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Assembly and Disassembly of RecA Protein Filaments Occur at Opposite Filament Ends

RELATIONSHIP TO DNA STRAND EXCHANGE*

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RecA protein primarily associates with and dissociates from opposite ends of nucleoprotein filaments formed on linear duplex DNA. RecA nucleoprotein filaments that are hydrolyzing ATP therefore engage in a dynamic process under some conditions that has some of the properties of treadmilling. We have also investigated whether the net polymerization of recA protein at one end of the filament and/or a net depolymerization at the other end drives unidirectional strand exchange. There is no demonstrable correlation between recA protein association/dissociation and the strand exchange reaction. RecA protein-mediated DNA strand exchange is affected minimally by changes in reaction conditions (dilution, pH shift, or addition of small amounts of adenosine-5′-*O*-(3-thiotriphosphate) that have large and demonstrable effects on recA protein association, dissociation, or both. Rather than driving strand exchange, these assembly and disassembly processes may simply represent the mechanism by which recA nucleoprotein filaments are recycled in the cell.

The recA protein of *Escherichia coli* promotes key steps in homologous recombination and recombinational DNA repair (for reviews, see Cox and Lehman, 1987; Kowalczykowski, 1987; Griffith and Harris, 1988; Radding, 1988). These processes are mimicked *in vitro* by recA protein-mediated DNA strand exchange reactions. The well-characterized three strand exchange reaction (Cox and Lehman, 1981a), utilizing circular ssDNA[1] and homologous linear duplex DNA derived from bacteriophages, involves the transfer of the complementary strand of the duplex to the ssDNA, resulting in a FII DNA product. Strand exchange requires stoichiometric amounts of purified recA protein (Shibata *et al.*, 1979; Cox and Lehman, 1981a, 1982; Weinstock *et al.*, 1981; West *et al.*, 1981a; Cox *et al.*, 1983). The active form of recA protein in these reactions is a nucleoprotein filament. There are three experimentally distinguishable steps in the strand exchange reaction: 1) recA protein binding to the ssDNA to form a nucleoprotein filament stabilized by SSB; 2) alignment of homologous DNA sequences between the ssDNA and the FIII DNA; and 3) transfer of the complementary strand of the duplex to the ssDNA as ATP is hydrolyzed. This third step is simply recA-mediated branch migration, and it proceeds unidirectionally, 5′ → 3′ relative to the ssDNA (Kahn *et al.*, 1981; Cox and Lehman, 1981b; West *et al.*, 1981b). In this paper we report on experiments done to test one hypothesis for the mechanism by which the recA nucleoprotein filament uses the energy of ATP hydrolysis to drive unidirectional branch migration.

Much is already known about the recA nucleoprotein filament. One recA protein monomer binds every three to four nucleotides or base pairs (for reviews see Cox and Lehman, 1987; Griffith and Harris, 1988; Radding, 1988). RecA protein binding in the presence of ATP or ATPγS extends ssDNA or dsDNA by approximately 50% (DiCapua *et al.*, 1982; Stasiak and DiCapua, 1982) and underwinds the DNA by approximately 40% relative to B form (Stasiak and DiCapua, 1982; Pugh *et al.*, 1989). RecA protein polymerizes in the 5′ to 3′ direction on ssDNA (Register and Griffith, 1985), which is one manifestation of the inherent polarity of the filament structure (Stasiak *et al.*, 1988). RecA protein filaments formed on dsDNA also have a clear axial polarity (Egelman and Stasiak, 1986).

Whereas binding to ssDNA is rapid, the binding of recA protein onto dsDNA at or above pH 7.0 is very slow due to a rate-limiting nucleation step (Pugh and Cox, 1987a, 1988; Kowalczykowski *et al.*, 1987). A ssDNA gap or tail provides a nucleation site and greatly stimulates the rate of recA protein binding to duplex DNA. RecA protein binds the two strands of the duplex asymmetrically; nuclease protection experiments have shown that recA protein protects the strand to which it initiated binding (*i.e.* the strand in the gap, referred to here as the initiating strand) more than it protects the complementary strand (Chow *et al.*, 1986, 1988; Lindsley and Cox, 1989). A 5′ tail stimulates the average rate of binding 5–10-fold more than a 3′ tail (Shaner and Radding, 1987; Shaner *et al.*, 1987; Lindsley and Cox, 1989). This is consistent with the addition of recA monomers primarily to the 3′ end of the filament, with respect to the initiating strand.

Association and/or dissociation of protein from filament ends has been suggested previously to explain the mechanism of action of recA protein (Cox *et al.*, 1983; Stasiak *et al.*, 1984; Howard-Flanders *et al.*, 1984; Cox *et al.*, 1984; Neuendorf and

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; FI DNA, supercoiled closed circular form of DNA as isolated from *E. coli* cells; FII DNA, the nicked circular form of the same molecule; FII DNA, the linear form of the same molecule; ATPγS, adenosine-5′-*O*-(3-thiotriphosphate); SDS, sodium dodecyl sulfate; bp, base pairs; MES, 4-morpholineethanesulfonic acid; SSB, Single-stranded DNA-binding protein.

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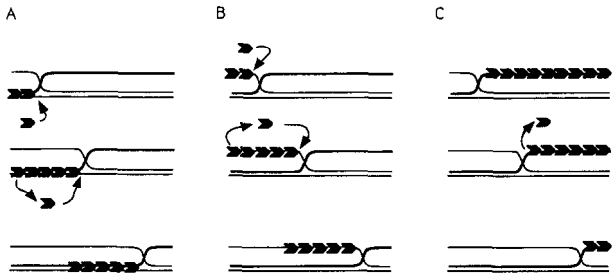


FIG. 1. **Three ways to couple directional branch migration to association/dissociation.** *A*, filament growth on the heteroduplex DNA. *B*, filament growth on the displaced single strand. *C*, dissociation of the filament in front of the branch point. RecA protein is represented by → and dsDNA is represented by the *parallel lines*.

Cox, 1986; Register *et al.*, 1987) and the analogous uvsX protein of bacteriophage T4 (Kodadek *et al.*, 1988). A clear link between association/dissociation and strand exchange has not been established, however. The stability of the filament directly correlates with the efficiency of strand exchange (Morrical and Cox, 1990; Cox and Lehman, 1987). ATP is hydrolyzed throughout the filament, with no detectable enhancement of hydrolysis at filament ends or at the branch point during strand exchange (Brenner *et al.*, 1987; Schutte and Cox, 1987; Kowalczykowski and Krupp, 1987). ATP enhances the affinity of recA protein for DNA, whereas ADP acts to lower the intrinsic binding constant (Menetski and Kowalczykowski, 1985). However, there is no direct evidence that recA protein dissociates from the DNA with each hydrolytic event. On the contrary, little exchange between a nucleoprotein filament formed on circular ssDNA and a free recA protein pool is detected in the presence of SSB and an ATP regenerating system (Neuendorf and Cox, 1986). When the ADP/ATP ratio is allowed to increase to 0.6–1.5 (depending on reaction conditions) the entire recA nucleoprotein filament dissociates cooperatively (Cox *et al.*, 1983).[2]

In the presence of ATP, an ATP regenerating system, and SSB, recA protein appears to bind stably to circular single-stranded and duplex DNA in a manner that is pH independent in the range 5.5–8.5. However, if the DNA is linearized, net protein dissociation can be measured (Shaner and Radding, 1987; Lindsley and Cox, 1989). Dissociation from linear ssDNA has been noted only in the presence of SSB.[3] Dissociation occurs primarily from one end of a linear nucleoprotein filament, but it is not clear which end is involved (Lindsley and Cox, 1989).

RecA protein preferentially binds to one end of the nucleoprotein filament (the 3′ end with respect to the initiating DNA strand). A reasonable scenario is that dissociation is primarily from the opposite end. Directional association and dissociation of recA monomers at filament ends potentially could drive polar branch migration coupled to ATP hydrolysis. This activity is similar to the "treadmilling" activity of actin and tubulin. RecA protein association and/or dissociation could drive branch migration by any of several mechanisms, three of which are diagrammed in Fig. 1. From the theory developed for treadmilling of actin and tubulin filaments (Wegner, 1976; Cleveland, 1982), we can make several testable predictions to determine if recA protein drives branch migration by a similar mechanism. 1) The filament should have both structural and kinetic polarity; the ends should be functionally distinguishable and the rates of assembly should differ at each end. 2) Head to tail polymerization, or a net polymerization at one end of a filament and depolymerization at the other, should be detectable. These polymerization and/or depolymerization rates should be comparable to the rate of strand exchange. 3) In the case of a true treadmilling reaction, an energy input is required for dissimilar equilibrium constants (for subunit addition) at the two ends of the filament. 4) There should be an opening in the filament near the branch point for polymerization and/or depolymerization to occur.

Predictions 1, 3, and 4 have been previously addressed to some extent and that work will be summarized in the Discussion. The focus of this paper is prediction 2. We show that recA protein primarily associates with and dissociates from opposite ends of a nucleoprotein filament on dsDNA. The presence of a 5′ tail stabilizes the linear nucleoprotein filament substantially better than a 3′ tail. This indicates that dissociation, like association, also occurs primarily in the 5′ to 3′ direction. To a first approximation, recA monomers add to the 3′ end and dissociate from the 5′ end (relative to the initiating strand) of the filament, suggesting a dynamic process with at least some properties of treadmilling. The results extend and refine the molecular interpretation of a series of related observations made by Shaner *et al.* (1987), Shaner and Radding (1987), and Lindsley and Cox (1989). However, reaction conditions that are known to affect association or dissociation of recA protein are shown to have little or no effect on recA protein-promoted branch migration. A model consistent with these results, using end-dependent dissociation as a way to recycle recA protein, and not as the driving force of branch migration, is presented.

MATERIALS AND METHODS

Reagents

E. coli recA protein was purified and stored as previously described (Cox *et al.*, 1981). The recA protein concentration was determined by absorbance at 280 nm, using a extinction coefficient of ϵ_{280} = 0.59 A_{280} mg^{-1} ml (Craig and Roberts, 1981). *E. coli* SSB protein was purified as described (Lohman *et al.*, 1986). The concentration of the SSB stock solutions was determined by absorbance at 280 nm, using an extinction coefficient of ϵ_{280} = 1.5 A_{280} mg^{-1} ml (Lohman and Overman, 1985). Restriction endonucleases were purchased from New England Biolabs. Pyruvate kinase, lactate dehydrogenase, NADH, DNase I, and ATP were purchased from Sigma. DNA grade hydroxylapatite was purchased from Bio-Rad Laboratories. MES buffer was from Research Organics Inc. ATPγS and Tris buffer were purchased from Boehringer Mannheim. The purity of the ATPγS was checked by thin layer chromatography (Shibata *et al.*, 1981) and was shown to be at least 90% pure, with the major contaminant being ADP.

The bacteriophage M13mp8.198 is the bacteriophage M13mp8 with a 198-bp sequence (*Rsa*I-*Rsa*I fragment from the *E. coli gal*T gene) inserted at the *Sma*I site. The plasmid pJEL3 (8036 bp) was developed for a separate set of experiments and contains sequences from: pBR322 (5052 bp; Bolivar *et al.*, 1977), the 2-μm plasmid of *Saccharomyces cerevisiae* (1599 bp), the *gal*T gene of *E. coli* (1041 bp), the kanamycin resistance gene from the transposon Tn 903 (268 bp; Oka *et al.*, 1981), and the φ10 promoter from bacteriophage T7 (76 bp; Tabor and Richardson, 1985). The DNA from pJEL3 was completely heterologous with the DNA from bacteriophage M13mp8 and was used here primarily as random sequence duplex DNA in heterologous controls for DNA pairing experiments.

Single-stranded and circular duplex DNA from bacteriophage M13mp8 (7229 bp; Messing, 1983) and M13mp8.198 were prepared using methods described (Neuendorf and Cox, 1986). Plasmid DNA was prepared as described (Davis *et al.*, 1980). Uniformly tritiated ssDNA was prepared by an established procedure (Julin *et al.*, 1986) and had a specific activity of 2–5 Ci mol^{-1}. The concentrations of ssDNA and dsDNA stock solutions were determined by absorbance at 260 nm, using 36 and 50 μg ml^{-1} A_{260}^{-1}, respectively, as conversion factors. DNA concentrations are expressed as total nucleotides. FII DNA was prepared from FI DNA using DNase I and ethidium bromide (Shibata *et al.*, 1981). This method produces 90–95% FII DNA, with a single random nick, and 5–10% FIII DNA. FIII DNA

[2] J. W. Lee and M. M. Cox, unpublished observations.
[3] J. E. Lindsley and M. M. Cox, unpublished observations.

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was derived from FI stocks by complete digestion with *Eco*RI or another restriction enzyme where noted. After digestion, residual protein was removed by extraction 1:1 sequentially with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), followed by ethanol precipitation.

Preparation of Gapped and Tailed Duplex DNA

Circular duplex DNA with a precisely defined gap was prepared as described (Lindsley and Cox, 1989). Briefly, FIII M13mp8 linearized with *Sma*I was denatured. To construct the gapped substrate used in this study (except for experiments in Fig. 2), the minus strand was annealed to a 15–20-fold excess of circular single-stranded M13mp8.198 DNA (the viral plus strand). The gapped duplex DNA that was formed was purified away from the remaining ssDNA on a small hydroxylapatite column. The resulting gapped duplex DNA was at least 90% pure, with the only contaminant being FIII M13mp8. Approximately 100 μg of gapped DNA was produced from each preparation. This gapped duplex was circular, with 7229 bp of duplex DNA and 198 nucleotides of ssDNA (see Fig. 4*b*). Tailed dsDNA molecules were made by digesting gapped duplexes with restriction enzymes that cut uniquely near the gap. The *Eco*RI site is 12 bp to the 5′ side of the gap and the *Hin*dIII site is 20 bp to the 3′ side of the gap, both relative to the (+) strand. Cleavage by either enzyme effectively results in a duplex DNA molecule with a ssDNA tail of 198 nucleotides at one end or the other, as shown in Fig. 4*b*. The cut DNA was extracted and ethanol precipitated, as previously described. The DNA was fully cut as judged by agarose gel electrophoresis. The concentration of the gapped duplex DNA was determined by absorption at 260 nm using the conversion factor for duplex DNA. No correction was introduced into the concentration measurement for the 198 nucleotide single-stranded region because it represented less than 3% of the total length of the DNA. The gapped duplex substrates used in Fig. 2 have been described previously (Lindsley and Cox, 1989) and they were prepared essentially as described above using appropriate circular ssDNAs.

Reaction Conditions

All reactions were carried out in 25 mM buffer (Tris acetate, 80% cation, pH 7.5, unless otherwise indicated), 10 mM $Mg(acetate)_2$, 5% glycerol, 1 mM dithiothreitol, 3 mM ATP, and an ATP regenerating system (4.5 units ml^{-1} pyruvate kinase, 3 mM phosphoenolpyruvate, and 3 mM K-glutamate). ATPase assays also included 4.5 units ml^{-1} lactate dehydrogenase and 1 or 3 mM NADH, as indicated for each experiment. DNA and recA protein concentrations are indicated for each experiment. Reactions were incubated at 37 °C for 10 min before ATP (and SSB, for strand exchange reactions) was added to start the reactions. All reactions were carried out at 37 °C. Strand exchange reactions followed by the S1 nuclease assay included ^{3}H-ssDNA and FIII DNA (both derived from bacteriophage M13mp8, except when FIII pJEL3 DNA was used as a heterologous control). ATPase reactions often had only ssDNA or duplex DNA as a cofactor; see figure legends for each experiment. For pH shift reactions, the reaction was diluted 1:1 at the indicated time with a buffer of appropriate pH (described in text) to give the desired final pH. The pH shift buffer included 10 mM $Mg(acetate)_2$, 5% glycerol, 1 mM dithiothreitol, 3 mM ATP, and ATP regenerating system, such that only the recA protein, the DNA and, where applicable, the SSB were diluted. The same was true for dilution experiments, except that the initial and final buffer was Tris acetate (80% cation, pH 7.5) and the dilution varied from 5- to 100-fold as indicated.

DNA-dependent ATPase Assay—This assay was used as a measure of recA protein binding to DNA. A Perkin-Elmer Lambda 7 double-beam recording spectrophotometer equipped with two thermojacketed cuvette holders, each capable of holding six cuvettes, was used for absorbance measurements. The cell path length and the band pass were 0.5 cm and 2 nm, respectively. The coupled assay used to measure ATP hydrolysis was previously described (Morrical *et al.*, 1986). The regeneration of ATP from ADP and phosphoenolpyruvate with the oxidation of NADH can be followed by the decrease in absorbance at 340 or 380 nm. Absorbances were measured at 380 nm, instead of 340 nm (the absorbance maximum for NADH), to remain within the linear range of the spectrophotometer when 3 mM NADH was used. NADH was used at 3 mM when the DNA and recA protein concentrations were above 5 and 0.8 μM, respectively, to insure that all reactions could reach a steady-state level of ATP hydrolysis before the NADH was exhausted. NADH extinction coefficients of ϵ_{340} = 6.23 mM^{-1} cm^{-1} and ϵ_{380} = 1.21 mM^{-1} cm^{-1} were used to calculate the rates of ATP hydrolysis. An increase in the concentration of ATP or any of the coupling system components did not change the observed ATPase rate; the data obtained reflect the true initial velocity of ATP hydrolysis at all times.

Linearization of Gapped Duplex DNA during ATPase (DNA-binding) Assays—During some ATPase assays a restriction endonuclease was added to linearize the gapped duplex DNA within the recA protein-DNA complexes. As a control, restriction endonuclease storage buffer (50% glycerol, 50 mM Tris acetate (80% cation, pH 7.5) and 150 mM NaCl) was added to a second cuvette containing an identical reaction. The rate of ATP hydrolysis occurring in these reaction mixtures was followed as described above. To follow the rate of linearization, aliquots (10 μl) of the reaction mixture were removed from the cuvettes at various time points, added to 5 μl of GED/SDS (25% glycerol, 5 mM EDTA, 0.005% bromphenol blue, 5% SDS), and electrophoresed overnight in a 0.8% agarose gel at 2–2.5 V cm^{-1}. The percentage of gapped duplex DNA linearized to tailed duplex DNA was determined by scanning photographic negatives of the gels stained in ethidium bromide.

S1 Nuclease Assay—This assay measures the conversion of ^{3}H M13mp8 ssDNA into an S1 nuclease resistant form (*i.e.* heteroduplex DNA). The assay was described in detail previously (Cox and Lehman, 1982). Basically, 50-μl aliquots of a reaction were removed at each time point, mixed with 4 μl of 10% SDS, and stored briefly on ice. S1 nuclease (83 Sigma units) in 620 μl of digestion buffer (500 mM NaCl, 50 mM sodium acetate, 50% cation, pH 4.6, 1 mM zinc acetate, and 32 μg/ml of heat-denatured calf thymus DNA) was incubated with each aliquot for 30 min at 37 °C. Reactions were stopped by addition of carrier DNA (40 μl of heat-denatured calf thymus DNA at 1 mg/ml) and 700 μl of cold 12% trichloroacetic acid. After at least 1 h on ice, the reaction mixtures were filtered through Whatman GF/B filters at a constant rate (1,270 mm Hg below atmospheric pressure; ~−67,966 Pa) on a 10-place filter holder equipped with individual Teflon valves (Hoeffer model FH 224V). Filters were rinsed with 3 ml of cold 12% trichloroacetic acid and 2 ml of 100% ethanol. Filters were then dried and the percentage of DNA bound to each was determined by liquid scintillation counting (Beckman LS 3801). To determine the total acid precipitable counts, 2 aliquots were treated the same as indicated above, except that no S1 nuclease was added. These two values were averaged to be the 100% value. The counts observed in the zero time point aliquot (taken prior to adding ATP and SSB) were always less than 5% of the 100% value and were subtracted from all data and the 100% control prior to calculating the fraction of ^{3}H-labeled ssDNA converted to heteroduplex.

Gel Assay of Strand Exchange

Aliquots (10 μl) of strand exchange reactions described above were removed at each time point and stopped by the addition of 5 μl of GED/SDS. These aliquots were stored on ice until after the last time point was taken. Samples were electrophoresed overnight in a 0.8% agarose gel at 2–2.5 V cm^{-1}. The percentage of DNA that had completed strand exchange to form FII DNA product was determined by scanning photographic negatives of the gels stained in ethidium bromide. The 100% value was defined as the amount of FIII DNA in the zero time point lane. The densitometric scans were performed on a Zeineh Soft Laser scanning densitometer, SL-504-XL, from Biomed Instruments, Inc.

For dilution reactions, the gel assay was performed slightly differently. Four strand exchange reactions were started in parallel under standard conditions; three were then diluted after the reaction was initiated. The method used (described below) was chosen such that both the total amount of DNA and final volumes of the reaction aliquots from each time point would be consistent among reactions. The glycerol concentration in the reaction mixtures was reduced to 2% to allow for the extensive volume reduction required by the sample preparation (lyophilization) described below. This reduction in glycerol concentration had no detectable effect on strand exchange. The initial volume of each reaction was 50 μl. Before the reactions were initiated with SSB and ATP, 5 μl was removed from each as a zero time point. This 5-μl aliquot was added to 495 μl of reaction buffer (25 mM Tris acetate at pH 7.5, 10 mM $Mg(acetate)_2$, 2% glycerol, 1 mM dithiothreitol, and 3 mM ATP) and immediately placed in liquid nitrogen. Four min after the reactions were initiated, 1.086 ml, 2.205 ml, or 4.455 ml of dilution buffer (reaction buffer plus ATP regenerating system) were added to 25-, 50-, or 100-fold dilution reactions, respectively. For each time point after dilution the volume of the

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aliquot removed was equal to 5 μl times the dilution factor. The aliquots were immediately added to enough reaction buffer to equal a total volume of 500 μl and frozen in liquid nitrogen. The aliquots were kept frozen in liquid nitrogen until all of the reactions were complete. They were then lyophilized to reduce the volume to about 10 μl. To each 10 μl sample, 10 μl of gel loading solution (5% SDS, 5 mM EDTA, 0.005% bromphenol blue) was added. The samples were loaded into an agarose gel and electrophoresed as described above.

RESULTS

The rate of DNA-dependent ATP hydrolysis catalyzed by the recA protein was used as a quantitative measure of recA protein bound to DNA. With the duplex DNA substrates used here, binding measurements derived from measurements of ATP hydrolysis correlate very well to binding estimates obtained by light scattering experiments, DNase protection, restriction enzyme protection, and DNA underwinding (Pugh and Cox, 1987a, 1987b; Lindsley and Cox, 1989). Binding of recA protein to duplex DNA is characterized by an intrinsic k_{cat} (rate of ATP hydrolysis/bound monomer) of 21–25 min^{-1}, assuming a binding site size of 1 recA monomer/4 bp. This k_{cat} varies little between pH 6 and 8.3 and over other conditions used in this work (Pugh and Cox, 1987a; Lindsley and Cox, 1989). The site size measured in recA protein titrations of gapped duplex DNAs is typically 1 recA/3.5–3.8 bp (Fig. 2). The percentage of DNA bound by recA protein at a given time can be estimated by dividing the rate of ATP hydrolysis at that time by the rate observed at saturation. The rate that reflects saturation is generally determined at pH 6.2, where binding to duplex DNA is optimal and stable, or in experiments such as that in Fig. 2. As noted previously (Pugh and Cox, 1988) recA protein binds slowly (on a timescale of hours) to fully duplex DNA at pH 7.5, and even more slowly at pH 8.1. In most of the experiments described below, recA protein is present in 1.5–2-fold excess relative to available DNA-binding sites (somewhat less if the binding site size is assumed to be 3 rather than 4).

The results presented here are divided into two sections: (*a*) presents results that demonstrate dissociation of recA nucleoprotein filaments from a unique filament end, and (*b*) presents experiments that examine the potential role of association and/or dissociation in the unidirectional strand exchange reaction promoted by recA protein.

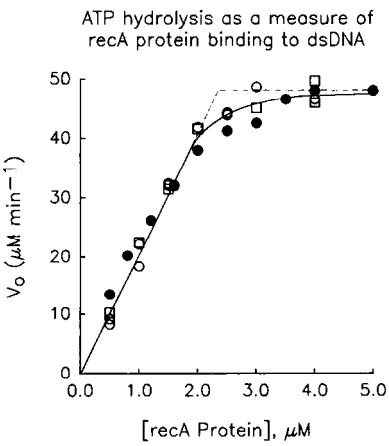


FIG. 2. **RecA protein titration of gapped duplex DNA.** Reactions were carried out at pH 7.5 as described under "Materials and Methods." Gapped duplex DNAs with gaps of 34 (○——○), 50 (□——□), or 1041 (●——●) nucleotides of ssDNA were present at 16, 16, and 15 μM, respectively. In each case the concentration of potential recA protein-binding sites is 2 μM if a site size of four nucleotides or 4 base pairs is assumed. The intersection of the asymptotic lines drawn through the vertical and horizontal portions of this curve give an apparent binding stoichiometry of 1 recA monomer/3.6 base pairs and an apparent k_{cat} of 22 min^{-1}.

(a) RecA Protein Binds Primarily at One End of the Nucleoprotein Filament Formed on Duplex DNA, and Dissociates Primarily from the Opposite End

As previously mentioned, recA protein binds primarily to the 3′ end of nucleoprotein filaments formed on duplex DNA, relative to the initiating strand. It binds stably to circular dsDNA but dissociates primarily from one end of filaments formed on linear duplex DNA at neutral pH. The experiments in this section were designed to determine from which end of the filament dissociation occurs. A ssDNA tail should prevent or slow recA protein dissociation from linear duplex DNA only if the tail is at the end of the filament from which dissociation occurs. RecA nucleoprotein filaments are oriented 5′ to 3′ along the initiating strand (Lindsley and Cox, 1989). If dissociation from a nucleoprotein filament occurs in the 5′ to 3′ direction, then a 5′ ssDNA tail should effectively block this process. Likewise, a 3′ ssDNA tail should block dissociation from the 3′ end of the filament (see Fig. 4*a*).

Parallel measurements of recA protein association with gapped duplex, 5′ ssDNA tailed duplex, 3′ ssDNA tailed duplex, FIII DNA and FII DNA, as measured by the rate of ATP hydrolysis, are shown in Fig. 3, *A* and *B*, for pH 7.5 and 8.1, respectively. The DNA and recA protein concentrations were 8 and 2 μM, respectively. The DNA ends of FIII DNA provided significant stimulation of binding as compared with FII DNA. However, recA protein remained stably bound to the FII DNA after overcoming the initial kinetic barrier but slowly dissociated from the FIII DNA. A ssDNA gap of as small as 24 nucleotides greatly enhanced the rate of recA protein binding to duplex DNA.[3] Optimal stimulation of binding is observed when the gap size is at least 50 nucleotides. The gap or tail size used in all of the remaining experiments in this report was 198 nucleotides. The binding lag time was decreased from >4 h (for FII DNA) to 1.5–2.5 min for the

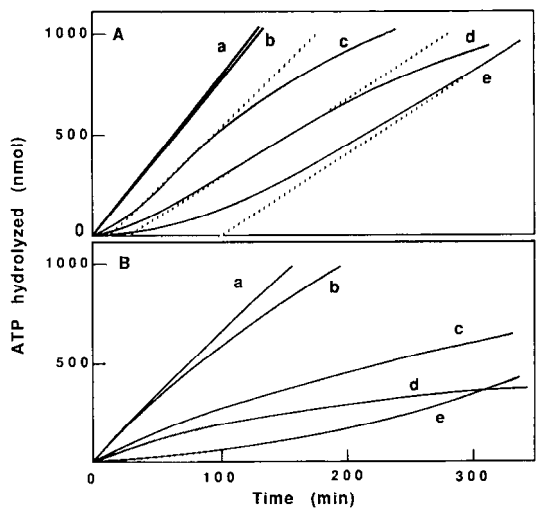


FIG. 3. **ATP hydrolysis as a function of time: gapped, tailed, and fully duplex DNA as cofactors.** Reactions were carried out as described under "Materials and Methods" at pH 7.5 (Tris acetate (80% cation)) (*A*) and pH 8.1 (Tris acetate (50% cation)) (*B*). The DNA cofactors used were gapped duplex (*a*), 5′ ssDNA-tailed duplex (*b*), 3′ ssDNA-tailed duplex (*c*), FIII (*d*), and FII DNA (*e*). The duplex portion of all DNA molecules was derived from M13mp8 (7229 bp) and the ssDNA gap or tail was 198 nucleotides from the *galT* gene of *E. coli*. See "Materials and Methods" for preparation of DNA substrates. In each reaction the DNA concentration was 8 μM, and the recA protein concentration was 2 μM. The *broken lines* were drawn at points of maximum velocity.

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gapped duplex DNA. As judged by the steady-state rate of ATP hydrolysis, recA protein remained stably bound to the gapped duplex throughout the duration of the experiment. In Fig. 3*A*, pH 7.5, the binding curve for 5′-tailed duplex as a cofactor was indistinguishable from that of the gapped duplex. The observed steady-state rate of ATP hydrolysis corresponded to that expected when the DNA is saturated with recA protein (v_0 = 23 μM min^{-1}). Assuming a binding stoichiometry of 1 recA monomer/4 bp, half of the available recA protein is bound at saturation. When 3′-tailed duplex DNA was used as a cofactor, recA protein nearly saturated the DNA lattice, with a binding lag of 13–15 min. After this point the rate of ATP hydrolysis began to decrease, reflecting a net dissociation of the protein. At 3 h, only about half of the available DNA-binding sites on the 3′-tailed duplex still had protein bound.

The curves for 3′-tailed duplex (*c*), linear duplex (*d*), and FII DNA (*e*) in Fig. 3*A* deserve further comment. In these experiments the rate of ATP hydrolysis never reached a level that would indicate saturation of the DNA. The lag in the progress curves reflects the slow nucleation of binding, and previous work indicates that a full-length filament is present very soon after each successful nucleation (Pugh and Cox, 1987a). The lags then reflect a slow accumulation of full-length or nearly full-length filaments. The effect of ends seen in the FIII:FII comparison (*curves d* and *e*) may indicate that transient strand separation or "breathing" at DNA ends facilitates nucleation at ends. As more and more filaments are formed, the rate of binding necessarily decreases due to depletion of both free recA protein and available nucleation sites (especially ends). In curves c and d, a dissociation process becomes evident before the binding is complete. As demonstrated previously (Lindsley and Cox, 1989), this dissociation occurs from one end. If all linear filaments dissociate at a constant rate, the net dissociation will increase with the total number of filaments formed. The sigmoidal shape of these curves evidently reflects a predictable change from net binding to net dissociation as the number of filaments increases. The rate of ATP hydrolysis never reaches zero (Lindsley and Cox, 1989 and data not shown). A low steady state is reached where association and dissociation are in balance. The final steady state must reflect many factors including the concentration of free recA protein and nucleation sites and the rate of dissociation. In the case of circular duplexes (*curve e*), the rate of ATP hydrolysis continues to increase even at the end of the experiment. The circular filaments formed on this DNA are stable (Lindsley and Cox, 1989; Pugh and Cox, 1987a) and the experiment is simply not carried out long enough for binding to reach completion.

The differences between DNA cofactors can be seen more clearly at pH 8.1, the pH at which the highest rates of net recA protein dissociation have been measured (Fig. 3*B*). As expected, recA protein bound more slowly to the FIII and FII DNA at the higher pH but again appeared to remain stably bound only to the FII DNA. RecA protein again binds very rapidly to the gapped duplex and the 5′-tailed duplex DNA. The binding lag for both was the same at pH 7.5 and 8.1; however, the final rate of ATP hydrolysis was approximately 15% lower at pH 8.1. This slower rate could be due to either a slightly lower k_{cat} value, reduced binding to contaminating FIII DNA at pH 8.1, or both. The protein remained stably bound to the circular gapped duplex DNA; the small amount of dissociation that was observed at late times in the reaction can be accounted for by dissociation from the contaminating FIII DNA in the gapped duplex preparation. Somewhat more recA protein dissociated from the 5′-tailed duplex than from the gapped duplex substrate at this elevated pH. After 3 h, approximately 30% of the recA protein had dissociated from the 5′-tailed duplex. When 3′-tailed duplex DNA was used as a cofactor much less of the DNA was bound and dissociation was much faster. Fifteen min after the reaction was started, an average of 40% of the DNA was saturated with recA protein. This value decayed to 20% by 3 h. From this experiment, it is impossible to distinguish whether the elevated pH decreased the rate of recA protein association onto, or increased the rate of dissociation from, the 3′-tailed duplex substrate. The next experiment was designed to answer that question.

To further compare the stability differences of recA nucleoprotein filaments arranged on the various duplex DNA forms, ATPase reactions that had reached steady state at pH 6.27 were shifted to pH 8.1. The final concentrations after the pH shift were 8 μM DNA and 2 μM recA protein. As judged by the rates of ATP hydrolysis prior to the pH shift, all DNA substrates were equally bound with recA protein (data not shown). At pH 6.27 the binding lag time for gapped duplex and 5′-tailed duplex DNA cofactors was still 1–2 min. The binding lag time for 3′-tailed duplex, FIII and FII reactions was approximately 4, 5, and 5 min, respectively (data not shown). After the shift to pH 8.1, recA protein dissociated from the FIII DNA with a dissociation lag time of 15 min and with an average of 12% of the DNA bound by recA protein at the final steady state (Fig. 4). The dissociation lag time is defined as the average length of time for half of the population of nucleoprotein filaments to dissociate to the final steady-state level. This agrees well with similar reactions described previously (Lindsley and Cox, 1989). RecA protein dissociated from the 3′-tailed duplex with a dissociation lag time of 18 min and an average of 20% of the DNA bound by recA protein at steady state. The dissociation of recA nucleoprotein filaments formed on linear duplex and 3′-tailed duplex substrates was very similar. A 5′ ssDNA tail provided significantly more stability to the linear nucleoprotein filament than the 3′ tail. After the pH shift, a dissociation lag time could not be accurately measured since the reaction never reached steady state, but it was >90 min. The ATPase rate at 3 h after the pH was shifted indicates that approximately 40% of the 5′-tailed duplex was still coated with recA protein. A small amount of recA protein dissociation occurred from the gapped duplex and FII DNA reactions, but again this can be ac-

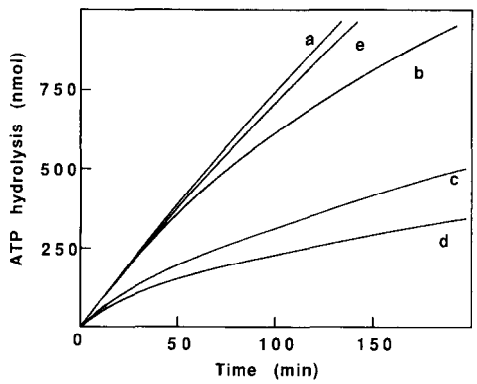


FIG. 4. **pH shift of ATPase reaction: gapped, tailed, and fully duplex DNA as cofactors.** ATPase reactions were started at pH 6.27 (MES/NaOH (56% anion)). After 15 min (time = 0 in the figure), 200 μl of each reaction was added to 200 μl of buffer (Tris acetate (20% cation, pH 8.95)) to give a final pH of 8.1. The DNA cofactors used were gapped duplex (*a*), 5′ ssDNA-tailed duplex (*b*), 3′ ssDNA-tailed duplex (*c*), FIII (*d*), and FII DNA (*e*). The final concentrations were 8 μM DNA and 2 μM recA protein.

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counted for by the 5–10% of contaminating FIII DNA in each preparation.

Assuming that recA protein binds in the 5′ to 3′ direction along the initiating strand of duplex DNA, there are two potential orientations for the protein filament along the tailed DNA (Fig. 5*a*). DNase protection experiments have shown that recA protein binds asymmetrically onto 5′-tailed duplex DNA; recA protein protects the tailed strand of 5′-tailed duplex DNA about twice as well as its complement (Lindsley and Cox, 1989). The DNase protection results, along with the ATPase results described earlier, indicate that the majority of recA protein filaments formed on 5′-tailed duplex DNA are oriented as shown in Fig. 5*a* (*i*). In this orientation the ssDNA tail provides significant stability to the filament. This asymmetry of binding is lost when 3′-tailed duplex DNA is used; recA protein shields the two strands approximately equally from DNase digestion (Lindsley and Cox, 1989). RecA protein may therefore bind the 3′-tailed duplex DNA in either orientation (Fig. 5*a* (*iii*) and (*iv*)). The ssDNA tail does not provide significant stability to that end of the filament, regardless of filament orientation.

When recA protein binds to circular gapped duplex DNA, the majority of the filaments are oriented along the strand that is single-stranded in the gap (Fig. 5*b*). If the recA protein-gapped duplex DNA complexes are then linearized with a restriction endonuclease, the protein filaments will be oriented primarily in one direction along the tailed DNA. This provides an additional method to examine the polarity of dissociation of recA protein from tailed duplex DNA molecules. For technical reasons only 3′ ssDNA tailed duplexes could be made by this procedure; *Eco*RI would not cut the recA protein-coated gapped duplex DNA. Two identical ATPase reactions with 2 μM recA protein and 12 μM GD_{198} were performed at pH 7.5. Twenty min after ATP was added to initiate recA protein binding, recA protein had saturated the gapped duplex DNA as indicated by the steady-state rate of ATP hydrolysis (Fig. 6). At this time, 4 μl of buffer (*cuvette 1*) or 4 μl of *Hin*dIII (80 units, *cuvette 2*) were added. The rate of ATP hydrolysis continued unperturbed for the reaction with only buffer added. Half of the gapped duplex DNA in cuvette 2 was linearized by the *Hin*dIII within 5 min; all of the DNA was linearized within 30 min. The rate of ATP hydrolysis in cuvette 2 began to noticeably drop 20 min after the *Hin*dIII was added. By the end of the reaction, the ATPase rate had dropped to below 50% of the initial rate, and was still dropping. This experiment demonstrated that even when the recA protein filaments were oriented along the strand with the ssDNA tail, a 3′ ssDNA tail could not prevent recA protein dissociation.

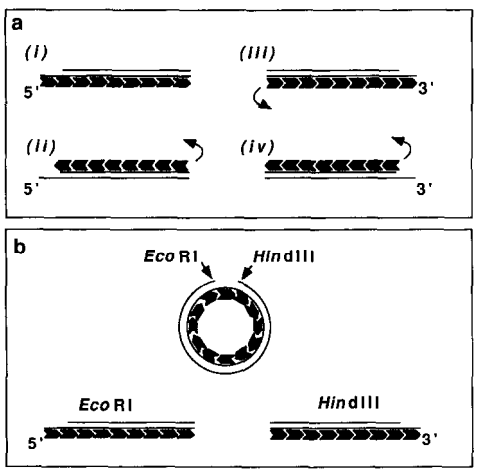


FIG. 5. **Schematic of recA protein filaments formed on gapped and tailed duplex DNA substrates.** Gapped and tailed duplex DNA was prepared as described under "Materials and Methods." *a*, recA protein filaments can align in either of two orientations on tailed duplex DNA. On 5′-tailed duplex DNA, orientation (*i*) is predominant, whereas on 3′-tailed duplex DNA both orientations appear to occur. The *arrows* indicate (−) or 5′ ends of filaments that are not stabilized by ssDNA tails and therefore are expected to dissociate. *b*, the recA nucleoprotein filaments align primarily in one orientation on circular gapped duplex DNA. Therefore, if the gapped duplex DNA is linearized *after* recA protein has bound, the protein filaments will primarily be aligned in one orientation relative to the DNA. The length of the gap is drawn approximately to scale, relative to the total DNA length. The recA protein filament is not drawn to scale.

The experiments from Figs. 3, 4, and 6 show that recA nucleoprotein filaments are polar with respect to both binding and dissociation. RecA protein binds more rapidly onto 5′-tailed duplexes than onto 3′-tailed duplexes; binding occurs primarily 5′ to 3′ with respect to the initiating strand. A 3′ ssDNA tail provided little stability to the linear nucleoprotein filaments. A 5′ ssDNA tail provided significantly more stability to the filament. This result indicates that dissociation also primarily occurs 5′ to 3′, with respect to the initiating strand. Therefore, polymerization occurs more rapidly at the 3′ end and depolymerization occurs more rapidly at the 5′ end of recA nucleoprotein filaments.

(b) Reaction Conditions That Affect the Association and/or Dissociation of RecA Protein Have Little or No Effect on Branch Migration

Is the association and dissociation that occurs at filament ends the driving force for recA protein-promoted strand exchange? This question was examined by determining the effect on strand exchange of three procedures that are known to alter the association or dissociation of recA protein to or from DNA: (i) dilution, (ii) pH shift, and (iii) addition of small quantities of ATPγS to ongoing strand exchange reactions. Dilution of the free recA protein concentration decreases the rate of recA protein binding (Chabbert *et al.*, 1987) and the amount of recA protein bound to the DNA lattice at steady state (Morrical *et al.*, 1986). A shift in pH from 6.8 to 8.0 increases the rate of net recA protein dissociation from linear nucleoprotein filaments at least 10-fold (Lindsley and Cox, 1989). Small quantities (<10 μM) of ATPγS, added along with ATP, prevent recA protein dissociation, while allowing

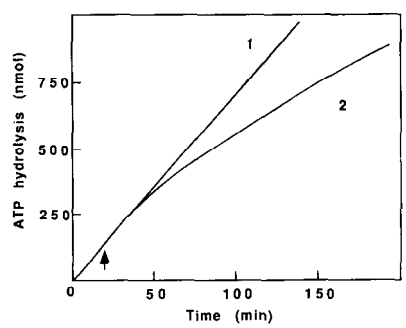


FIG. 6. **Linearization of gapped duplex DNA in recA protein-DNA complexes: ATP hydrolysis.** ATPase reactions were carried out as described under "Materials and Methods" except that sodium acetate was added to the reaction to a final concentration of 50 mM to aid in restriction enzyme digestion. Twenty min after the reaction was initiated by the addition of ATP, 4 μl of *Hin*dIII storage buffer (50% glycerol, 50 mM Tris acetate (80% cation, pH 7.5), and 150 mM NaCl) was added to *cuvette 1* and 4 μl of the restriction endonuclease *Hin*dIII (80 units) was added to *cuvette 2*. The GD_{198} DNA concentration was 12 μM, and the recA protein concentration was 2 μM.

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most ATP hydrolysis to continue (Lindsley and Cox, 1989). If recA protein binding and/or dissociation are required for branch migration, then the rate or extent of branch migration should be affected by one or more of these three variations. In each case a change in the appropriate reaction condition was made 4 min after ATP and SSB were added simultaneously to initiate strand exchange. This time was chosen such that the pairing phase of the reaction would be minimally affected by the change and so that branch migration would still be in an early stage. Only 10–15% of the input DNA had been converted to heteroduplex at this time, as judged by the S1 nuclease assay, and product FII DNA molecules were not observed before 15 min. The effects of dilution and ATPγS addition were also compared for recA nucleoprotein filaments involved in strand exchange and those not involved. The effect of pH shifts on recA filaments has already been documented (Lindsley and Cox, 1989).

(i) Dilution—The free recA protein concentration is known to affect the binding of recA protein to single-stranded and double-stranded DNA. Using ATP hydrolysis as a measure of recA protein binding to ssDNA, Morrical *et al.* (1986) and Kowalczykowski and Krupp (1987) found that the steady-state level of binding decreased below 1.2 and 0.4 μM recA protein, respectively. SSB was included in both experiments. Using a fluorescent single-stranded polynucleotide, for which recA protein has a higher affinity than natural ssDNA, Chabbert *et al.* (1987) showed that the rate of recA protein binding decreased below a protein concentration of 0.4 μM. As determined by ATP hydrolysis, the optimum recA protein concentration for binding to gapped duplex DNA is above 0.6 μM.[3] Therefore, dilution of the recA protein concentration below 0.4–1.2 μM will affect its binding to both ssDNA and dsDNA.

The effect of dilution on ongoing strand exchange reactions is shown in Fig. 7. The dilution buffer contained ATP, ATP regenerating system, glycerol, Mg acetate, dithrothreitol, and Tris acetate so that only the DNA and protein components of the reaction were diluted. The initial protein and DNA concentrations were 8 μM recA protein, 2.4 μM SSB, 24 μM ssDNA, and 48 μM FIII DNA. The strand exchange reaction is completely homology dependent; ^{3}H-ssDNA remained S1 sensitive when the FIII DNA was heterologous (Fig. 7*a*). Dilution of the strand exchange reaction by 5– or 10-fold, to recA protein concentrations of 1.6 or 0.8 μM, respectively, had no detectable effect on the reaction as measured by the S1 nuclease assay (Fig. 7*a*). For technical reasons, a 10-fold dilution was the maximum dilution possible for reactions followed by the S1 nuclease assay. Reactions diluted to a greater extent were followed by gel assay (Fig. 7*b*). Again at 4 min, strand exchange reactions were diluted: 1) not at all (8 μM recA), 2) 25-fold (0.32 μM recA), 3) 50-fold (0.16 μM recA), 4) 100-fold (0.08 μM recA), and 5) 100-fold with recA protein added back to a total final concentration of 0.8 μM. Dilution up to and including 50-fold had little effect on the strand exchange reaction. However, a 100-fold dilution of the reaction largely inhibited the formation of the FII product of complete strand exchange. The reaction was not inhibited if recA protein is supplemented back to 0.8 μM at the time of dilution. Therefore, it was the recA protein concentration change that causes the decreased strand exchange in the 100-fold dilution reaction. There was no clear correlation between binding and rates of strand exchange. The strand exchange reaction was insensitive to dilution of the recA protein below concentrations known to affect recA binding, until the concentration was diluted to 0.08 μM.

The ATPase assay was used to determine if and when recA protein dissociated from the DNA upon dilution of the strand

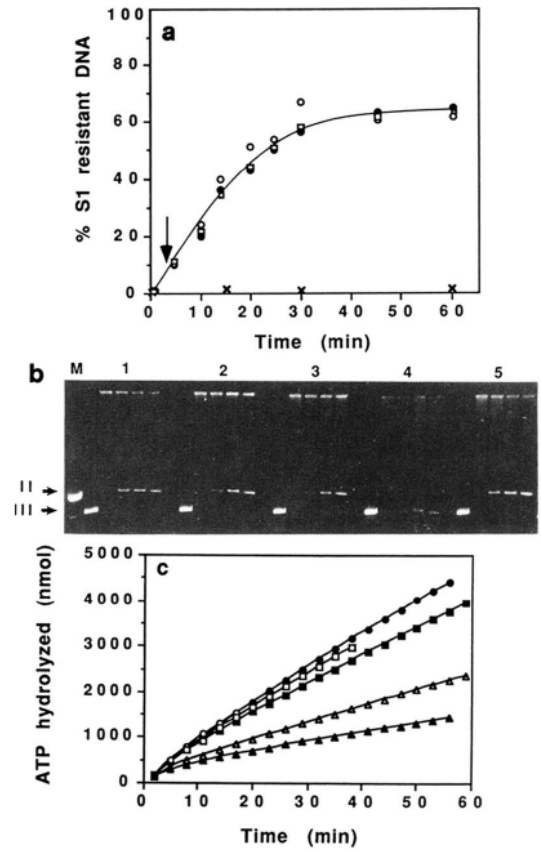


FIG. 7. **Effect of dilution on ongoing recA protein promoted strand exchange.** Strand exchange reactions were performed as described under "Materials and Methods." The initial concentration of reactants was 8 μM recA protein, 24 μM ssDNA, 48 μM FIII DNA, 2.4 μM SSB, and 3 mM ATP. *a*, the S1 nuclease assay was used to follow the progress of strand exchange; ^{3}H-ssDNA (derived from M13mp8) becomes resistant to S1 nuclease as it is incorporated into heteroduplex DNA. Reactions were diluted 10-fold (□——□), 5-fold (●——●), or not at all (○——○) 4 min after they were started, as indicated by the *arrow*. FIII pJEL3 DNA was used for a heterologous control (×——×). *b*, strand exchange reactions were followed by gel assay as described under "Materials and Methods." The time points at which aliquots of the reaction were stopped were 0, 15, 30, 45, and 60 min. Four min after the reactions were initiated, they were diluted not at all (*1*), 25-fold (*2*), 50-fold (*3*), 100-fold (*4*), and 100-fold (*5*), with recA protein added back to a final total concentration of 0.8 μM. A FII DNA was run in the first lane as a marker for the final product of the strand exchange reaction. *c*, the effect of dilution on recA binding to DNA:ATPase. Strand exchange reactions were performed under the same conditions as *a* and *b*. Four min after the reactions were initiated they were diluted: not at all (○——○); 5-fold (●——●); 10-fold (□——□); 25-fold (■——■); 50-fold (△——△); and 100-fold (▲——▲). The time course of ATP hydrolysis was followed from the time of dilution as described under "Materials and Methods."

exchange reaction (Fig. 7*c*). The strand exchange reactions were performed under the same conditions as in Fig. 7*a* and *b*, except that the reactions were in cuvettes. Aliquots from a single starting reaction were diluted 4 min after initiation; the amount of ATP hydrolyzed was followed from the point of dilution. The resulting rates of ATP hydrolysis were corrected for the dilution factor. For example, for reactions diluted 50-fold, the amount of ATP hydrolyzed at each time point was multiplied by 50. In this way, changes in the amount of recA protein bound to the DNA with time could be directly compared between the various dilution assays. The rate of ATP hydrolysis for the non-diluted strand exchange reaction decreased slightly as the reaction progressed (Fig. 7*c*). This may be due to the conversion of ssDNA (to which recA protein

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is bound initially) to heteroduplex DNA (Pugh and Cox, 1987b); the k_{cat} for ATPase is 30% lower when dsDNA is the cofactor than when ssDNA is the cofactor (Schutte and Cox, 1987). The 5- and 10-fold dilutions had little effect on the ATPase rate; the 25-fold dilution caused a slight decrease. The rate of ATP hydrolysis dropped significantly more when the strand exchange reaction was diluted 50- or 100-fold (Fig. 7*c*). The drop was nearly complete within 5 min of the dilution, indicating that a majority of the recA nucleoprotein filaments rapidly dissociated upon dilution. Therefore, the inhibition of strand exchange upon 100-fold dilution (Fig. 7*b*) can be explained by the immediate dissociation of much of the recA protein in the filaments driving the reaction. The more limited dissociation in the other dilution experiments had no obvious effect on strand exchange.

(ii) pH Shift—A shift in pH has large effects on the rate and extent of net recA protein dissociation from duplex DNA (Lindsley and Cox, 1989; this work). The measured rate of net dissociation exhibits a strong, linear dependence on increasing pH from pH 6.8 to 8.1. If recA protein dissociation is essential for strand exchange, the pH of the reaction should affect the rate of strand exchange. Aliquots of a strand exchange reaction started at pH 6.8 were diluted 2-fold after 4 min with reaction buffers (at pH 6.8, 7.8, and 8.3), and the final pH values of the reactions were 6.8, 7.4, and 8.0. Fig. 8 shows the results of S1 nuclease assays used to follow the reactions. There was no detectable difference in the course of the reactions as determined by the S1 nuclease assay (or by gel assay, data not shown). The final pH did not affect the branch migration phase of recA protein-promoted strand exchange in the range of pH 6.8 to 8.0.

(iii) Addition of Small Quantities of ATPγS—The nonhydrolyzable ATP analogue, ATPγS, in high concentration (0.1–1.0 mM) completely blocks recA protein-catalyzed ATP hydrolysis and strand exchange. ATPγS in small quantities (1–10 μM, depending on the ATP concentration), added in addition to ATP, completely blocks the dissociation of recA protein from the ends of nucleoprotein filaments formed on duplex DNA, while allowing ATP hydrolysis to continue at near its maximal rate. At these low concentrations the ATPγS acts by arresting dissociation of the recA protein and not by promoting rebinding or providing a stable nucleation site (Lindsley and Cox, 1989). The amount of ATPγS needed to arrest the dissociation directly depends on the concentration of ATP in the reaction and not on the recA protein or DNA concentrations (Lindsley and Cox, 1989). The amount of ATPγS necessary to block the dissociation of recA protein from FIII DNA in the presence of 3 mM ATP was examined systematically (data not shown). The minimum amount of ATPγS required to completely block dissociation in the presence of 3 mM ATP was 3.5 μM (Fig. 9*a*). The rate of ATP hydrolysis was again used as a measure of how much recA protein was bound to the DNA at any given time, corrected for the small decrease in rate caused by competitive inhibition by the ATPγS observed at pH 6.27. The stability of recA nucleoprotein filaments formed at pH 6.27 on FIII DNA and shifted to pH 8.1 was compared for reactions with 3.5 μM, 7.0 μM, or no ATPγS. The ATPγS was added along with the pH shift buffer, at time = 0 in the figure. The final concentrations of other components in the reactions were 3 μM recA protein, 13 μM FIII DNA, and 3 mM ATP. Without ATPγS, the majority of the recA protein dissociated from the DNA within 30 min, as shown by the decrease in the rate of ATP hydrolysis. In the presence of 3.5 μM ATPγS, the initial ATPase rate was inhibited by 15%, but that rate was maintained for 1 h, indicating no dissociation. The 15% decrease in ATPase rate was caused by the ATPγS competitive inhibition, and not by recA protein dissociation, as previously determined (Lindsley and Cox, 1989). ATPγS added to a final concentration of 7.0 μM decreased the initial ATPase rate by 30% but also effectively prevented dissociation.

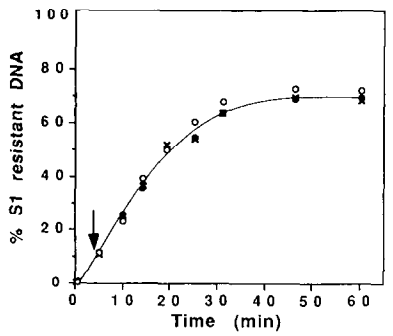


FIG. 8. **Effect of pH shift on ongoing recA protein-promoted strand exchange.** Strand exchange reactions were performed as described under "Materials and Methods" and followed using the S1 nuclease assay. Reactions were started at pH 6.85 (50% MES/NaOH (56% anion), 50% Tris acetate (70% cation)). After 4 min, reactions were diluted 1:1 with buffer to yield a final pH of: 6.85 (●——●); 7.45 (○——○); and 8.0 (×——×). The final concentrations were 4 μM recA protein, 12 μM ssDNA, 24 μM FIII DNA (both forms of DNA were derived from M13mp8), 1.2 μM SSB, and 3 mM ATP.

The effect of 3.5 or 7.0 μM ATPγS on the rate of ATP hydrolysis in strand exchange reactions is shown in Fig. 9*b*. ATPγS was added to two of the three reactions 4 min after the reactions were started by the addition of ATP and SSB. Along with the other components listed for the experiment whose results are shown in Fig. 9*a*, 7.5 μM ssDNA and 0.75 μM SSB were included. The rate of ATP hydrolysis remained constant throughout the reaction when no ATPγS was added. When ATPγS was added to a final concentration of 3.5 μM, the ATPase rate initially dropped but then remained steady at about 88% of the maximum rate. The drop in ATP hydrolysis caused by the added ATPγS was completed within 5 min. When 7 μM ATPγS was added to the reaction, the ATPase activity quickly dropped to about 70% of the maximum rate, then remained stable. These rate changes correspond well to changes in the rates of ATP hydrolysis obtained from reactions with only FIII DNA as a cofactor (Fig. 9*a*). The addition of ATPγS increases, rather than decreases, the DNase protection of the DNA (Pugh and Cox, 1987a). Therefore, the changes in ATP hydrolysis are attributed to competitive inhibition by ATPγS rather than recA protein dissociation.

When the S1 nuclease assay was used to measure the progress of branch migration in identical reaction mixtures, the effect of ATPγS was quite modest (Fig. 9*c*). Either 0, 3.5, or 7.0 μM ATPγS was again added to strand exchange reactions 4 min after initiation. The concentrations of other components in the reactions were 6 μM recA protein, 15 μM ^{3}H-ssDNA, 26 μM FIII DNA, 1.5 μM SSB, and 3 mM ATP. With no ATPγS present, the reaction proceeded with an initial rate of 260 bp min^{-1} of heteroduplex formation, to a final average of 74% of the ssDNA becoming S1 nuclease resistant. When ATPγS was added to a final concentration of 3.5 μM, the reaction proceeded with the same initial rate and to the same final extent (within error). Both the initial rate (200 bp min^{-1}) and the final extent of heteroduplex formation (52%) were decreased somewhat by 7.0 μM ATPγS. Therefore, as judged by the S1 nuclease assay, concentrations of ATPγS that were able to completely block the dissociation of recA protein from the ends of nucleoprotein filaments had little effect on the rate or extent of branch migration.

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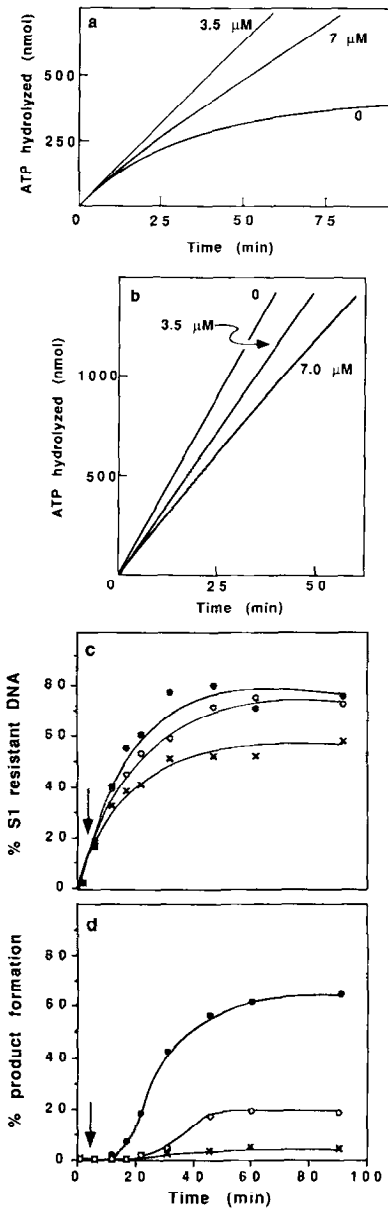


FIG. 9. **Effect of small amounts of ATPγS on recA protein-promoted strand exchange.** *a*, pH shift of ATPase reactions. ATPγS prevents dissociation of recA protein from linear duplex DNA. ATPase reactions were started at pH 6.27 (MES/NaOH (56% cation)). After 20 min (time = 0 in the figure), 200 μl of each was added to 200 μl of buffer (Tris acetate (30% cation)) to give a final pH of 8.1. Included in the pH shift buffer was 0, 7, or 14 μM ATPγS, to give final concentrations of 0, 3.5, and 7 μM ATPγS. The final concentrations of the other reaction components were 3 μM recA protein, 13 μM FIII DNA, and 3 mM ATP. *b*, ATP hydrolyzed as a function of time for strand exchange reactions. Reactions were performed as described under "Materials and Methods." Four min after the reactions were started 0, 3.5, or 7.0 μM ATPγS (final concentration) was added. The reactions included 7.5 μM ssDNA, and 0.75 μM SSB, with the concentrations of other reactants being the same as in part *a*. *c*, strand exchange assays performed as described under "Materials and Methods" were followed by the S1 nuclease assay. Four min after the reactions were started, 0 μM (●——●), 3.5 μM (○——○), or 7 μM (×——×) ATPγS (final concentration) was added. The concentrations of other reactants were 6 μM recA protein, 15 μM ^{3}H-ssDNA, 26 μM FIII, 1.5 μM SSB, and 3 mM ATP. *d*, aliquots were removed from strand exchange reactions described in *c*, stopped with GED/SDS and run on an agarose gel. The amount of FII DNA product was measured by scanning photographic negatives of the gel stained with ethidium bromide. A value of 100% would mean that 100% of the starting FIII DNA was converted to final FII DNA product.

Aliquots of the same reactions used in the S1 nuclease assay (Fig. 9*c*) were stopped with SDS and run through an agarose gel (Fig. 9*d*). A photographic negative of the gel was scanned to determine the amount of FII DNA product that was visible at each time point for the three reactions. The results are shown in Fig. 9*d*. In the absence of ATPγS, 63% of the starting FIII DNA was converted into FII heteroduplex DNA. With the addition of 3.5 or 7.0 μM ATPγS, the amount of FII DNA product dropped to 19 and 5%, respectively. About the same amount of FIII DNA in each of the reactions was paired with the ssDNA as seen by the disappearance of the FIII DNA and appearance of slowly migrating intermediate bands. These results taken together with those in Fig. 9*c* suggest that ATPγS in small quantities has an effect on the completion of the strand exchange reaction that is minimally reflected in measurements of net heteroduplex formation.

DISCUSSION

RecA protein binds primarily to one end and dissociates primarily from the opposite end of nucleoprotein filaments formed on duplex DNA. This suggests that under some conditions recA protein filaments, hydrolyzing ATP, may treadmill or undergo some similar process. The observed association and dissociation processes, however, do not drive recA protein-promoted strand exchange. Dilution of strand exchange reactions to recA protein concentrations (0.16–0.4 μM) that inhibit binding of free recA protein does not affect an ongoing reaction. A shift in pH from 6.8 to 8.0, which increases net recA protein dissociation from linear nucleoprotein filaments, has no effect on strand exchange. And finally, addition of small amounts of ATPγS blocks recA protein dissociation but has only modest effects on recA-mediated branch migration.

Experiments reported here indicate that dissociation of recA protein from nucleoprotein filaments (where it happens at all) occurs primarily at the 5′ end relative to the initiating strand. Shaner and Radding (1987) and Lindsley and Cox (1989) previously described the decay of ATP hydrolysis from linear duplexes explained by the present experiments. RecA protein binds to ssDNA rapidly but to duplex DNA very slowly at neutral pH. Filaments formed on linear duplex DNA are unstable. RecA protein forms a complete nucleoprotein filament on 5′ single-stranded DNA-tailed duplex at least five times more quickly than on 3′-tailed duplex. The nucleoprotein filament formed on the 5′-tailed duplex is essentially stable at pH 7.5, indicating that the ssDNA tail prevents net dissociation from the linear filament. At higher pH values, these filaments do slowly dissociate, indicating that they are not completely stabilized. Conversely, a 3′ ssDNA tail provides little stability for the filaments; recA protein dissociates from 3′-tailed duplexes almost as rapidly as from fully duplex linear DNA. A ssDNA tail only has a significant stabilizing effect if it is on the 5′ end of the filament. This indicates that recA protein dissociation is primarily from the 5′ end of the filament (relative to the initiating strand), whereas binding occurs primarily at the 3′ end.

An asymmetric polymer may inherently have different rates of association and dissociation at each end. However, unless the assembly process is coupled to a thermodynamically irreversible step it is constrained by the principle of microscopic reversibility to have equivalent equilibrium constants for subunit addition at the two ends. Wegner (1976) proposed that the irreversible step could be nucleoside triphosphate hydrolysis for actin filaments. If ATP or GTP hydrolysis is coupled to the assembly (or disassembly) of the polymer, then net association at one end and net dissociation at the other end

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is possible. Some portion of the energy derived from ATP hydrolysis by recA protein could potentially be used for treadmilling.

It should be emphasized, however, that we have almost certainly not observed a steady-state treadmilling reaction in the experiments reported here. The assembly processes we observe are rapid once nucleation occurs (Pugh and Cox, 1987a) and limited by the length of the DNA. Dissociation is relatively slow. It is therefore unlikely that any of the filaments on linear DNAs are ever treadmilling in the sense that assembly at one end is just balanced by disassembly at the other under these conditions. More work is clearly needed to demonstrate a classical treadmilling process, if indeed one occurs. The stability of the filaments on circular DNAs could be interpreted as representing incomplete and treadmilling filaments with the "head chasing the tail" indefinitely. While we cannot rule this out, the different rates of association and dissociation argue against it. We also note that in the case of recA filaments on circular ssDNA, recA monomers are not exchanged between the filaments and a free pool of recA protein (Neuendorf and Cox, 1986).

In most *in vitro* experiments done with recA protein, conditions are such that the protein completely coats a circular DNA. There is no evidence for filament ends and therefore no movement of the filament due to treadmilling has been identified. The calculated cooperativity constant for recA protein binding to ssDNA ($\omega = 10^4$, Takahashi, 1989) indicates that the average cluster size at half-saturation would be 300 nucleotides; electron microscopy studies showed an average cluster size of 600 nucleotides at subsaturating recA concentrations (Dunn *et al.*, 1982; Flory and Radding, 1982). When recA protein binding to nicked circular duplex DNA was studied by a ligation assay, no DNA molecules with recA protein partially bound were identified (Pugh and Cox, 1987a). This suggests that the cooperativity constant for recA protein binding to duplex DNA may also be high. Although treadmilling by recA protein has not been detected previously in *in vitro* reactions, it might be important in the cell where the filament must have ends. The uninduced *E. coli* cell has approximately 1300 recA monomers or a recA protein concentration of about 2–3 μM (Salles and Paoletti, 1983). Within this concentration range, we have shown that recA protein will preferentially bind to the 3′ end of a filament (relative to the initiating strand) and dissociate from the 5′ end. This net polymerization at one end and depolymerization at the other would result in a net directional movement of the filament. This process could recycle the protein, allowing strand exchange to proceed farther than one unit length of the nucleoprotein filament (see Fig. 10).

The final three sets of experiments reported here were carried out in the expectation that a link between treadmilling and strand exchange should be manifested by a demonstrable correlation between rates of strand exchange and rates of the association/dissociation processes we are measuring. If association of recA protein from the bulk solution drives branch migration, then diluting the free recA protein concentration should decrease the rate of the reaction accordingly. Branch migration was negatively affected in the 100-fold dilution experiments, but this dilution was much greater than that required to affect recA protein binding to DNA. The other dilution experiments indicate that DNA strand exchange reactions are relatively unimpeded by dilution to final recA protein concentrations that have significant effects on nucleoprotein filament formation.

The branch migration phase of the strand exchange reaction is also not tightly coupled to recA protein dissociation. A

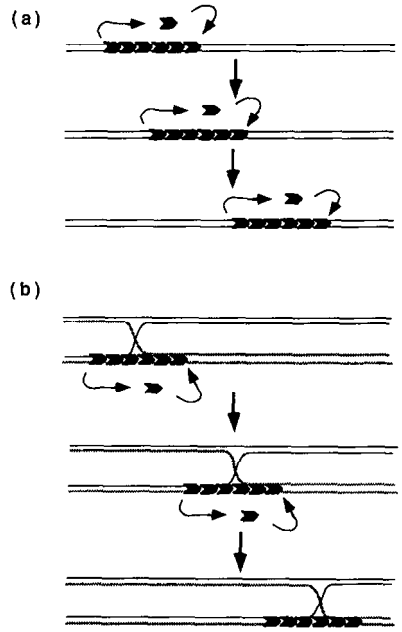


FIG. 10. **Models.** *a*, treadmilling of a short recA nucleoprotein filament along duplex DNA. *b*, recA protein-promoted branch migration driven by a filament surrounding the branch point. Treadmilling moves the nucleoprotein filament in the same direction as branch migration. Unidirectional branch migration is driven by an unknown process, separate from treadmilling.

pH shift that enhances the rate of net recA protein dissociation more than 10-fold (and presumably should affect any microscopic dissociation process operative at the branch point) has no effect on branch migration. The rate and final extent of strand exchange is constant over the pH range 6.8–8.1.

Finally, a low concentration of ATPγS that blocks recA protein dissociation does not significantly affect branch migration. This again indicates that dissociation of the recA nucleoprotein filament is not required for substantial branch migration to proceed. However, formation of the final FII DNA product is inhibited to a degree by the small concentration of ATPγS. There are several possible explanations for the differences seen in these two assays. 1) ATPγS only inhibits the final separation of the FII DNA and linear ssDNA products. 2) Branch migration is inhibited if a critical number of contiguous recA monomers are bound to ATPγS. The block in FII product formation could be readily explained if branch migration were halted by such a continuous tract, and the probability of forming the necessary tract would increase with ATPγS concentration. The timing of the strand exchange reaction might be such that only near the end of the reaction do enough ATPγS molecules bind to contiguous recA monomers to block branch migration. It is important to note, however, that ATPγS blocks the dissociation of recA protein from linear filaments and slightly decreases the observed rate of ATP hydrolysis in these strand exchange reactions within 5 min of its addition. Therefore, the lack of inhibition of branch migration is not simply due to slow binding of ATPγS. 3) Another possibility is that the S1 nuclease assay is not a true measure of branch migration and heteroduplex formation. Any DNA structure formed that is resistant to S1 nuclease digestion will be measured as heteroduplex DNA. If in fact a three-stranded DNA intermediate is formed that is resistant to S1 nuclease, formation of this intermediate, instead of heteroduplex DNA, could potentially be measured by the assay. However, SDS is added to remove the proteins before S1 nuclease is added. This hypothetical non-hetero-

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duplex, S1 nuclease-resistant intermediate would have to be extensive and stable during the 30-min S1 nuclease incubation period. Since no stable, extensive, random sequence triple-stranded DNA structures have yet been described, we prefer explanation 1 or 2.

These experiments all indicate the absence of a coupling between recA association/dissociation and strand exchange, but none are definitive on their own. It can be argued, for example, that the end-dependent dissociation from linear duplex DNA we are measuring here (affected by ATPγS and pH) is not the same process as a microscopic dissociation that might be postulated to occur at the branch point. This data, however, reinforces a lengthening series of experiments indicating that a link between strand exchange and association or dissociation does not exist. The analysis of classical treadmilling systems is based, in part, on the implicit assumption that once a protein monomer dissociates, it will be in equilibrium with an existing pool of free monomers. Using this criteria, recA protein filaments formed on circular ssDNA in the presence of ATP and SSB do not exhibit treadmilling (Neuendorf and Cox, 1986). Similar exchange experiments with DNA substrates undergoing strand exchange have not yet been performed. In addition, if association or dissociation of recA monomers were driving branch migration, one would expect an opening at or near the branch point where polymerization and/or depolymerization could occur and possibly a higher level of ATPase activity at the branch point. An extension of the filament through the branch point is indicated by DNase protection experiments of three and four strand exchange reactions (Chow *et al.*, 1986; Chow *et al.*, 1988). The branch point and surrounding DNA (at least within 50 base pairs) were equally protected from DNase digestion. When SSB is present, the rate of recA-mediated ATP hydrolysis remains constant during strand exchange and long after the reaction is complete (Schutte and Cox, 1987), and the heteroduplex product remains extensively underwound long after the reaction is complete (Pugh and Cox, 1987b). These two results provide unambiguous evidence for continued recA protein binding to the product heteroduplex and indicate that no net dissociation of recA is required for strand exchange. The recA nucleoprotein filament hydrolyzes ATP throughout the filament, with no enhancement of hydrolysis at filament ends (Brenner *et al.*, 1987). The rate of ATP hydrolysis is also unaffected by the number of branch points in a reaction (Brenner *et al.*, 1987). As indicated above, recA-mediated ATP hydrolysis is not tightly coupled to an exchange of recA protein between free and bound forms (Neuendorf and Cox, 1986). Together these results provide a compelling case against the idea that recA protein association or dissociation at the branch point (Fig. 1) is the driving force for unidirectional branch migration. The only evidence for a dissociative process comes from some studies in which strand exchange was examined by electron microscopy (Stasiak and Egelman, 1988; Register *et al.*, 1987; Stasiak *et al.*, 1984). In each case recA protein was not bound to heteroduplex product DNA and dissociation was indicated. These studies, however, directly conflict in some cases with unambiguous solution measurements cited above (continued ATP hydrolysis and underwinding of the heteroduplex DNA) that indicate recA remains bound to heteroduplex at least when SSB is present. The discrepancy could reflect an artifact arising from the documented (Shaner *et al.*, 1987) difficulty in preserving recA·dsDNA complexes during spreading when ATP is present.

A model showing recA protein association/dissociation distant from the branch point is shown in Fig. 10. In this model, a treadmilling-like process recycles the recA protein, allowing branch migration to extend further than a unit length of the protein filament. The driving force for branch migration would come from within the filament, possibly by one of two mechanisms described in detail elsewhere (Howard-Flanders *et al.*, 1984; Cox, 1989). In the cell, a true treadmilling process may occur on the *E. coli* chromosome, when filament assembly ties up most of the recA and further assembly is limited by the free recA made available by the slow dissociation process.

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