕX174 complexes.

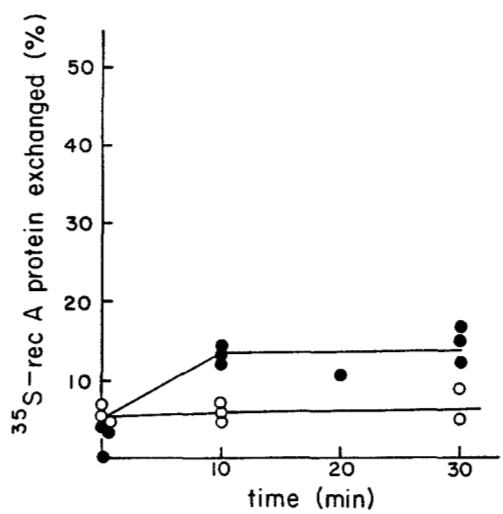

FIG. 6. **Lack of exchange between bound and free recA protein.** Points represent the per cent of total ^{35}S-labeled recA protein which has migrated from recA·ssDNA complexes to the free pool of recA protein (O——O), or from the free pool to complexes (●——●) at the time indicated. Experimental procedures and conditions are described under "Materials and Methods."

cannot conclude that the approach to equilibrium follows first order kinetics or accurately determine the half-time for such a process. If the assumption is made that first order kinetics applies, we estimate from many experiments that the half-time falls between 3 and 7 min under the standard set of conditions employed in these experiments. If a half-time of 4 min is used in the calculations, an apparent first order rate constant of 0.09 min^{-1} for transfer of recA protein from one complex to another is obtained under this single set of conditions. The calculation employs the additional assumption that the rate of transfer is the same in both directions, so that the observed rate constant (0.18 min^{-1}) is the sum of the forward and reverse rate constants (33).

Exchange between Complexes and a Pool of Free recA Protein—The recA protein exchange between complexes could occur via a free recA protein solution intermediate or by a direct transfer between adjacent complexes. Results shown in Fig. 6 indicate that most of the observed exchange occurs between adjacent complexes. In this set of experiments, recA·ssDNA complexes were formed with ϕX174(+) as described above. In the following discussion, this will be designated as solution 1. Solution 2 was identical in all respects except that it contained no ssDNA or SSB. Mixing equal volumes of solutions 1 and 2 results in a mixture containing approximately equal concentrations of free and complex-bound recA protein. Equal concentrations of ATP and buffer components in solutions 1 and 2 ensure that these parameters do not change upon mixing. As described under "Materials and Methods," exchange of recA protein between the complexes and the free pool was monitored by employing ^{35}S-labeled recA protein for solution 1 and unlabeled recA protein for

solution 2, or vice versa. Any labeled recA protein which dissociates completely from the complexes should be diluted in the pool of unlabeled free recA protein. Reactions were again halted by the addition of ATPγS to a final concentration of 0.2 mM at various times after mixing solutions 1 and 2. Controls to determine the extent of exchange at $t = 0$ are described under "Materials and Methods."

As shown in Fig. 6, very little exchange was observed between recA·ssDNA complexes and the free recA protein pool. A modest level of exchange (≦5%) was usually observed in the $t = 0$ control. About 10% of the recA protein in the free pool generally migrated to the recA·ssDNA complexes in the first 10 min when labeled recA protein was employed in solution 2. No significant increase in this exchange was observed in subsequent time points collected 20 and 30 min after combining solutions 1 and 2. When labeled recA protein was employed in solution 1 in a parallel experiment, little or no migration of recA protein from complexes to the free pool was observed. In these experiments the proportion of labeled recA protein present in the free pool was comparable to that observed in the $t = 0$ controls even 30 min after combining solutions 1 and 2. The limited migration of recA protein from the free pool to complexes may reflect an uncharacterized aggregation of free recA protein with complexes. In any case, exchange of recA protein between free and complex-bound forms cannot account for the exchange of recA protein between complexes described above. We conclude that most or all of the observed exchange between complexes does not proceed via a free recA protein solution intermediate. Instead, most exchange between complexes must occur between adjacent complexes. Complete equilibration between free and complex-bound recA protein was never observed.

ATP Hydrolysis during Exchange Reactions—The rate of ATP hydrolysis was also measured in these experiments. The apparent turnover number (k_{cat}) for recA protein-promoted ATP hydrolysis under the set of conditions employed to observe exchange between complexes is 21.4 min^{-1} (average of 5 measurements). Under the conditions employed to observe exchange between free and complex-bound recA protein, the apparent k_{cat} for ATP hydrolysis was 12 min^{-1}. This reflects the fact that only one-half of the recA protein in these reactions has access to the ssDNA and again agrees well with other measurements.[2]

Given the rate of recA monomer exchange between complexes described above, approximately 200 ATP molecules are hydrolyzed for every observable transfer of recA monomer from one complex to another. While this estimate may be in error by a factor of 2–3 (due to the uncertainty in the exchange rate calculation), it is not in error by two orders of magnitude. The excess hydrolysis may indicate that exchange is not tightly coupled to ATP hydrolysis. Alternatively, the excess hydrolysis may reflect repeated dissociation and reassociation with the same complex.

We have not attempted to make the large extrapolations and assumptions necessary to compare ATP hydrolysis and exchange of recA protein between free and bound forms. The rate of exchange is at least an order of magnitude slower than that observed between complexes and this must be compared with the rate of ATP hydrolysis described above. In the 30 min following the combination of solutions 1 and 2, approximately 360 ATPs are hydrolyzed for every recA monomer in the reaction mixture. It is clear that a tight coupling does not exist between ATP hydrolysis and association or dissociation of recA protein if dissociation is taken to mean the production of recA monomers with complete diffusional freedom. The hydrolysis of thousands of ATPs must occur to produce the complete dissociation of 1 recA monomer from a recA·ssDNA complex formed in the presence of SSB under these conditions.

[2] S. Morrical, unpublished data.

DISCUSSION

Our principal conclusion is that recA protein does exchange between recA·ssDNA complexes even though it will not efficiently migrate from a complex to a free molecule of ssDNA. This exchange does not generally proceed via a free recA protein intermediate, but instead usually involves adjacent complexes. Since the rate of ATP hydrolysis exceeds the rate of observable exchange by two orders of magnitude, either the exchange is not coupled to ATP hydrolysis or rapid exchange (impossible to detect in these experiments) is occurring within single complexes. Every complex involves more than 1000 recA monomers, and the latter possibility does not seem unreasonable since a hypothetical intracomplex exchange should be much more efficient than the intercomplex exchange we are measuring. We speculate that a rapid rearrangement of recA monomers coupled to ATP hydrolysis may be occurring within these complexes. The same reaction would permit exchange of recA monomers between different complexes at times when two complexes are adjacent. Complete dissociation of recA protein from a complex appears to be a rare event under these conditions.

While the functional significance of such an exchange within a complex is unclear in the context of a DNA strand exchange reaction, these results provide arguments against several models currently under consideration. Particularly important in this discussion is the role of ATP hydrolysis in recA protein-promoted reactions. It has been suggested (13, 15, 18) that ATP hydrolysis is coupled to association/dissociation of recA protein from filamentous complexes formed on DNA, and that this in turn is coupled in some way to DNA strand exchange. In these experiments, the rate of ATP hydrolysis exceeds the rate of exchange between free and bound forms of recA protein by at least three orders of magnitude. Complete dissociation of recA protein from a complex is therefore not a required feature of any step of the ATP hydrolytic cycle. This in turn implies that treadmilling is not occurring in these complexes. All quantitative treatments of treadmilling in tubulin or actin filaments (34–38) are based, in part, on the implicit assumption that dissociation of a monomer from a filament results in equilibration with an existing pool of free monomer. In the case of recA protein, measurable exchange occurs only between adjacent complexes. recA protein-promoted ATP hydrolysis is, therefore, not coupled to treadmilling under these conditions. If a modified form of treadmilling occurs in recA complexes, some mechanism exists to keep most of the migrating recA protein within the complex. We have postulated above that an ATP-driven rearrangement of recA protein occurs within a complex. Whatever form this may take, it does not appear to be treadmilling in the classical sense.

Even though the conditions used in these experiments are optimal for recA protein-promoted DNA strand exchange, we cannot yet directly extrapolate our results to the complete strand exchange reaction. The possibility exists that rapid dissociation and reassociation of recA protein might occur when homologous duplex DNA is added to these complexes and DNA strand exchange is allowed to proceed. If we assume this does not occur, then the results constitute evidence against models for DNA strand exchange which involve treadmilling of recA monomers in a complex or any other form of association/dissociation coupled to ATP hydrolysis.

Several additional characteristics of the reaction must be better understood before the significance of this exchange reaction can be properly evaluated. We do not know whether the exchange involves recA monomers at the ends of complexes or whether every recA monomer in the complex has an equal probability of exchange. Indeed, it is not clear that stable complexes formed on circular ssDNA molecules have distinct ends. Electron micrographs of such complexes generally reveal a uniform filamentous structure which coats the entire circle (39, 40). Recent evidence indicates that ATP hydrolysis is not restricted to recA monomers located at the ends of filamentous complexes.[3]

The coupling of ATP hydrolysis to any other molecular event which could begin to explain the overall process of recA protein-promoted branch migration remains to be demonstrated. A coupling of ATP hydrolysis to an undefined rearrangement of recA protein within a complex is only one of many possibilities. If our rationalization of these results is correct, the measurable rate of exchange of recA monomers between complexes and the apparent efficiency of this exchange in terms of ATP hydrolyzed should increase as a function of the concentration of complexes in the solution. Given the limitations encountered in these experiments, this is a prediction we cannot yet test quantitatively.

The properties of stable complexes revealed in these experiments are consistent with those defined by other methods (18, 41). The stoichiometry of recA protein is 1 monomer/4 nucleotides of ssDNA, which is similar to previous reports. The amount of SSB required to produce detectable complexes (1/20 nucleotides) is also consistent with other measurements (5). SSB has not been detected in these complexes in experiments similar to ours (3), but studies utilizing spectroscopic methods indicate it remains bound to the complex (5). The lack of detection in sedimentation experiments indicates it may not be bound to the complex tightly enough to survive centrifugation.

A secondary aspect of this work is the further characterization of one effect of SSB on recA protein complexed to ssDNA. SSB is required simply to observe recA·ssDNA complexes in these experiments. This implies that SSB affects the stability of the complexes in some manner. In principle, this could reflect the removal of secondary structure in ssDNA as suggested elsewhere (7). A complex in which the ssDNA is completely coated with recA protein may be intrinsically more stable than the shorter recA filaments which might form between regions of secondary structure in the absence of SSB. The amount of recA protein incorporated into recA·ssDNA complexes in these experiments, however, was linearly dependent upon the concentration of SSB present until a saturating level was reached (at 1 SSB monomer/20 nucleotides) which is sufficient to saturate the ssDNA with SSB under these conditions (5). If SSB acted only to eliminate secondary structure in ssDNA and was then displaced by recA protein, the reaction might be expected to exhibit a less stringent requirement for lower, catalytic levels of SSB. Recent evidence indicates that SSB remains continuously associated with recA·ssDNA complexes under some conditions (5) and suggests that SSB may directly enhance the cooperative interactions in the complex. Taken together, these results suggest that the role of SSB is not limited to the removal of ssDNA secondary structure. More work is required to fully elucidate the mechanism by which SSB enhances the stability of recA·ssDNA complexes and the efficiency with which they react.

[3] Brenner *et al.*, unpublished observations.

REFERENCES

1. Cox, M. M., and Lehman, I. R. (1982) *J. Biol. Chem.* **257,** 8523–8532
2. Radding, C. M., Flory, J., Wu, A., Kahn, R., DasGupta, C., Gonda, D., Bianchi, M., and Tsang, S. S. (1982) *Cold Spring Harbor Symp. Quant. Biol.* **47,** 821–828
3. Soltis, D. A., and Lehman, I. R. (1983) *J. Biol. Chem.* **258,** 6073–6077
4. Cox, M. M., Soltis, D. A., Livneh, A., and Lehman, I. R. (1983) *J. Biol. Chem.* **258,** 2577–2585
5. Morrical, S. W., Lee, J., and Cox, M. M. (1986) *Biochemistry* **25,** 1482–1494
6. Kahn, R., and Radding, C. M. (1984) *J. Biol. Chem.* **259,** 7495–7503
7. Muniyappa, K., Shaner, S. L., Tsang, S. S., and Radding, C. M. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 2757–2761
8. Griffith, J. D., Harris, L. E., and Register, J., III (1984) *Cold Spring Harbor Symp. Quant. Biol.* **49,** 553–559
9. Cox, M. M., Soltis, D. A., Lehman, I. R., DeBrosse, C., and Benkovic, S. J. (1983) *J. Biol. Chem.* **258,** 2586–2592
10. Glassberg, J., Meyer, R. R., and Kornberg, A. (1979) *J. Bacteriol.* **140,** 14–19
11. Whittier, R. F., and Chase, J. W. (1981) *Mol. Gen. Genet.* **183,** 341–347
12. Whittier, R. F., and Chase, J. W. (1983) *Mol. Gen. Genet.* **190,** 101–111
13. Cox, M. M., Morrical, S. W., and Neuendorf, S. K. (1984) *Cold Spring Harbor Symp. Quant. Biol.* **49,** 525–533
14. Cleveland, D. N. (1982) *Cell* **28,** 689–691
15. Howard-Flanders, P., West, S. C., and Stasiak, A. (1984) *Nature* **309,** 215–219
16. Register, J. C., and Griffith, J. (1985) *J. Biol. Chem.* **260,** 12308–12312
17. Weinstock, G. M., McEntee, K., and Lehman, I. R. (1981) *J. Biol. Chem.* **256,** 8845–8849
18. Menetsky, J. P., and Kowalczykowski, S. C. (1985) *J. Mol. Biol.* **181,** 281–295
19. West, S. C., Cassuto, E., Mursalim, J., and Howard-Flanders, P. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 2569–2573
20. Newport, J. W., Lanberg, N., Kowalczykowski, S. C., and Von Hippel, P. H. (1981) *J. Mol. Biol.* **145,** 105–121
21. Kaguni, J., LaVerne, L. S., and Ray, D. S. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 6250–6254
22. Cox, M. M., McEntee, K., and Lehman, I. R. (1981) *J. Biol. Chem.* **256,** 4676–4678
23. Weiner, J. H., Bertsch, L. L., and Kornberg, A. (1975) *J. Biol. Chem.* **250,** 1972–1980
24. Cox, M. M., and Lehman, I. R. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 3433–3437
25. Pagano, J. S., and Hutchinson, C. A., III (1971) *Methods Virol.* **V,** 79–123
26. Craig, N. L., and Roberts, J. W. (1981) *J. Biol. Chem.* **256,** 8039–8044
27. Lohman, T. M., and Overman, L. B. (1985) *J. Biol. Chem.* **260,** 3594–3603
28. Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., and Candia, O. A. (1979) *Anal. Biochem.* **100,** 95–97
29. Weinstock, G. M., McEntee, K., and Lehman, I. R. (1981) *J. Biol. Chem.* **256,** 8829–8834
30. Snyder, L. R. (1972) *J. Chromatogr. Sci.* **10,** 200–212
31. Weinstock, G. M., McEntee, K., and Lehman, I. R. (1981) *J. Biol. Chem.* **256,** 8850–8855
32. Bryant, F. R., Taylor, A. R., and Lehman, I. R. (1985) *J. Biol. Chem.* **260,** 1196–1202
33. Jencks, W. P. (1982) *Adv. Enzymol. Relat. Areas Mol. Biol.* **51,** 75–106
34. Bergen, L. G., and Borisy, G. G. (1980) *J. Cell Biol.* **84,** 141–150
35. Caplow, M., Langford, G. M., and Zeeberg, B. (1982) *J. Biol. Chem.* **257,** 15012–15021
36. Wegner, A. (1982) *J. Mol. Biol.* **161,** 607–615
37. Brenner, S. L., and Korn, E. D. (1983) *J. Biol. Chem.* **258,** 5013–5020
38. Bonder, E. M., Fishkind, D. J., and Mooseker, M. S. (1983) *Cell* **34,** 491–501
39. Dunn, K., Chrysogelos, S., and Griffith, J. (1982) *Cell* **28,** 757–765
40. Flory, J., and Radding, C. M. (1982) *Cell* **28,** 747–756
41. Morrical, S. W., and Cox, M. M. (1985) *Biochemistry* **24,** 760–767