

## Properties of recA441 Protein-catalyzed DNA Strand Exchange Can Be Attributed to an Enhanced Ability to Compete with SSB Protein\*

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Polly E. Lavery and Stephen C. Kowalczykowski

From the Department of Molecular Biology, Northwestern University Medical School, Chicago, Illinois 60611

We have investigated the recombinase activity of recA441 protein by comparing its *in vitro* DNA strand exchange activity to that of wild-type recA protein. Consistent with its proficiency in recombination *in vivo*, recA441 protein is able to catalyze the *in vitro* exchange of a circular single-stranded DNA molecule for a homologous strand in a linear double-stranded DNA molecule. Under conditions optimal for wild-type recA protein, the rates of joint molecule formation are the same for the two recA proteins, but the wild-type protein converts these intermediate species to gapped circular heteroduplex DNA product molecules more rapidly than recA441 protein. In the recA441 protein reaction, joint molecules are instead converted to extensive homology-dependent DNA networks via presumed reinitiation reactions. Under some conditions, the DNA strand exchange activity of recA441 protein is enhanced relative to the wild-type. These conditions include when single-stranded DNA•SSB protein (where SSB is *Escherichia coli* single-stranded DNA-binding protein) complexes are formed prior to the addition of recA protein, at low magnesium ion concentration in the presence of spermidine, and at low ATP concentrations. Under the conditions examined, recA441 protein competes more effectively with SSB protein for DNA-binding sites; thus, the differences between the strand exchange activities of the wild-type and recA441 proteins can be attributed to this enhanced ability in SSB protein competition.

The recA protein of *Escherichia coli* has been shown to be necessary for both genetic recombination (for reviews see Radding, 1988; Cox and Lehman, 1987; Kowalczykowski, 1987) and induction of the genes that comprise the SOS system (for reviews see Little and Mount, 1982; Walker, 1984). Induction of the SOS system occurs when recA protein stimulates the cleavage of the LexA repressor protein in response to treatments that cause DNA damage or inhibit DNA replication (Witkin, 1976). The lytic repressors of lambdaoid phage are also targets of this protease activity of recA protein, accounting for the recA-dependent induction of prophage (Roberts *et al.*, 1978). *In vitro*, recA protein becomes active as a protease when it binds ssDNA<sup>1</sup> and ATP or dATP, suggesting that activation *in vivo* results from the binding of recA

protein to single-stranded regions or gaps in damaged DNA (Craig and Roberts, 1980; Phizicky and Roberts, 1981).

There are mutations in the *recA* gene that affect the regulation of the SOS response (Tessman and Peterson, 1985). Some mutant recA proteins are constitutively activated for repressor cleavage, *i.e.* they do not require DNA damaging treatment for SOS or prophage induction. The recA441 protein exhibits constitutive protease activity under some conditions, most notably at elevated temperature (Goldthwait and Jacob, 1964; Kirby *et al.*, 1967). There are two mutations in *recA441*, one that changes amino acid 38 from glutamic acid to lysine and another that changes amino acid 298 from isoleucine to valine (Knight *et al.*, 1984). In a previous report (Lavery and Kowalczykowski, 1988), we compared biochemical properties of recA441 protein to those of wild-type recA protein in an effort to account for the constitutive protease activity observed in *recA441* strains. We found that, compared with recAwt protein, recA441 protein does not appear to have a greater equilibrium binding affinity for ssDNA, nor a slower rate of dissociation from ssDNA. However, recA441 protein is more capable of using ssDNA that has been complexed with SSB protein as a cofactor for ATPase and protease activities, implying that the recA441 protein is more proficient at displacing SSB protein from ssDNA. We hypothesized that the enhanced ability of recA441 protein in the competition with SSB protein for ssDNA sites must be related to an increased rate of association with ssDNA. Additionally, we concluded that recA441 protein can be activated to cleave repressors in the absence of DNA damage by displacing SSB protein from the limited ssDNA that occurs naturally in *E. coli*.

*In vitro*, recA protein catalyzes the unique reaction of DNA strand exchange (Cox and Lehman, 1981). In this reaction, one strand of a linear duplex DNA molecule is exchanged for a homologous circular ssDNA molecule, ultimately producing a gapped circular heteroduplex DNA product molecule and a displaced linear ssDNA molecule. This reaction is generally thought to be a good model for *in vivo* recombination. Consistent with this, examination of mutant recA proteins has shown that proteins that are incapable of recombination *in vivo* are unable to catalyze the DNA strand exchange reaction *in vitro* (see Kowalczykowski and Roman, 1990) *e.g.* recA1 protein (Rusche *et al.*, 1985), recA56 protein,<sup>2</sup> and recA142 protein (Kowalczykowski *et al.*, 1989). Additionally, mutant recA proteins that are capable of at least some level of recombination *in vivo* are able to catalyze the DNA strand exchange reaction *in vitro*, *e.g.* recA430 protein (Menetski and Kowalczykowski, 1990b) and recA803 protein.<sup>3</sup>

The effect of the *recA441* mutation on *in vivo* recombination has been examined, and varying conclusions have been

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<sup>1</sup> The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; recAwt protein, wild-type recA protein; SSB protein, *E. coli* single-stranded DNA-binding protein.

<sup>2</sup> S. D. Lauder and S. C. Kowalczykowski, unpublished observations.

<sup>3</sup> M. V. V. S. Madiraju, P. E. Lavery, S. C. Kowalczykowski, and A. J. Clark, unpublished observations.

reached. From an examination of both conjugal recombination and recombination between  $\lambda$  phage deficient in their autonomous recombination systems, Castellazzi *et al.* (1972) suggested that the *recA441* mutation does not affect the recombinase activity of the *recA* protein either at 30 °C or after a temperature pulse at 42 °C for 40 min. Lloyd (1978) studied conjugal recombination in strains where synthesis of *recA* protein is increased, *e.g.* due to the *recA441* mutation at 37 °C or due to the *lexA51* mutation, which yields a defective LexA repressor protein. He demonstrated that, while recombination proficiency is only slightly elevated by an increase in the level of wild-type *recA* protein or by the *recA441* mutation, the frequency of genetic exchanges/unit length of chromosome (as inferred from the reduced linkage of multiple donor alleles) is increased by each. Additionally, the linkage of donor alleles was reduced more dramatically when the recipient strain carried the *recA441* mutation in addition to the *lexA51* mutation as compared with the *lexA51* mutation alone. Thus, Lloyd (1978) concluded that the *recA441* mutation may indeed have some stimulatory effect on the recombinase activity of the *recA* protein.

In this study we address the recombinase activity of the *recA441* protein by comparing its *in vitro* DNA strand exchange activity to that of the wild-type *recA* protein under a variety of experimental conditions, particularly those where we have previously seen enhanced ssDNA-dependent ATPase and LexA-protease activity by *recA441* protein (Lavery and Kowalczykowski, 1988). We find that, under optimal conditions, *recA441* protein appears to be no more proficient at joint molecule formation than wild-type *recA* protein, and in fact, produces gapped circular heteroduplex DNA product molecules more slowly than wild-type *recA* protein (for reasons discussed below). However, under suboptimal conditions, *recA441* protein does show enhanced DNA strand exchange activity relative to wild-type *recA* protein. This enhanced activity correlates with the enhanced ability of *recA441* protein in the displacement of SSB protein from ssDNA.

#### MATERIALS AND METHODS

**Chemicals and Buffers**—All chemicals were reagent grade and solutions were made using glass-distilled water. ATP was purchased from Boehringer Mannheim and was dissolved as a concentrated stock at pH 7.5. Reactions were performed in TD buffer (25 mM Tris-acetate, pH 7.5, and 1 mM dithiothreitol) and contained magnesium acetate as indicated in the figure legends.

**Proteins**—Wild-type *recA* protein was purified from strain JC12772 (Uhlen and Clark, 1981) using a preparative procedure<sup>4</sup> based on spermidine precipitation (Griffith and Shores, 1985). *RecA441* protein was purified from strain BEU397 (kindly provided by A. John Clark of the University of California, Berkeley), using the procedure described by Cox *et al.* (1981). Strain BEU397 is strain JC10289 containing pBEU54, a derivative of pBEU28 (Uhlen *et al.*, 1983), into which the *Bam*HI fragment with the *recA441* gene has been inserted. SSB protein was purified from strain RLM727 using a preparative protocol provided by Roger McMacken of The Johns Hopkins University. Protein concentrations were determined using molar extinction coefficients of  $2.7 \times 10^4$  M<sup>-1</sup> cm<sup>-1</sup> for *recA* protein and  $3 \times 10^4$  M<sup>-1</sup> cm<sup>-1</sup> for SSB protein, both at 280 nm (Ruyechan and Wetmur, 1976).

Pyruvate kinase was purchased from Sigma as an ammonium sulfate suspension. A working solution of this enzyme was made by centrifuging a homogenous sample of the suspension and dissolving the protein pellet in reaction buffer.

**DNA**—Single and double-stranded DNA were prepared from bacteriophage M13mp7 using the procedures described by Messing (1983). The duplex DNA was linearized by digestion with *Eco*RI restriction endonuclease. pBEU41 was prepared from strain BEU293 (kindly provided by A. John Clark of the University of California, Berkeley); pBEU41 is a derivative of pBEU2 (Uhlen *et al.*, 1979)

containing the cloned *recA56* gene.  $\lambda$  DNA was purchased from U. S. Biochemical Corporation. Molar nucleotide concentrations were determined using extinction coefficients of 6500 M<sup>-1</sup> cm<sup>-1</sup> for duplex DNA and 8780 M<sup>-1</sup> cm<sup>-1</sup> for single-stranded DNA, at 260 nm.

**DNA Strand Exchange Assay**—The agarose gel assay for DNA strand exchange was conducted as described previously (Cox and Lehman, 1981; Roman and Kowalczykowski, 1986). However, in this study, reaction time points were subjected to electrophoresis in 0.8% agarose gels in the absence of ethidium bromide. The gels were then stained with 2  $\mu$ g/ml ethidium bromide for 1 h to visualize the DNA bands. This procedure allows for the separation of the gapped circular heteroduplex DNA product molecules from intermediate species (Menetski and Kowalczykowski, 1989). Intermediates in this reaction are homologically paired plectonemic joint molecule structures containing both strands of the linear duplex DNA molecule and the invading ssDNA molecule (Cox and Lehman, 1981).<sup>5</sup>

The stained gels were photographed, and the negatives were scanned using a Zeineh soft laser scanning densitometer. The amount of intermediate species and product molecules present at each time point was computed using a Hewlett-Packard 3390A integrator. The percentage of product and joint molecule intermediates formed were determined as the amount of each species present divided by the total amount of dsDNA present at each time point. The rates of joint molecule and product formation reported represent the most rapid rate of appearance observed for a particular species.

Unless otherwise indicated, DNA strand exchange reactions were performed at 37 °C in TD buffer containing 8 mM magnesium acetate, 1 mM ATP, 7.5 mM phosphoenolpyruvate, 10 units of pyruvate kinase/ml, 6  $\mu$ M *recA* protein, 0.9  $\mu$ M SSB protein, 9.9  $\mu$ M ssM13 DNA, and 16.8  $\mu$ M dsM13 DNA. *RecA* protein was incubated for 1 min in reaction buffer containing ssDNA prior to the addition of SSB protein. The reaction was then started by the addition of dsDNA.

**Coaggregation Assay**—Coaggregation, as defined by Tsang *et al.* (1985), of ssM13 DNA and pBEU41 dsDNA or  $\lambda$  dsDNA was measured using an agarose gel assay (Kowalczykowski *et al.*, 1989). Reactions were performed by preincubating (5 min at 37 °C) *recA* protein with ssDNA in TD buffer, in the presence of magnesium acetate, spermidine, ATP, and SSB protein, as indicated. The dsDNA was added, the reaction mixture was incubated at 37 °C for 10 more min and then subjected to centrifugation in an Eppendorf centrifuge for 10 min. The amount of DNA present in both the supernatant and the pellet (resuspended in 1% SDS) was assayed by agarose gel electrophoresis.

#### RESULTS

***RecA441* Protein Can Promote DNA Strand Exchange**—The *recA441* protein is able to catalyze the exchange of a circular ssM13 DNA molecule for the homologous strand in a linear dsM13 DNA molecule (Fig. 1). Since our agarose gel assay for DNA strand exchange permits separation of heteroduplex DNA product molecules from plectonemic joint molecule intermediate species, both can be quantitated in a single experiment. Fig. 2 compares the time course for the appearance of joint molecules and final product molecules in the wild-type protein and *recA441* protein reactions, as derived from densitometric scanning of data like that in Fig. 1. In this reaction, the rates of joint molecule appearance are essentially the same for the two *recA* proteins, suggesting that the *recA441* protein-ssDNA complex and the *recAwt* protein-ssDNA complex interact comparably with dsDNA.

While the initial rates of joint molecule formation are the same for both proteins, the wild-type protein converts these intermediate species to product molecules about six times more rapidly than *recA441* protein (Fig. 2). However, in the *recA441* protein reaction, DNA species that do not migrate into the gel are formed instead (Fig. 1). The time course for the appearance of these species is also shown in Fig. 2. Their appearance coincides with the disappearance of joint molecule intermediate species, suggesting that they are formed from the joint molecules. The DNA molecules that fail to enter the

<sup>4</sup> S. C. Kowalczykowski, manuscript in preparation.

<sup>5</sup> J. P. Menetski, D. G. Bear, and S. C. Kowalczykowski, manuscript in preparation.

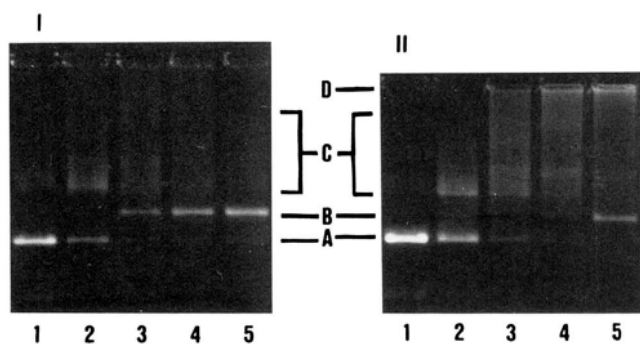


FIG. 1. RecAwt protein and recA441 protein-catalyzed DNA strand exchange. Reactions were performed as described under "Materials and Methods." The bands are A, linear dsM13 DNA substrate molecules; B, gapped circular heteroduplex DNA product molecules; C, joint molecule intermediate DNA species; D, homology-dependent DNA networks. Lanes 1-5 are 5, 10, 20, 30, and 60 min, respectively. Panel I is wild-type recA protein and Panel II is recA441 protein.

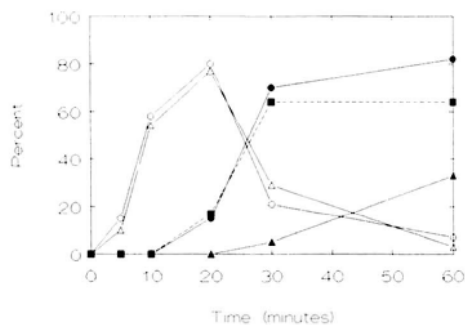


FIG. 2. Time course for recAwt protein and recA441 protein-catalyzed DNA strand exchange reactions. Reactions were performed as described under "Materials and Methods." For recAwt protein, open circles indicate joint molecules and filled circles indicate product molecules. For recA441 protein, open triangles indicate joint molecules, filled triangles indicate product molecules, and squares indicate DNA networks.

gel probably represent extensive networks of single-stranded and double-stranded DNA molecules formed via reinitiation of pairing by the ssDNA that is partially displaced from the initial joint molecules (Chow *et al.*, 1988). These reactions were performed at recA protein concentrations 2-fold in excess of available ssDNA-binding sites. When recA protein concentration was decreased to 3 or 1.5  $\mu\text{M}$ , homology-dependent DNA networks still appeared in the recA441 protein reaction; however, at 1  $\mu\text{M}$  recA protein and below, no networks were formed (data not shown).

**The Order of SSB Protein Addition Affects DNA Strand Exchange**—DNA strand exchange is affected by the order in which recA protein and SSB protein are added to the reaction mixture, *i.e.* the reaction occurs much less efficiently when recA protein is added to ssDNA after SSB protein (Cox and Lehman, 1982; Menetski and Kowalczykowski, 1989). Compared with recAwt protein, recA441 protein is more capable of using ssDNA that has been complexed with SSB protein as a cofactor for ATP hydrolysis and repressor cleavage, implying that it can displace SSB protein from ssDNA more quickly and completely than recAwt protein (Lavery and Kowalczykowski, 1988). To determine whether this enhanced ability is significant to the DNA strand exchange reaction, this activity was examined using ssDNA complexed with SSB protein. The results in Fig. 3 indicate that the rates of appearance of both joint molecules and final product molecules are much greater in the recA441 protein-catalyzed reaction.

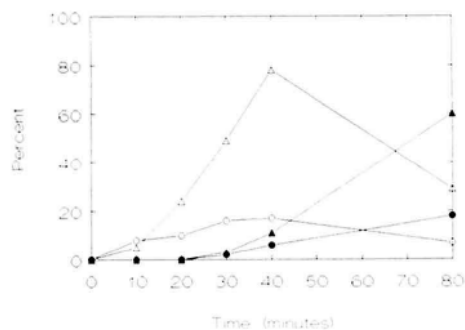


FIG. 3. Time course for DNA strand exchange reactions using ssDNA that has been complexed with SSB protein, (SSB-first). Reactions were performed as described under "Materials and Methods," except that SSB protein was incubated for 1 min in reaction buffer containing ssDNA prior to the addition of recA protein. The reaction was then started by the addition of dsDNA. For recAwt protein, open circles indicate joint molecules, and filled circles indicate product molecules. For recA441 protein, open triangles indicate joint molecules, and filled triangles indicate product molecules.

TABLE I

The effect of temperature on DNA strand exchange

All reactions were performed in TD buffer and contained 8 mM magnesium acetate, 1 mM ATP, 10 units of pyruvate kinase/ml, 7.5 mM phosphoenolpyruvate, 6  $\mu\text{M}$  recA protein, 0.9  $\mu\text{M}$  SSB protein, 9.9  $\mu\text{M}$  ssM13 DNA, and 16.8  $\mu\text{M}$  linear dsM13 DNA.

T	Protein	Rate of species appearance (%/min) <sup>a</sup>				
		SSB second <sup>b</sup>		SSB first <sup>c</sup>		
		Intermediate	Product	Networks	Intermediate	Product
°C						
30	recAwt	2.4	2.6	0	<0.1	0
30	recA441	2.5	0.3	2.8	0.6	0
37	recAwt	8.6	5.5	0	0.6	0.3
37	recA441	8.8	0.9	4.7	2.9	1.2
42	recAwt	15.6	7.0	0	1.1	6.0
42	recA441	14.2	1.7	8.8	6.0	5.2

<sup>a</sup> The rates of species appearance reported represent the maximum rate of appearance of each species, as deduced from a reaction time course (like that shown in Fig. 2).

<sup>b</sup> SSB second designates reactions in which recA protein was added to ssDNA in reaction buffer. After 1 min SSB protein was added, and then dsDNA was added to start the strand exchange reaction.

<sup>c</sup> SSB first designates reactions in which SSB protein was added to ssDNA in reaction buffer. After 1 min recA protein was added, and then dsDNA was added to start the strand exchange reaction.

Nearly all of the input linear dsDNA substrate is taken up into stable heteroduplex DNA structures (*i.e.* either joint molecule intermediate species or product molecules) within 80 min in the recA441 protein reaction. In contrast, less than 30% of the linear dsDNA is found in stable heteroduplex DNA structures after 80 min in the recAwt protein-catalyzed reaction.

A comparison of Figs. 2 and 3 shows that the rate of joint molecule formation by recA441 protein in the SSB-first reaction is only about one-third of that observed when SSB protein is added after recA protein (see Table I). Additionally, in the SSB-first reaction, homology-dependent DNA networks are not formed by recA441 protein. The slower rate of joint molecule formation presumably restricts their accumulation and subsequent involvement in the putative reinitiation reactions that lead to extensive DNA network formation.

**The Effect of Temperature on the DNA Strand Exchange Reaction**—The enhanced activity of recA441 protein in ATP hydrolysis and repressor cleavage is strongly dependent on temperature (Lavery and Kowalczykowski, 1988). To deter-

mine if differences in the strand exchange activities of the recA441 and wild-type recA proteins were also temperature-dependent, we examined their activities at various temperatures (Table I). For reactions using the SSB-second protocol, the trends observed are similar at all temperatures (Table I). The rates of joint molecule formation are essentially the same for the two recA proteins. In all cases, the wild-type protein converts these joint molecules to final product molecules at a significantly greater rate than recA441 protein. However, instead of producing gapped circular product molecules, recA441 protein produces homology-dependent DNA networks at a rate comparable to the rate at which recAwt protein produces product molecules.

When the SSB-first protocol is followed, neither recA441 protein nor recAwt protein is able to promote significant DNA strand exchange at 30 °C (Table I). At 42 °C, like 37 °C, joint molecules appear more rapidly in the recA441 protein reaction than in the recAwt protein reaction (Table I). A comparison of recAwt protein activity in the SSB-first and SSB-second reactions at 42 °C shows that product molecules begin to appear about 15 min later in the reaction time course of the SSB-first reaction (data not shown), presumably due to the slower rate of joint molecule formation under these conditions. However, the maximum rates at which joint molecules are converted to gapped circular product molecules are essentially the same in both of these reactions (Table I). Thus, it appears that once joint molecules are formed, branch migration is unaffected by the order in which recA protein and SSB protein were added to the reaction.

In the SSB-first reaction at 42 °C, homology-dependent DNA networks are not formed by recA441 protein, and the rate at which product molecules appear approaches that observed for recAwt protein. This rate is significantly greater than that observed in the recA441 protein SSB-second reaction, where homology-dependent DNA networks are formed. Thus, the formation of these DNA networks from joint molecules appears to be inhibitory to the completion of DNA strand exchange.

**The Effect of Varying Magnesium Ion Concentration on DNA Strand Exchange**—Because ATP hydrolysis and repressor cleavage activities of recA441 protein are enhanced considerably at low (1 mM) magnesium ion concentration (Lavery and Kowalczykowski, 1988), we compared the DNA strand exchange activities of the wild-type and recA441 proteins at various magnesium acetate concentrations. At 1 mM magnesium acetate, neither wild-type recA protein nor recA441 protein is able to catalyze DNA strand exchange (Fig. 4). Since the ATPase activity of recAwt protein is inhibited by SSB protein at this magnesium ion concentration, it has been proposed that this contributes to the deficiency in DNA strand exchange (Roman and Kowalczykowski, 1986). However, the ATPase activity of recA441 protein is not inhibited by SSB protein at 1 mM magnesium ion and still strand exchange does not occur (Lavery and Kowalczykowski, 1988). This suggests that elevated magnesium ion concentration is required for more than just allowing recA protein to compete with SSB protein, *i.e.* magnesium ion fulfills some other requirement in DNA strand exchange.

Above 1 mM magnesium ion, both the wild-type and recA441 proteins are able to carry out DNA strand exchange (Fig. 4). However, at 2 mM magnesium acetate, neither protein catalyzes DNA strand exchange very effectively. As magnesium ion concentration increases, the rates at which joint molecule intermediate species appear increase more or less equivalently for both recA proteins. The rate of product molecule appearance also increases with increasing magne-

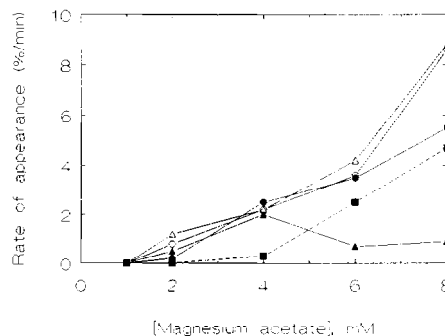


FIG. 4. **Magnesium acetate concentration dependence of DNA strand exchange.** Reactions were performed as described under "Materials and Methods," except that magnesium acetate concentration was varied as indicated. The *maximum* rates of species appearance are represented as follows: for recAwt protein, *open circles* indicate joint molecules and *filled circles* indicate product molecules; for recA441 protein *open triangles* indicate joint molecules, *filled triangles* indicate product molecules, and *squares* indicate homology-dependent DNA networks.

sium acetate concentration for recAwt protein. However, for recA441 protein, the rate of product molecule appearance only increases up to 4 mM magnesium ion. Beyond this concentration, a significant amount of homology-dependent DNA networks are produced, and subsequently the rate of product molecule formation remains low (Fig. 4). The combined rates of network formation plus product molecule formation by recA441 protein parallel product molecule formation by recAwt protein, *i.e.* networks seem to be formed in lieu of product molecules in the recA441 protein reaction.

**Spermidine Reduces the Magnesium Requirement for DNA Strand Exchange**—Buffer containing 1 mM magnesium ion does not support DNA strand exchange by either recAwt protein or recA441 protein. However, if spermidine is added to this buffer, both recA proteins are able to catalyze DNA strand exchange (Fig. 5). At least 0.5 mM spermidine is required in order for any joint molecules to be formed in the recAwt protein-catalyzed reaction. As spermidine concentration increases to 0.75 mM, the rate of joint molecule formation saturates at a value that is less than 20% of that observed under optimal DNA strand exchange conditions (*i.e.* at 8 mM magnesium acetate). Additionally, the rate at which product molecules appear is only about 30% of that observed at 8 mM magnesium acetate, suggesting that joint molecule formation limits product molecule formation by recAwt protein.

In contrast, some joint molecule formation occurs in the recA441 protein reaction when as little as 0.05 mM spermidine is present (Fig. 5). Above 0.05 mM spermidine, the rate of joint molecule formation increases, saturating at a rate comparable to that observed at 8 mM magnesium acetate. Additionally, at saturating spermidine concentration (1 mM), the profile for the conversion of these intermediate species to product molecules is similar to that seen at 8 mM magnesium acetate; homology-dependent DNA networks are produced, and product molecules appear at approximately equivalent rates. Thus, at 1 mM magnesium acetate, the presence of 1 mM spermidine can completely supplant the requirement for elevated magnesium ion concentration in the recA441 protein-catalyzed DNA strand exchange reaction, but not in the recAwt protein-catalyzed reaction.

**RecA441 Protein Can Catalyze DNA Strand Exchange at Low ATP Concentrations**—DNA strand exchange normally requires the presence of ATP or dATP (Cox and Lehman, 1981; Menetski and Kowalczykowski, 1989). Phizicky and Roberts (1981) have shown that, compared with the wild-type recA protein, recA441 protein has a slightly lower apparent

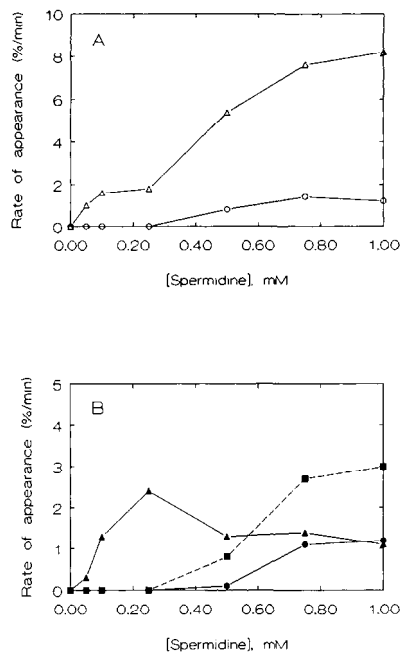


FIG. 5. Spermidine concentration dependence of DNA strand exchange. Reactions were performed as described under "Materials and Methods," except that magnesium acetate concentration was 1 mM, ATP concentration was 0.5 mM, and spermidine concentration was varied as indicated. The maximum rates of species appearance are represented as follows: A, open circles and open triangles indicate joint molecules for recAwt protein and recA441 protein, respectively; B, filled circles and filled triangles indicate product molecules for recAwt protein and recA441 protein, respectively; squares indicate homology-dependent DNA networks for recA441 protein.

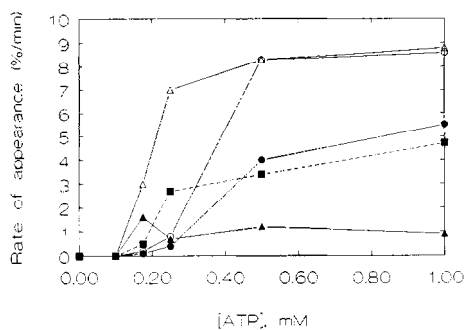


FIG. 6. ATP concentration dependence of DNA strand exchange. Reactions were performed as described under "Materials and Methods," except that ATP concentration was varied as indicated. The maximum rates of species appearance are represented as follows: for recAwt protein, open circles indicate joint molecules and filled circles indicate product molecules; for recA441 protein, open triangles indicate joint molecules, filled triangles indicate product molecules, and squares indicate homology-dependent DNA networks.

$K_m$  value for ATP in the hydrolysis reaction. Consistent with this, the results in Fig. 6 show that recA441 protein is more proficient than recAwt protein in catalyzing DNA strand exchange at low concentrations of ATP. At 0.1 mM ATP and below, neither recA protein displays strand exchange activity. As ATP concentration increases, both proteins begin to be able to catalyze DNA strand exchange, with the activity of the recA441 protein increasing more quickly. The apparent  $K_m$  values for ATP in joint molecule formation are about 0.2 mM for recA441 protein and about 0.35 mM for recAwt protein.

Coaggregation of ssDNA and dsDNA under DNA Strand Exchange Conditions—Tsang *et al.* (1985) have shown that

TABLE II

## The Coaggregation of ssDNA and dsDNA

All reactions were performed in TD buffer and contained 1 mM ATP, 10 units of pyruvate kinase/ml, 7.5 mM phosphoenolpyruvate, 6  $\mu$ M recA protein, 0.9  $\mu$ M SSB protein, 9.9  $\mu$ M ssM13 DNA, 16.8  $\mu$ M dsDNA, and magnesium acetate and spermidine as indicated. RecA protein was added to reaction buffer containing ssDNA 1 min prior to the addition of SSB protein and then coaggregation was assayed as described under "Materials and Methods."

Protein	dsDNA	Percentage of dsDNA in coaggregates <sup>a</sup>		
		8 mM magnesium	1 mM magnesium	1 mM magnesium-1 mM spermidine
recAwt	pBEU41	30	<10	<10
recA441	pBEU41	60	<10	40
recAwt	Lambda	<10	<10	<10
recA441	Lambda	40	<10	<10

<sup>a</sup> The percentage of supercoiled pBEU41 dsDNA or linear  $\lambda$  dsDNA found in the pellet after centrifugation.

recA protein can form homology-independent coaggregates of ssDNA and dsDNA, that pellet rapidly upon centrifugation and have proposed that coaggregates are intermediates in homologous pairing. In an attempt to interpret the observed effects of magnesium and spermidine ions on DNA strand exchange, we examined the coaggregation of ssM13 DNA and non-homologous dsDNA. When the coaggregation of ssM13 DNA and pBEU41 dsDNA is assayed under optimal conditions for DNA strand exchange (rather than conditions optimal for coaggregation (Tsang *et al.*, 1985)), about 30% of the supercoiled pBEU41 DNA is found in coaggregates in the recAwt protein reaction, and about 60% in the recA441 protein reaction (Table II). At 1 mM magnesium acetate, less than 10% of the dsDNA is found in the pellet in both the recAwt protein and recA441 protein reactions. At 1 mM magnesium acetate, the addition of 1 mM spermidine does not change the amount of dsDNA found in the pellet in the recAwt protein reaction, but does result in coaggregation of about 40% of the supercoiled DNA in the recA441 protein reaction.

Since our DNA strand exchange reaction utilizes linear dsM13 DNA as a substrate, we also examined the coaggregation of ssM13 DNA and linear dsDNA. Compared with the reactions using supercoiled dsDNA, less coaggregation of ssM13 DNA and  $\lambda$  dsDNA is observed (Table II). The only instance where greater than 10% of the dsDNA is found in the pellet is in the recA441 protein reaction at 8 mM magnesium acetate. When linear pBR322 dsDNA was used in place of  $\lambda$  dsDNA, comparable results were obtained (data not shown). Thus, there does not appear to be a correlation between the conditions where strand exchange occurs and coaggregation of ssM13 DNA and linear dsDNA is observed.

## DISCUSSION

The recA441 protein is competent in recombination *in vivo* (Castellazzi *et al.*, 1972), and in fact, may have recombinase activity that is enhanced relative to the wild-type recA protein (Lloyd, 1978). Consistent with its recombination proficiency *in vivo*, we show that recA441 protein is able to catalyze DNA strand exchange *in vitro*. Under conditions that are optimal for recAwt protein, recA441 protein forms joint molecules at essentially the wild-type rate. However, recAwt protein converts these intermediate species to heteroduplex DNA product molecules more rapidly than recA441 protein, while the recA441 protein reaction produces extensive homology-dependent DNA networks instead. Under suboptimal conditions, the recA441 protein does show increased strand exchange activity relative to recAwt protein, which is compatible

with recA441 protein displaying enhanced recombinase activity *in vivo*.

An interesting observation of the recA441 protein-catalyzed DNA strand exchange reaction is that, although the initial rate of joint molecule formation is the same as for recAwt protein under optimal conditions, conversion to gapped circular heteroduplex DNA product molecules occurs about six times more slowly than in the recAwt protein reaction. In the recA441 protein reaction, DNA species that do not migrate into the gel are produced instead. The time course for the appearance of these species coincides with the disappearance of the initial joint molecules, suggesting that they are formed from the joint molecules. Along these lines, Chow *et al.* (1988) have reported the formation of extensive homology-dependent DNA networks during DNA strand exchange and hypothesized that they result from reinitiation of pairing and strand exchange by the DNA strands partially displaced from the initial joint molecules. They have also suggested that this reinitiation might limit the completion of strand exchange, which is consistent with the slow appearance of product molecules seen in our recA441 protein reaction.

The reason we observe these homology-dependent DNA networks in the recA441 protein reaction, but not the recAwt protein reaction, is most likely due to the fact that our DNA strand exchange reactions are performed in the presence of SSB protein. The irreversible formation of product molecules in the strand exchange reaction has been hypothesized to result from the binding of SSB protein to the 5' end of the displaced linear ssDNA (Roman and Kowalczykowski, 1986). Chow *et al.* (1988) have shown that SSB protein suppresses the formation of homology-dependent DNA networks, presumably by coating the displaced ssDNA and inhibiting reinitiation. By more effectively competing with SSB protein for the displaced strands of the initial joint molecules (Lavery and Kowalczykowski, 1988), recA441 protein would be more competent at reinitiating pairing between them and consequently form extensive DNA networks. Consistent with this interpretation, under conditions where the wild-type recA protein is more effective at displacing SSB protein from ssDNA (*i.e.* at reduced phosphoenolpyruvate concentration and/or increased magnesium ion concentration), the recAwt protein-catalyzed strand exchange reaction produces a significant amount of DNA species that do not enter the gel as well<sup>6</sup> (Kowalczykowski and Krupp, 1989).

When conditions are suboptimal for joint molecule formation (*i.e.* at low magnesium ion concentration, at low ATP concentration, at low spermidine concentration, at low recA protein concentration, or in the SSB-first reaction), homology-dependent DNA networks are not formed by recA441 protein. Under these conditions, joint molecules appear slowly, and as a result, few accumulate and become involved in the reinitiation reactions that lead to the formation of these extensive networks. The concentration dependence profiles for magnesium ion, spermidine, and ATP all exhibit an optimum for product molecule formation. Below the optimal concentration, joint molecule formation is slow and appears to limit product formation. Above this concentration, the rate of joint molecule formation is more rapid, but homology-dependent DNA networks begin to appear at the expense of product molecules.

Under conditions where homology-dependent DNA networks are formed in the recA441 protein reaction, they appear at a rate comparable to the rate at which product molecules appear in the recAwt protein reaction. Thus, the rate-limiting process for branch migration in the recAwt protein reaction

and for reinitiation of joint molecule formation in the recA441 protein reaction appears to be the same. Thus, a certain length of ssDNA must be produced by a rate-limiting branch migration step before rapid reinitiation events can occur, or both branch migration and reinitiation are limited by a common step. This step could include a redistribution of the recA protein-DNA complex, requiring dissociation from and reassociation with ssDNA.

Under suboptimal conditions, differences in the DNA strand exchange activities of the wild-type and recA441 proteins become apparent, and generally recA441 protein displays enhanced activity. Consistent with its greater ATPase and protease activities using ssDNA that has been complexed with SSB protein (Lavery and Kowalczykowski, 1988), the DNA strand exchange activity of the recA441 protein is impeded less in the SSB-first reaction. The difference between the two recA proteins under these reaction conditions is most pronounced at 37 °C, where the ability of recAwt protein in SSB protein displacement is relatively poor, and DNA strand exchange activity is limited. At 42 °C, the ability of recAwt protein in SSB protein displacement is sufficiently improved that significant DNA strand exchange occurs; final product molecules appear as rapidly as they do in the recA441 protein reaction.

Although the rate at which joint molecules appear at 42 °C in the SSB-first recAwt protein reaction is still quite a bit less than in the SSB-second reaction, the conversion of joint molecules to final product molecules occurs at a rate approaching that observed in the SSB-second reaction. In the SSB-second reaction, joint molecules appear rapidly and accumulate, indicating that branch migration is the limiting step to complete DNA strand exchange (Cox and Lehman, 1981). In the SSB-first reaction, joint molecule formation is much slower and few accumulate. As a result, product molecules appear about 15 min later in the time course of the SSB-first reaction. However, the maximum rate at which joint molecules are converted to final product molecules, *i.e.* the rate of branch migration, appears to be independent of the rate at which joint molecules were formed.

Neither the wild-type recA protein nor the recA441 protein is able to catalyze DNA strand exchange at 1 mM magnesium acetate. Since SSB protein displaces recAwt protein from ssDNA at this magnesium ion concentration, strand exchange cannot occur (Roman and Kowalczykowski, 1986). However, recA441 protein is able to displace SSB protein from ssDNA at 1 mM magnesium ion (Lavery and Kowalczykowski, 1988), and still strand exchange does not occur. This implies a role for elevated magnesium ion concentration in DNA strand exchange in addition to enabling recA protein to compete with SSB protein. One possibility is that the requirement for elevated magnesium ion concentration may be related to coaggregation, since the ability of recA protein to form coaggregates of ssDNA and dsDNA has been shown to be dependent on elevated magnesium ion concentration (Tsang *et al.*, 1985). However, our examination of coaggregation has shown no correlation between the conditions where DNA strand exchange occurs and those where coaggregation of ssM13 DNA and non-homologous linear dsDNA is observed.

It is known that magnesium ion concentration has an effect on the aggregation state of the recA protein (Brenner *et al.*, 1988; Cotterill and Fersht, 1983; Roman and Kowalczykowski, 1986) and increased concentrations will induce recA protein precipitation (Cotterill *et al.*, 1982). Increased concentrations of spermidine will also induce the precipitation of recA protein (Griffith and Shores, 1985), and its presence has been shown to enhance D-loop formation by the recA protein (Shibata *et*

<sup>6</sup> P. E. Lavery and S. C. Kowalczykowski, unpublished observation.

al., 1980). These observations, together with our finding that the presence of spermidine reduces the magnesium ion concentration required for DNA strand exchange, suggest that the requirement for elevated magnesium ion concentration in strand exchange could be related to a requirement for a certain degree of recA protein aggregation.

Lloyd (1978) observed slightly heightened recombinase activity for recA441 protein *in vivo*. However, under optimal conditions for DNA strand exchange, we do not see increased activity by recA441 protein. Thus, these conditions may not be stringent enough to allow for the detection of slight differences between the activities of the two recA proteins. However, the increased activity that we observe under suboptimal conditions (*i.e.* at low ATP concentration, at low magnesium ion concentration in the presence of spermidine, or in the SSB first reaction), is consistent with the idea that these conditions are necessary to provide a window for observing an enhancement in recA441 protein strand exchange activity. This enhancement would be manifest in heightened recombinase activity in the more demanding *in vivo* situation.

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