Quantitative RecA Protein Binding to the Hybrid Duplex Product of DNA Strand Exchange†

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ABSTRACT: Following a DNA strand exchange reaction, RecA protein remains bound to the hybrid DNA product. DNA strand exchange reactions were carried out under optimal conditions in the presence of both RecA protein and SSB protein. As monitored by a sensitive DNA underwinding assay, all of the RecA protein present in the RecA nucleoprotein filament that initiates the strand exchange reaction can be accounted for on the hybrid DNA. As shown elsewhere, the SSB is bound to the displaced single DNA strand. Previous studies showed that RecA protein will dissociate from dsDNA when ADP levels build up, or transfer from dsDNA to ssDNA when the latter is not bound by SSB. The present work (done with ATP regeneration and SSB) shows that efficient strand exchange occurs in the absence of a net dissociation or transfer of RecA monomers from the filament. Such a dissociation or transfer is therefore not a mechanistic requirement for DNA strand exchange. The results provide evidence against some models proposed for the DNA strand exchange mechanism.

In a bacterial cell, the RecA protein is required for recombinational DNA repair, homologous genetic recombination, chromosome partitioning at cell division, and the induction of the S.O.S. response to heavy DNA damage (Little, 1991; Zyskind *et al.*, 1992; Clark & Sandler, 1994; Kowalczykowski *et al.*, 1994). In vitro, the RecA protein promotes a set of DNA strand exchange reactions that provide experimental models for its repair and recombination functions. The mechanism of RecA-mediated DNA strand exchange is the focus of research efforts in many laboratories (Clark & Sandler, 1994; Cox, 1994; Kowalczykowski *et al.*, 1994; Roca & Cox, 1990; West, 1992).

The most common in vitro model system is the three-strand exchange reaction illustrated in Figure 1A. The DNA substrates for this reaction are usually derived from bacteriophage DNAs such as ϕX174 or M13. Several characteristics of the reaction mechanism have been established. The active species in the reaction is a RecA nucleoprotein helical filament, which forms on the single-stranded substrate as the first step in the reaction and contains one RecA monomer per three nucleotides of DNA. RecA protein has a DNA-dependent ATPase activity, and ATP is hydrolyzed uniformly throughout this filament. A homologous linear duplex DNA is aligned with the single-stranded DNA (ssDNA)[1] in the filament, and a limited strand exchange occurs in a reaction phase that does not require ATP hydrolysis (Menetski *et al.*, 1990; Rosselli & Stasiak, 1990; Rehrauer & Kowalczykowski, 1993; Jain *et al.*, 1994; Shan *et al.*, 1995; Bedale & Cox, 1995). The nascent hybrid DNA is extended in a final reaction phase that does require ATP hydrolysis (Bedale & Cox, 1995; Jain *et al.*, 1994; Shan *et al.*, 1995). Branch movement in this final phase is unidirectional, 5′ to 3′ with respect to the single strand to which the RecA protein initially binds. This parallels the direction of filament assembly, which also occurs 5′ to 3′ (Register & Griffith, 1985). Since disassembly of filaments is localized to the filament end opposite to that at which assembly occurs (Lindsley & Cox, 1990), disassembly of filaments also occurs 5′ to 3′. Unidirectional branch movement is especially important to RecA function in recombinational DNA repair (Cox, 1993), and it represents a demonstrated function of ATP hydrolysis (Jain *et al.*, 1994). This is one of a series of observations that demonstrate a direct coupling between ATP hydrolysis and the last phase of DNA strand exchange (Bedale & Cox, 1995; Cox, 1994; Shan *et al.*, 1995).

A number of mechanisms for RecA protein-mediated DNA strand exchange have been proposed. These can be distinguished in part by the manner in which they explain how the reaction is rendered unidirectional (Figure 1). Several of these can be thought of as dynamic filament models, sharing the property that strand exchange is coupled in some manner to association and/or dissociation of RecA protein monomers. In one version (Howard-Flanders *et al.*, 1984), unidirectionality is achieved by the progressive dissociation of RecA monomers from one end of the RecA filament as hybrid DNA is formed (Figure 1, model B1). As noted above, the disassembly of a RecA nucleoprotein filament

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[1] Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FI DNA, supercoiled closed-circular form of plasmid or bacteriophage DNA as isolated from *E. coli* cells; FII DNA, circular duplex DNA with a nick in one strand; FIII DNA, linear double-stranded DNA; FIV DNA, topologically relaxed circular duplex DNA with both strands covalently closed; SDS, sodium dodecyl sulfate; bp, base pair(s); kbp, kilobase pair(s); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); SSB, single-stranded DNA binding protein of *E. coli*; PAGE, polyacrylamide gel electrophoresis; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

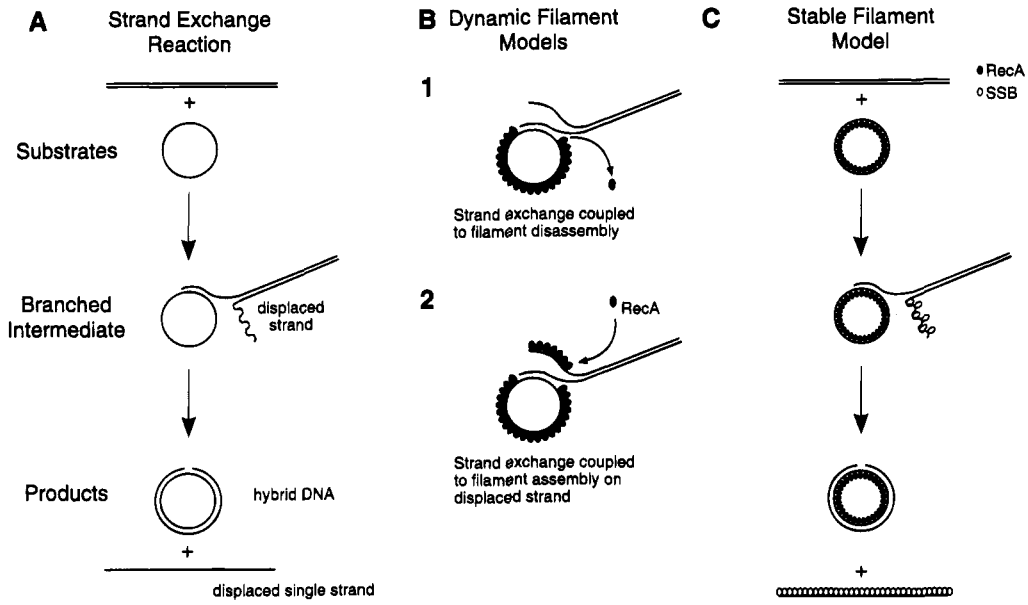

FIGURE 1: Models for DNA strand exchange. (A) RecA protein-mediated DNA strand exchange reaction as described in the text. (B) Models for RecA promotion of DNA strand exchange via assembly or disassembly of RecA filaments. (C) DNA strand exchange promoted by a stable filament of RecA protein.

(when it occurs) takes place with the required polarity. Another version postulates that the assembly of a new RecA filament on the displaced DNA strand (Figure 1, model B2), which would also occur in the 5′ to 3′ direction, confers unidirectionality (Konforti & Davis, 1992). A final model in this class postulates that DNA strand exchange occurring independently of ATP hydrolysis is limited only by discontinuities in the filament, and a redistribution of RecA monomers is the function of ATP hydrolysis in the final phase of the reaction (Menetski *et al.*, 1990; Rehrauer & Kowalczykowski, 1993). In this case, the polarity of the filament assembly and disassembly reactions would produce unidirectional branch movement. We have suggested a second class of model, in which the RecA filament remains intact throughout the reaction, and is bound to the hybrid DNA product after the reaction is complete. In this case, unidirectional branch movement in the final phase of DNA strand exchange would result from a directed rotation of the DNA substrates coupled to ATP hydrolysis (Cox, 1994). Distinguishing among these mechanistic alternatives requires an unambiguous determination of the disposition of RecA protein during the reaction.

This question has been addressed in a wide range of studies, often with conflicting results. The answer obtained experimentally depends in large measure on the activity of another protein, the single-stranded DNA binding protein or SSB, which greatly stimulates RecA-mediated DNA strand exchange and is therefore often added to the reaction. Several studies done with electron microscopy are consistent with models B1 or B2 in Figure 1. When SSB is omitted from the reaction, the RecA protein is bound to the displaced single strand. When SSB is present, SSB is bound to the displaced single strand, and a general disassembly of the RecA filament appears to occur as strand exchange proceeds (Stasiak *et al.*, 1984; Register *et al.*, 1987; Stasiak & Egelman, 1988, 1994).

The binding of SSB to the displaced strand is supported by solution measurements (Lavery & Kowalczykowski, 1992; B. C. Schutte and M. M. Cox, unpublished observations). However, the disassembly of RecA filaments during strand exchange in reactions containing SSB is not supported by solution measurements. When the homologous linear duplex DNA is added to initiate DNA strand exchange, the measured k_{cat} for ATP hydrolysis drops abruptly about 30% from the rate typical of complexes on ssDNA (30 min^{-1}) to a rate similar to that seen for RecA complexes on dsDNA (about 20 min^{-1}). The new rate is maintained throughout the ensuing strand exchange reaction, with no decline that might signal filament disassembly (Schutte & Cox, 1987). In addition, the hybrid duplex product of strand exchange is highly underwound in a manner consistent with RecA protein binding, long after strand exchange is complete (Pugh & Cox, 1987a). The hybrid duplex DNA product is also protected from DNase and restriction enzyme cleavage to an extent consistent with RecA binding (Chow *et al.*, 1986). Two studies (Register & Griffith, 1988; Shaner *et al.*, 1987) have shown that the fixation and spreading conditions used in much of the EM work will not preserve RecA complexes bound to dsDNA in the presence of ATP, potentially explaining the discrepancy between solution and EM observations. However, the DNA underwinding and DNase protection results are themselves not definitive, since the gels used in the former either do not specify the degree of DNA underwinding or were done with RecA in sufficient excess that it could be bound to both the hybrid DNA and the displaced single strand, and RecA confers only partial DNase protection on the DNA. It is nevertheless likely that at least some RecA remains bound to the hybrid DNA.

The results described above also indicate that RecA protein location after DNA strand exchange can be affected by a number of variables. The task is to determine which observations reflect events that are part of the fundamental mechanism of DNA strand exchange. RecA protein binding to the hybrid DNA product of strand exchange has not been quantified sufficiently to determine its significance. A sensitive method to measure RecA binding to duplex DNA can be developed based on a more quantitative assessment of the degree of DNA underwinding conferred on dsDNA

by RecA binding. In this report, we use a DNA underwinding assay and rates of ATP hydrolysis to examine RecA protein binding to the hybrid DNA under conditions that produce an optimal strand exchange reaction.

MATERIALS AND METHODS

Enzymes, Chemicals, and DNA. RecA protein was purified to homogeneity as described (Cox *et al.*, 1981). The concentration of RecA protein in stock solutions was determined by the absorbance at 280 nm, using an extinction coefficient of $\epsilon_{280} = 0.59\ A_{280}\ mg^{-1}$ mL (Craig & Roberts, 1981). Bacteriophage T4 DNA ligase was purified following published procedures (Davis *et al.*, 1980), and the concentration was determined by comparison against a commercial standard. *E. coli* SSB protein was prepared, characterized, and stored as described elsewhere (Lohman *et al.*, 1986). SSB protein stock concentrations were determined by the absorbance at 280 nm, using an extinction coefficient of $\epsilon_{280} = 15.1\ mg^{-1}$ mL (Lohman & Overman, 1985). Restriction enzymes were purchased from New England Biolabs and Promega Biotech. All other chemicals and enzymes were purchased from Sigma.

The plasmid pEAW3 (2426 bp) is derived from pBR322. It was constructed by removing the 18 bp *Bam*HI−*Eco*RI fragment from plasmid pT7-5 (2404 bp) (S. Tabor, personal communication) and inserting a 40 bp fragment containing a recognition site for the yeast FLP recombinase (E. A. Wood and M. M. Cox, unpublished results). Plasmid purification was carried out as described (Davis *et al.*, 1980). ϕX174 FI DNA and ϕX174 ssDNA (virion DNA) were obtained from New England Biolabs. Plasmid pUC119 ssDNA and dsDNA (3.16 kbp) (Vieira & Messing, 1987) were purified according to published procedures (Sambrook *et al.*, 1989). The bacteriophage M13mp8.1037 is the bacteriophage M13mp8 with a 1037 bp sequence (*Eco*RV fragment) from the *E. coli galT* gene inserted into the *Sma*I site. The ssDNA and supercoiled circular duplex (FI) DNA derived from M13mp8.1037 were purified as described (Messing, 1983). The concentrations of DNA stock solutions were determined by measuring the absorbance at 260 nm, using 50 $\mu g\ mL^{-1}\ A_{260}^{-1}$ and 36 $\mu g\ mL^{-1}\ A_{260}^{-1}$ as conversion factors for dsDNA and ssDNA, respectively. DNA concentrations are expressed as total nucleotides.

Nicked circular duplex (FII) DNA was prepared from FI DNA by digesting with DNase I (Sigma, type II) in the presence of ethidium bromide according to the method of Shibata *et al.* (1981). The concentration of the ethidium bromide stock solution was determined at 480 nm, using an extinction coefficient of $\epsilon_{480} = 5600\ M^{-1}\ cm^{-1}$ (Baase & Johnson, 1979; Waring, 1965). This method generates circular duplex DNA containing a single nick. Linear duplex (FIII) DNA was prepared from FI DNA by digestion with the appropriate restriction endonuclease. Circular, covalently closed, relaxed circular duplex DNA (FIV) was prepared by ligating the nicked form of the DNA with T4 DNA ligase under standard conditions (Sambrook *et al.*, 1989).

Preparation of pEAW3 FX DNA at Different pHs. DNA in which the underwinding conferred by RecA protein binding was preserved (form X or FX DNA) was prepared by a modification of the pH-shift method described by Pugh and Cox (1987b). In all cases, 20 μM FII pEAW3 DNA and 15 μM RecA protein were incubated at 37 °C for 15 min in a 1−2 mL reaction mixture containing 10 mM magnesium acetate, 1.1 mM ATP, 1 mM dithiothreitol, 5% glycerol, an ATP regenerating system (3 units/mL pyruvate kinase, 3 mM phosphoenolpyruvate, and 3 mM potassium glutamate), and 20 mM PIPES buffer (pH 6.1). After 15 min, 187.5 μL aliquots were removed, and the reaction pH of each was raised by a 1:1 addition of a solution (preincubated at 37 °C) containing 20 mM of shifting buffer with concentrations of all remaining components held constant expect RecA and DNA. The buffers used to shift the reaction from pH 6.1 to pHs 6.3, 6.7, 7.1, 7.5, 7.8, 8.1, and 8.5 were, respectively, PIPES (pH 6.4), PIPES (pH 7.1), PIPES (pH 11.7), TES (pH 12.0), TES (pH 12.3), Tricine (pH 9.6), and Tricine (pH 12.5). After 10 min, T4 DNA ligase (500 units/μL) was added, and the incubation was continued for 20 min. The amount of ligase added was varied from 3 μL at pH 8.5 to 18 μL at pH 6.1 to compensate for a decrease in ligase activity encountered as the pH decreased. The DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, by volume). To this was added 0.1 volume of 3 M sodium acetate and 3 volumes of ethanol, and the DNA was precipitated overnight at −20 °C (Sambrook *et al.*, 1989). The DNA was pelleted by centrifugation, washed with 70% ethanol, and resuspended in 20 μL of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and 5 μL of gel loading buffer [60 mM EDTA, 5% SDS, 25% (v/v) glycerol, and 0.2% bromophenol blue].

Preparation of FX DNAs at pH 7.5. FX DNAs used as a reference for heteroduplex DNA underwinding were prepared by incubation of the indicated concentrations of FII DNA, RecA protein, and ATP in a 100 μL reaction mixture containing 20 mM PIPES buffer, pH 6.1, 1 mM DTT, 5% glycerol, 10 mM MgOAc, 3 mM potassium glutamate, and an ATP regenerating system (10 mM phosphocreatine and 10 units/mL creatine phosphokinase) for 20 min at 37 °C. The pH was then shifted to 7.5 by 1:1 addition of a solution (preincubated for at least 5 min at 37 °C) containing 20 mM TES buffer, pH 12.0, with concentrations of all other components held constant expect for RecA protein and DNA. Eight minutes after shifting, 7 μL of T4 DNA ligase (500 units/μL) was added, and incubation was continued for 20 min. Reactions were stopped with a phenol/chloroform/isoamyl alcohol (25:24:1) mixture, then ethanol-precipitated, and prepared for electrophoresis according to the procedure described above.

Strand Exchange and Hybrid DNA Ligation Reactions. All reactions were carried out at 37 °C in a standard reaction mix (175 μL total volume) containing 1 mM DTT, 5% glycerol, 10 mM MgOAc, 3 mM ATP, 20 mM buffer (a mixture of equimolar amounts of PIPES buffer, pH 6.1, and TES buffer, pH 12.0, to a final pH of 7.5; see above), and an ATP regenerating system (8 mM phosphocreatine and 10 units/mL creatine phosphokinase, unless otherwise indicated). The concentrations of ssDNA, dsDNA, RecA protein, and SSB protein are as indicated in the text and figure legends. Strand exchange was initiated by addition of ATP and SSB protein. After 30 min, 7 μL of T4 DNA ligase (500 units/μL) was added and incubation continued for 20 min. Reactions were stopped with phenol/chloroform/isoamyl alcohol, ethanol-precipitated, and prepared for electrophoresis as described above.

Agarose Gel Electrophoresis. Agarose gel electrophoresis was performed in TAE buffer [40 mM Tris−acetate (pH 7.5),

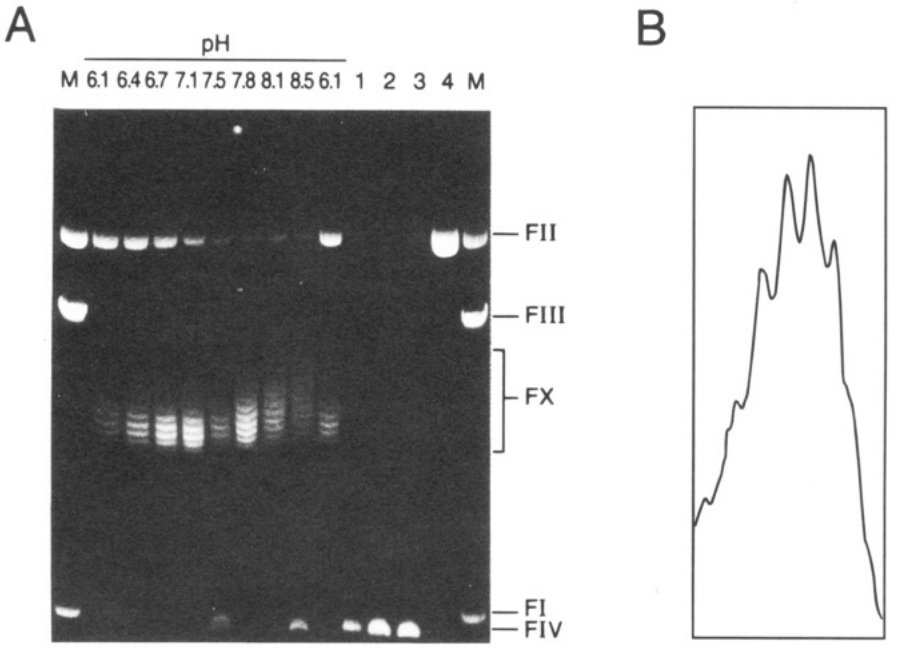

FIGURE 2: (A) pH dependence of DNA underwinding mediated by RecA protein binding. Reactions were carried out as described under Materials and Methods. Samples were subjected to electrophoresis on a 1.5% agarose gel in the presence of 9.5 μM ethidium bromide. The pH marked above 9 lanes reflects the final pH at which the DNA was ligated following the pH-shift described under Materials and Methods. Lanes marked M contain markers for FI, FII, and FIII versions of pEAW3 DNA. Lanes labeled 1 through 3 contain FIV DNA derived from the ligation of FII pEAW3 DNA in the absence of RecA at pHs 6.1, 7.1, and 8.5, respectively. Lane 4 contains unligated FII DNA. (B) Densitometric scan of the resolved topoisomers shown in panel A at pH 7.1.

1 mM EDTA] with the indicated concentrations of agarose and ethidium bromide. Ethidium bromide was added to both the gel and running buffer, with the buffer circulated at a rate of 2 mL/min. The gel was run in darkness for 25−40 h at 3.75−5 V/cm with additional buffer being added when appropriate to compensate for loss of volume due to evaporation. Following electrophoresis, gels were destained for 1−5 h, depending on the ethidium bromide and agarose concentrations, then illuminated with short-wave ultraviolet radiation, and photographed. Other gels were stained with 2 μM ethidium bromide for 45 min before photography. Photographic negatives of the gel were scanned with a Zeineh Soft Laser scanning densitometer, SL-504-XL, obtained from Biomed Instruments, Inc.

ATPase Assay. DNA-dependent hydrolysis of ATP by RecA protein was measured by a coupled enzyme assay (Lindsley & Cox, 1990; Morrical *et al*., 1986). 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 band-pass were 0.5 cm and 2 nm, respectively. The coupled assay used to measure ATP hydrolysis was described previously (Morrical *et al*., 1986). The regeneration of ATP from ADP and phosphoenolpyruvate with the oxidation of NADH can be followed by the decrease in the absorbance at 380 nm. Absorbance was measured at 380 nm rather than 340 nm (the absorbance maximum for NADH), to remain within the linear range of the spectrophotometer. In addition to the phosphoenolpyruvate/pyruvate kinase regeneration system, reactions contained 3 mM NADH and 2.5 units mL^{-1} lactic acid dehydrogenase. An NADH extinction coefficient of ϵ_{380} = 1.21 mM^{-1} cm^{-1} was used to calculate the rate of ATP hydrolysis. An increase in the concentration of the coupling system did not change the observed ATPase rate. Reactions also included 1 mM DTT, 5% glycerol, 10 mM MgOAc, 3 mM potassium glutamate, 3 mM ATP, 20 mM buffer (PIPES, pH 6.1, or Tris−acetate, pH 7.5), and the indicated concentrations of DNA, RecA protein, and SSB protein. Reactions were started by addition of ATP, mixed with SSB protein in cases where SSB was present. Where RecA protein in excess of that required to saturate available binding sites in the DNA was present, reported k_{cat} values are based on dividing the observed initial rate of ATP hydrolysis by the bound RecA protein only, assuming 1 bound monomer per 3 bp of DNA.

RESULTS

Experimental Design. When RecA protein binds to dsDNA, the DNA is highly underwound (Di Capua *et al*., 1982; Shibata *et al*., 1981). This property can be used to develop a sensitive and quantitative DNA binding assay for RecA (Lee & Cox, 1990; Pugh *et al*., 1989). Binding of RecA protein to duplex DNA occurs on a time scale of hours at neutral pH, with the rate limited by a slow nucleation step (Kowalczykowski *et al*., 1987; Pugh & Cox, 1987b, 1988). Growth of a nucleated filament is rapid. Once bound, the RecA nucleoprotein filament is stable as long as ATP is regenerated. The rate of nucleation is highly pH-dependent, and direct binding to duplex DNA can be accelerated greatly by carrying out the reaction at pH 6.1 and then shifting the pH to 7.5 (Pugh & Cox, 1987b, 1988).

When ATP is used as a cofactor, and the DNA is saturated with RecA protein, the DNA is underwound by 39.6% (Pugh *et al*., 1989), slightly less than the underwinding of 43% observed when ATP hydrolysis is blocked by substituting ATPγS (Stasiak & Di Capua, 1982). If RecA is bound to a nicked circular duplex, the DNA can be ligated without removing RecA, thereby converting the underwinding into superhelicity. The very highly supercoiled DNA that results is called form X (FX) (Shibata *et al*., 1981). The distribution

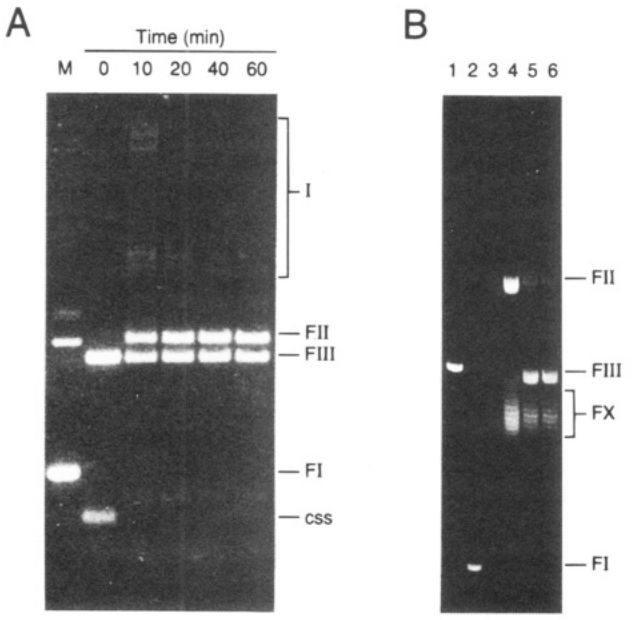

FIGURE 3: Underwinding of the hybrid DNA product of DNA strand exchange. (A) DNA strand exchange reaction carried out with pUC119 ssDNA circles (css) and linear pUC119 duplex DNA (FIII). The marker lane (M) contains supercoiled (FI) and nicked circular (FII) DNA. Minor bands migrating above the FII DNA are reaction intermediates (I). The reaction was carried out as described under Materials and Methods, with 15 μM pUC119 ssDNA, 15 μM pUC119 linear duplex DNA, 5 μM RecA protein, and 1.5 μM SSB. No DNA ligase was used in this experiment. (B) Underwinding of the hybrid DNA product. DNA samples were run in a 1.5% agarose gel, in the presence of 10 μM ethidium bromide, for 40 h at 4 °C. Lanes 1, 2, and 3 contain FIII, FI, and FII markers, respectively. In this gel system, the FI DNA is positively supercoiled. Lane 4 contains pUC119 FX DNA, generated as described under Materials and Methods. Lanes 5 and 6 contain the products of a DNA strand exchange reaction such as in panel A, in which DNA ligase was added at 30 min into the reaction as described under Materials and Methods. Reactions were identical to those described in panel A, except that 6 μM (instead of 5 μM) RecA protein was used in the reaction in lane 6.

of topoisomers in FX DNA is small, indicating a high degree of structural uniformity in the RecA filaments that produced it (Pugh *et al.*, 1989). The 39.6% underwinding corresponds to 1 helical turn of the DNA removed for every 8.7 RecA monomers bound. The topoisomers of this highly underwound DNA can be resolved via agarose gel electrophoresis if the gel is run in the presence of sufficiently high concentrations (about 10 μM) of ethidium bromide. With relatively small (3.5 kbp or less) DNAs, the resolution of individual topoisomers permits the detection of a loss or gain in the range of $\pm 1\%$ of the 1000 RecA monomers that can be bound to a 3 kbp DNA.

The general strategy is to make FX DNA in the presence of ATP, and compare its superhelical density quantitatively to that of the ligated hybrid DNA product of DNA strand exchange. We did not remeasure the superhelical density of FX DNA in this study. We assume that the superhelical density is equivalent to that measured previously [$\sigma = -0.396$ (Pugh *et al.*, 1989)]. As verification, the amount of ethidium bromide that must be present in the agarose gel to partially relax the FX DNA and resolve its topoisomers (about 9−10 μM) is consistent with all previous studies (Pugh & Cox, 1987a,b; Pugh *et al.*, 1989). In comparison, normally supercoiled FI DNA [$\sigma = -0.075$ (Schutte & Cox, 1988)] is completely relaxed when run in the presence of 0.2−0.4 μM ethidium bromide (Pugh & Cox, 1987b). In addition, we were unable to increase the superhelical density of the FX samples by increasing the concentration of RecA protein used to generate them (data presented below and data not shown). To ensure that the DNA strand exchange reactions proceeded efficiently and in a manner consistent with previous results, we monitored ATP hydrolysis during the reactions, and examined the reactions themselves on agarose gels. ATP hydrolysis also serves as a less sensitive but complementary method for monitoring the status of RecA during DNA strand exchange.

The Superhelical Density of FX DNA Is pH-Independent. We wish to use FX DNA as a standard for RecA protein binding. In our scheme to make FX DNA, we bind excess RecA protein to duplex DNA at pH 6.1 and then shift the pH to 7.5 before ligating. We wished to determine if there are any pH-dependent changes in the underwinding of RecA-bound DNA that might signal a change in RecA binding or filament structure. At pH 6.1, RecA protein binds relatively quickly to dsDNA, with binding curves exhibiting stoichiometry points at or about 1 RecA monomer per 3 bp. When saturated with RecA, the complex hydrolyzes ATP with a monomer k_{cat} of 20 ± 2 min^{-1} (assuming 1 bound RecA monomer per 3 bp), and the rate of ATP hydrolysis is independent of pH as long as the RecA protein remains bound to DNA (Pugh & Cox, 1987b, and data not shown).

Excess RecA protein was bound to nicked circular pEAW3 at pH 6.1, and the pH was then raised as described under Materials and Methods. The DNA was ligated in the presence of bound RecA and the results shown in Figure 2A. This gel was run in the presence of 9.5 μM ethidium bromide, which resolves the topoisomers of FX DNA. Both normally supercoiled (FI) DNA and relaxed but covalently closed circular (FIV) DNA are positively supercoiled under these conditions, and migrate much faster than the FX DNA. The requirement for 9.5−10 μM ethidium bromide in the gel to resolve the FX DNA in all of the experiments described in this study is consistent with the levels used previously to quantify the degree of underwinding in FX DNA (Pugh *et al.*, 1989). The distribution of topoisomers is very similar in all the samples. The experiment was repeated 3 times with the same result. An increase in the amount of unligated DNA (FII) at the lower pHs reflects the lower efficiency of T4 DNA ligase as the pH decreases. In the pH 7.5 and 8.5 lanes, a small amount of FIV DNA is evident, which must arise from ligation of DNA to which RecA is not bound. This species occurred sporadically in these experiments, probably due to occasional disruption of RecA complexes due to transient exposure to high pH during the pH-shift procedure. The topoisomers found at pH 7.1 were scanned densitometrically with the results presented in Figure 2B. More than 80% of the ligated DNA was found in just four topoisomers, suggesting a high degree of structural uniformity in the RecA complexes from which they arose. The results show that the degree of underwinding in RecA−dsDNA complexes formed in the presence of ATP is independent of pH over the range of pH 6.1−8.5.

The Hybrid DNA Product of DNA Strand Exchange Is Bound Quantitatively by RecA Protein. DNA strand exchange reactions were carried out with DNA substrates derived from pUC119 DNA. This plasmid contains an M13 intergenic region, and a circular single-stranded version can be obtained with the help of an M13 helper phage (Vieira

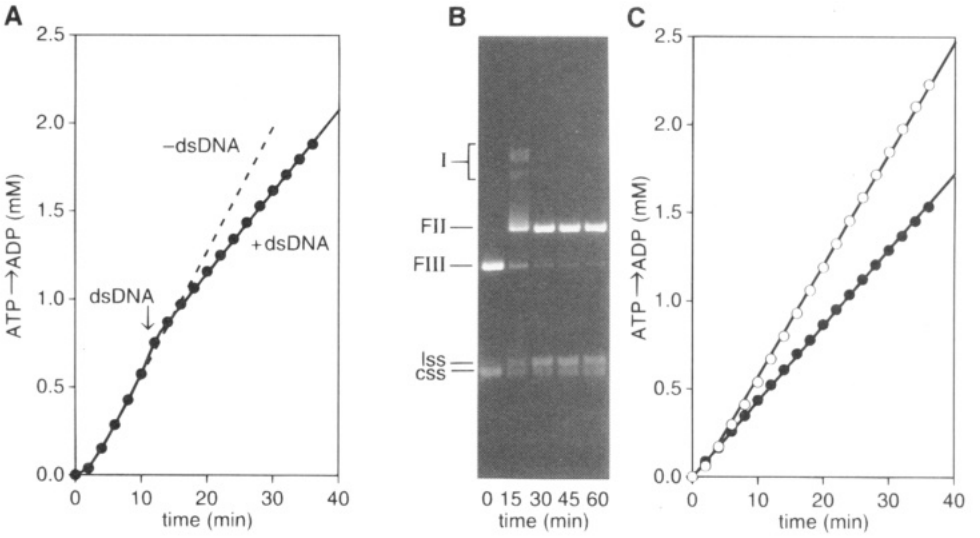

FIGURE 4: ATP hydrolysis during DNA strand exchange. (A) Reactions were carried out as described under Materials and Methods and contained 7.5 μM ϕX174 ssDNA, 4 μM RecA protein, and 0.75 μM SSB. Reactions were started with ATP and SSB. After 11 min (arrow), 15 μM ϕX174 linear duplex DNA was added to one of the two reactions, and both were continued as shown. The k_{cat} values reported in the text are based on assuming 2.5 μM bound RecA protein. (B) DNA strand exchange reaction with ϕX174 DNA substrates. Conditions are as in panel A, except that the RecA concentration was 2.5 μM. (C) ATP hydrolysis during strand exchange under the conditions of panel B. The apparent k_{cat} values for these experiments are 26.2 and 15.9 min^{-1} for the reactions with ssDNA alone (○) or the strand exchange reaction (●), respectively. In this experiment, both reactions (with or without dsDNA) were initiated with a mixture of ATP and SSB after preincubation of all other components at 37 °C, and monitored from the initiation point which defines $t = 0$. These rates are a little lower than normal because of the somewhat incomplete binding of RecA to the DNA when exactly stoichiometric amounts of RecA are employed (see Figure 5).

& Messing, 1987). A typical reaction is shown in Figure 3A. Because of the small size of the DNA, nicked circular hybrid DNA products are apparent after only 10 min. Additional DNA strand exchange reactions were carried out under the same conditions, except that DNA ligase was added to the reaction mixture at 30 min as described under Materials and Methods. The resulting DNA is compared with FX DNA derived from pUC119 in Figure 3B. The experiment was done twice with slightly different concentrations of RecA protein. In each case, the DNA comigrated with the FX DNA. The FIII DNA in lanes 5 and 6 is unreacted linear duplex substrate DNA; the FII DNA is hybrid DNA product which was not ligated. Note that all of the ligated DNA is as underwound as FX. FIV DNA, which would result from ligation of FII hybrid DNA unbound by RecA, was not produced in significant amounts. The underwinding in the hybrid DNA is equivalent, to the topoisomer, to that which reflects binding saturation by RecA in the generation of FX DNA. This binding persists for at least 10−20 min beyond the point where the strand exchange reaction reaches completion. This experiment was carried out 3 times with the same result.

Rates of RecA-Mediated ATP Hydrolysis Do Not Change after Strand Exchange Is Initiated. We also followed the ATPase activity of RecA protein as the strand exchange reactions progressed. In the experiment shown in Figure 4A, the RecA protein was left to hydrolyze ATP in the presence of ϕX174 ssDNA for 11 min before addition of the homologous duplex DNA. As reported previously, the rate of ATP hydrolysis drops upon addition of the homologous DNA in a homology-dependent fashion (Schutte & Cox, 1987), reaching a new rate about one-third lower in 2−3 min. The rate of ATP hydrolysis then continues linearly during the ensuing strand exchange reaction. The rate during strand exchange in Figure 4A translates into an apparent k_{cat} of 17.6 min^{-1}. A parallel ATPase assay with 15 μM pUC119 FII DNA and 10.5 μM RecA protein done at pH 6.1 yielded a dsDNA-dependent k_{cat} of 17.9 min^{-1} (data not shown; k_{cat} based on assumption of 5 μM bound RecA protein). In the reaction shown in Figure 4, panels B and C, the strand exchange reaction was monitored concurrently with ATP hydrolysis to demonstrate that there is no change in ATP hydrolysis even during an optimally efficient DNA strand exchange reaction.

Effects of SSB and RecA Concentration on Underwinding of Hybrid DNA. The results described above may leave open the possibility that a small fraction of the RecA protein is being transferred to the displaced single strand at least transiently during DNA strand exchange. The concentration of SSB available to bind the displaced single strand should affect a RecA transfer such that higher SSB concentrations should more effectively block it. This would leave more RecA on the hybrid DNA with an accompanying increase in the observed level of DNA underwinding. We carried out a number of experiments to examine the effects of changing the concentration of the protein components, and we also wished to determine if RecA protein binding on hybrid DNA was maintained to an equivalent degree on larger DNA substrates. Reactions were carried out with ϕX174 DNA (5386 bp), and the underwinding of the hybrid DNA following strand exchange was examined. The results are shown in Figure 5. All of these reactions proceeded essentially to completion, with little unreacted FIII DNA. Because of the larger size of the DNA, individual topoisomers cannot be resolved in these DNA molecules with their high superhelical density, and the DNA appears as a compact smear. The only variable that appears to affect the underwinding of the hybrid DNA is the RecA protein concentration. When exactly stoichiometric concentrations were used, the DNA is underwound somewhat less than FX DNA (lanes 4, 8, 12, and 14). Increasing the amount of RecA by just 20% produced hybrid DNA as underwound as FX. The

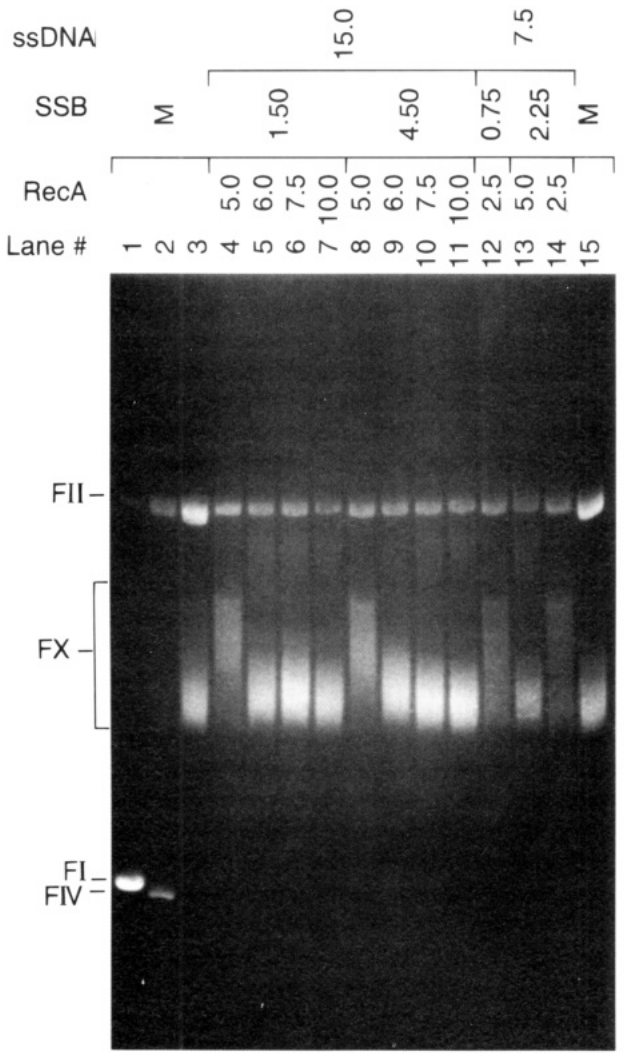

FIGURE 5: Effects of RecA and SSB concentration on the underwinding of FX DNA. All DNAs were derived from ϕX174. Samples were run in a 1.0% agarose gel in the presence of 10 μM ethidium bromide. FX DNA was prepared from FII DNA as described under Materials and Methods. Lanes 1, 2, and 3 contain FI, FIV, and FX DNA markers, respectively. Lanes 4−14 contain the products of DNA strand exchange reactions after ligation, carried out as described under Materials and Methods. Lane 15 contains another FX marker. All reactions contained 15 μM linear dsDNA substrate. Concentrations of ssDNA substrate and proteins (in μM) are indicated in the table.

results are consistent with a standard binding curve in which saturation is not observed until the protein concentration slightly exceeds the point at which it is stoichiometric with available binding sites. A clear saturation effect is observed, since higher amounts of RecA do not increase the degree of underwinding. This provides additional evidence that the degree of underwinding of the hybrid DNA that we observe following DNA strand exchange reflects saturation by RecA protein. Note that these gels are capable of resolving DNA species with superhelical densities (σ) in the range of approximately −0.33 to −0.45. The shift in the bands from lanes 5 to 4 (or lanes 9 to 8) in Figure 5 reflects a decrease in σ of less than 10%, which should parallel the decrease in RecA protein binding. The gels are sufficiently sensitive that small changes in RecA protein binding are readily monitored.

The amount of SSB did not affect the results, even when RecA binding to the hybrid DNA was somewhat below saturation, providing evidence that no significant transfer of RecA from the hybrid DNA to the displaced single strand is occurring. The amounts of SSB used were in all cases sufficient to produce an efficient strand exchange, and the effect of omission of SSB was not examined because of the severe reduction in strand exchange that results.

DISCUSSION

Our primary conclusion is that RecA protein remains quantitatively bound to the hybrid DNA product of DNA strand exchange long after strand exchange is complete. This is the case when optimal conditions for DNA strand exchange are used which include SSB protein. Independent studies have shown that the SSB is bound to the displaced single strand (Lavery & Kowalczykowski, 1992; B. C. Schutte and M. M. Cox, unpublished observations). The underwinding of the hybrid DNA is identical to that of FX DNA produced by established methods, and indicates that a saturating level of RecA (1 monomer per 3 bp) is bound. We can therefore account for all of the RecA protein in the nucleoprotein filament that initiated DNA strand exchange. The linear rates of ATP hydrolysis observed during strand exchange provide additional evidence against either transfer of RecA to the displaced single strand (which would result in a gradual 50% increase in the rate of ATP hydrolysis as the reaction progressed) or disassembly of the filament (which would lead to a complete reduction of the rate of ATP hydrolysis). The actual rate of ATP hydrolysis observed during DNA strand exchange is identical to the rate characteristic of RecA−dsDNA complexes.

The use of DNA underwinding to monitor RecA binding to duplex DNA is highly quantitative. Complete RecA protein binding followed by ligation reduces the linking number of a 3.16 kbp DNA such as that used in Figure 3 by about 120. The resolution of the gel used in Figure 3 permits the detection of a change in the average linking number of 1 or 2 units, so that we could readily detect the loss of as few as 10−15 RecA monomers out of the more than 1000 that must be bound to produce FX DNA. The effects of small reductions in RecA binding are evident in Figure 5.

The only caveat to this measurement is the assumption that the 39.6% underwinding reflects binding saturation by RecA. When ATPγS replaces ATP as cofactor, the underwinding is somewhat greater (43%) (Stasiak & Di Capua, 1982). We have assumed that the lower value obtained with ATP simply reflects transient conformation changes by subsets of the monomers in the filament as ATP is hydrolyzed. The degree of underwinding does not exceed 39.6% as the RecA protein concentration is increased beyond levels required to saturate the DNA. At worst, the 39.6% underwinding could mean that only 92% of the dsDNA can be bound when ATP is hydrolyzed, if the 43% value is taken as the limit. In this scenario, we could only account for 92% of the RecA protein on the hybrid DNA product. Given the cooperativity of RecA binding to dsDNA, we do not see how 8% of the DNA could be refractory to RecA protein binding even in the presence of a large excess of RecA protein.

These data should not be taken to imply that the RecA filament is extraordinarily stable. Two factors in these experiments are crucial to maintaining the RecA protein on the hybrid DNA, the presence of SSB and ATP regeneration. A buildup of ADP results in disassembly of RecA filaments

(Lee & Cox, 1990; Soltis & Lehman, 1983). The binding of RecA to the hybrid DNA and the DNA underwinding associated with it last only as long as the ATP regeneration system does. Also, if excess ssDNA is added to a solution containing RecA−dsDNA complexes formed with ATP, RecA protein transfers from the dsDNA to the ssDNA (Pugh & Cox, 1987a). During strand exchange, this transfer is blocked when SSB is present on the displaced strand. RecA filament disassembly or transfer to ssDNA can clearly be brought about if the reaction conditions are manipulated appropriately. Our results do show that disassembly or transfer is not a mechanistic requirement for DNA strand exchange, since a very efficient DNA strand exchange reaction can occur in their absence.

On the basis of these and earlier results cited above, we argue that mechanisms that couple unidirectional DNA strand exchange to the transfer of RecA monomers to the displaced strand, or to the dissociation of RecA monomers as the strand exchange proceeds, are no longer viable. These results do *not* eliminate the proposal of Kowalczykowski and colleagues that the last phase of DNA strand exchange depends upon an ATP-dependent redistribution of RecA monomers in the filament (Menetski et al., 1990; Rehrauer & Kowalczykowski, 1993). However, strand exchange can also be envisioned without RecA redistribution. Models have been proposed in which the filament remains intact throughout the reaction, in which unidirectional branch movement in strand exchange is brought about by a facilitated rotation of the DNA substrates coupled to ATP hydrolysis (Burnett *et al.*, 1994; Cox, 1994). Evidence is provided elsewhere that ATP hydrolysis is coupled to branch movement in DNA strand exchange (Kim *et al.*, 1992a,b; Jain et al., 1994; Shan et al., 1995; Bedale & Cox, 1995), and that a torsional stress is generated in the DNA consistent with the facilitated rotation concept (Jwang & Radding, 1992).

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