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Formation of Heteroduplex DNA Promoted by the Combined Activities of *Escherichia coli* recA and recBCD Proteins*

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We have established an *in vitro* reaction in which heteroduplex DNA formation is dependent on the concerted actions of recA and recBCD proteins, the major components of the recBCD pathway of genetic recombination *in vivo*. We find that heteroduplex DNA formation requires three distinct enzymatic functions: first, the helicase activity of recBCD enzyme initiates heteroduplex DNA formation by unwinding the linear double-stranded DNA molecule to transiently form single-stranded DNA (ssDNA); second, recA protein traps this ssDNA before it reanneals; third, recA protein catalyzes the pairing of this ssDNA molecule with another homologous ssDNA molecule, followed by the renaturation of these molecules to form heteroduplex DNA. The first two functions should be important to all *in vitro* reactions involving recA and recBCD proteins, whereas the third will be specific to the DNA substrates used. The rate-limiting step of heteroduplex DNA formation is the trapping by recA protein of the ssDNA produced by recBCD enzyme. A model for this reaction is described.

Homologous genetic recombination is a complex cellular process involving many different proteins. Mutational analyses have shown that both recA protein and recBCD enzyme play central roles in this process (Clark, 1973). RecA protein has been studied in great detail *in vitro* (for review, see Radding, 1982; Cox and Lehman, 1987; Kowalczykowski, 1987) and has been shown to catalyze two major reactions: the ATP-stimulated renaturation of complementary ssDNA[1] (Bryant and Lehman, 1985; McEntee, 1985) and the ATP-dependent invasion of dsDNA by homologous ssDNA (for review, see Kowalczykowski, 1987; Cox and Lehman, 1987). Although DNA strand invasion is thought to be an important activity *in vivo*, renaturation activity may also be important. Of the mutant recA proteins examined, those defective in genetic recombination are also defective in renaturation activity. RecA629 protein, the product of a mutant which exhibits a cold-sensitive phenotype for recombination, is also cold-sensitive for renaturation activity *in vitro* (Weinstock *et al.*, 1979). Similarly, recA1 protein is both recombination-deficient *in vivo* (Clark and Margulies, 1965) and deficient in renaturation activity *in vitro* (Bryant and Lehman, 1986; see below).

RecBCD enzyme is a complex multifunctional enzyme consisting of three nonidentical subunits which together possess ATPase, nuclease, and helicase activities (for review, see Telander-Muskavitch and Linn, 1981; Smith, 1988; Taylor, 1988). The nuclease activities of recBCD enzyme are inhibited by high concentrations of ATP (Mackay and Linn, 1976), SSB protein (Mackay and Linn, 1976), and calcium ions (Rosamond *et al.*, 1979), all of which are present *in vivo*. Helicase activity, on the other hand, is relatively unaffected (Roman and Kowalczykowski, 1989), suggesting that recBCD helicase activity is active and may play a significant functional role *in vivo*.

The helicase activity of recBCD enzyme has been described (Taylor and Smith, 1980; Muskavitch and Linn, 1982) and biochemically characterized (Roman and Kowalczykowski, 1989). RecBCD enzyme enters a linear dsDNA molecule at an end and moves rapidly through the molecule, unwinding the DNA. In the absence of SSB protein, the ssDNA produced by recBCD helicase activity rapidly reanneals behind the enzyme. In the presence of SSB protein, reannealing of the ssDNA is prevented, resulting in the production of fully ssDNA molecules coated with SSB protein, provided the dsDNA is less than 50 kilobase pairs in length.[2]

Although the genetic evidence is ambiguous, it indicates that at least one role for recBCD enzyme occurs early in recombination (*i.e.* before recA protein action; Yancey and Porter, 1985; Porter *et al.*, 1978), suggesting that the role of recBCD enzyme is to create a DNA substrate suitable for use by recA protein (Smith *et al.*, 1984). To fully understand how recA and recBCD proteins function together *in vivo* to promote homologous recombination, *in vitro* reactions requiring both recA and recBCD functions for heteroduplex DNA formation are essential. Such studies will eventually permit the reconstitution of an *in vitro* recombination system using purified biochemical components.

In this paper, we describe a reaction which focuses on potential initiation events catalyzed by recBCD enzyme *in vitro*. We use this assay, consisting of linear dsDNA, circular ssDNA, and recA and recBCD proteins, to examine the ability of recA and recBCD proteins to catalyze the formation of heteroduplex DNA *in vitro*. We find that, for recBCD-dependent heteroduplex DNA formation to occur, recBCD enzyme must first unwind the dsDNA substrate. RecA protein

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; SSB protein, *E. coli* ssDNA binding protein; RF, replicative form; SDS, sodium dodecyl sulfate; ATPγS, adenosine 5′-*O*-(thio)triphosphate.

[2] L. J. Roman and S. C. Kowalczykowski, manuscript in preparation.

must trap the unwound single strands of DNA which it then uses to catalyze the formation of heteroduplex DNA; the renaturation activity of recA protein is essential for this process. Thus, recBCD enzyme creates a DNA substrate upon which recA protein is able to act. These results are consistent with the genetic evidence and models (Smith *et al.*, 1984) in which the helicase activity of recBCD enzyme is used early (*i.e.* in initiation) in the recBCD pathway of homologous recombination.

EXPERIMENTAL PROCEDURES

Protein and DNA Isolation—M13 ssDNA and dsDNA were isolated from bacteriophage M13 mp7 by using the procedure of Messing (1983). Heterologous DNA (pBEU41) was prepared from strain BEU293 (kindly provided by A. John Clark of University of California, Berkeley); pBEU41 is plasmid pBEU2 (Uhlin *et al.*, 1983) containing the cloned recA56 gene. The molar concentration of nucleotides was determined by using an extinction coefficient of 6500 M^{-1} cm^{-1} for dsDNA and 8784 M^{-1} cm^{-1} for ssDNA. The M13 dsDNA was linearized by digestion with *Eco*RI and the BEU dsDNA was digested with *Bam*HI. The dsDNA referred to as M13 circular dsDNA is a mixture of approximately 50% supercoiled and 50% relaxed RF DNA.

RecA protein was purified using a procedure based on spermidine precipitation,[3] and protein concentration was determined using an extinction coefficient of 2.7×10^4 M^{-1} cm^{-1} at 280 nm. Mutant recA proteins were kindly provided by Renee Krupp (recA142), Polly Lavery (recA441), Joseph Menetski (recA430), and Owen Murray (recA1), all from this laboratory.

RecBCD enzyme was purified as described (based on the procedures of Dykstra *et al.* (1984) as modified by Roman and Kowalczykowski (1989)). Protein concentration was determined using an extinction coefficient of 4.0×10^5 M^{-1} cm^{-1} at 280 nm (Roman and Kowalczykowski, 1989), and the specific activity of the preparation used was 3.5×10^4 nuclease units/mg protein or 8×10^3 helicase units/mg. Nuclease and helicase units were measured as described by Eichler and Lehman (1977) and Roman and Kowalczykowski (1989), respectively.

SSB protein was isolated from strain RLM727 using a protocol provided by Dr. Roger McMacken of The Johns Hopkins University. Protein concentration was determined using an extinction coefficient of 3.0×10^4 M^{-1} cm^{-1} at 280 nm (Ruyechan and Wetmur, 1975).

Reaction Conditions—The standard reaction mixture consisted of 25 mM Tris acetate, pH 7.5, 1 mM dithiothreitol, 1 mM ATP, 1 mM magnesium acetate, 2 mM spermidine acetate, 1.5 mM phosphoenolpyruvate, approximately 8 units/ml pyruvate kinase, 10 μM dsDNA, 5 μM tritium-labeled ssDNA, 5 μM recA protein, and 13.9 nM recBCD enzyme (35.8 helicase units/ml, as defined by Roman and Kowalczykowski, 1989), unless otherwise indicated. The amount of recBCD enzyme present is sufficient to saturate approximately 60% of the dsDNA ends with functional enzyme. Assays were performed at 25 °C (or 37 °C, where indicated) and were begun with the addition of recA protein followed by recBCD enzyme after preincubation of all other components for a few minutes. Denatured dsDNA was created just prior to use by heating intact dsDNA at 95 °C in a water bath for 5 min. Reactions which involve denatured dsDNA were begun by the addition of recA protein after preincubation of all other components for a few minutes.

S1 Nuclease Assay for Heteroduplex DNA Formation—Aliquots (50 μl) of the above reaction mixtures were stopped at various times by the addition of 0.1 volume of 10% SDS and placement on ice. S1 nuclease digest buffer (300 μl; 60 mM sodium acetate, 100 mM sodium chloride, and 2 mM zinc chloride, pH 4.6) was added to each aliquot along with approximately 40 units of S1 nuclease. After incubation at 37 °C for 30 min, calf thymus DNA (15 μl of a 2.5 mg/ml stock) and 15% trichloroacetic acid (300 μl) were added. The sample was put on ice for 15–30 min, spun down in a tabletop centrifuge for 10 min, and the supernatant (500 μl) counted. These data are normalized by the amount of S1 nuclease-sensitive material at zero time (*i.e.* 100% sensitive) to yield the percent of DNA sensitive to S1 nuclease. The rates were measured from the slope of the linear region of the time course.

Aggregation Assay—The aggregation assay used was based on that of Tsang *et al.* (1985) with the following modifications. Aliquots (30 μl) consisted of 15 μM ssDNA and 5 μM recA protein in the standard buffer described above. The aliquots were incubated at 25 °C for 30 min, then spun in a tabletop microfuge for 5 min. The supernatant (30 μl) was removed and added to 3 μl of 10% SDS, while the pellet was resuspended in 33 μl of 1% SDS. Both the pellet and the supernatant aliquots were loaded onto a 0.8% agarose gel and electrophoresed in TAE buffer (40 mM Tris acetate, 2 mM EDTA). Single-stranded DNA present in the pellet is indicative of aggregation.

[3] S. C. Kowalczykowski, manuscript in preparation.

RESULTS

RecA and recBCD Proteins Can Catalyze the Formation of Heteroduplex DNA—Formation of heteroduplex DNA by recA and recBCD proteins was measured using linear duplex M13 DNA and tritium-labeled circular ssDNA substrates. Upon heteroduplex DNA production, the labeled ssDNA molecule is protected from degradation by S1 nuclease. As shown in Fig. 1, a time-dependent increase in the amount of ssDNA protected is observed. The total amount of this protection, or heteroduplex DNA formation, is 35% (±5%). The initial rate of heteroduplex DNA formation is 57 nM nucleotides/min.

This increase in protection is dependent on the presence of both recA and recBCD proteins. If either protein is omitted, the total amount of protection observed is 5% (±5%) (Fig. 1). If either M13 circular dsDNA, which cannot be unwound by recBCD enzyme because it has no end (Taylor and Smith, 1985), or nonhomologous linear dsDNA, is substituted for the M13 linear dsDNA (Fig. 1), the total amount of protection observed is also only 5% (±5%) in 60 min (or 4.2 ± 4.2 nM nucleotides/min). Thus, the formation of heteroduplex DNA is dependent on: 1) recA protein, 2) recBCD enzyme, 3) the presence of a linear dsDNA molecule, and 4) homologous DNA substrates. Unless stated in the text, the amount of recBCD enzyme-independent heteroduplex DNA formation under any particular set of conditions at 25 °C was found to be 5% (±5%) in 60 min or 4.2 (± 4.2) nM nucleotides/minute.

Electrophoresis of deproteinized reaction products in an agarose gel showed that both ssDNA and dsDNA were retained in the wells in a time-dependent fashion (not shown), indicating that complex DNA structures too large to enter the gel were being formed. This is similar to what was observed

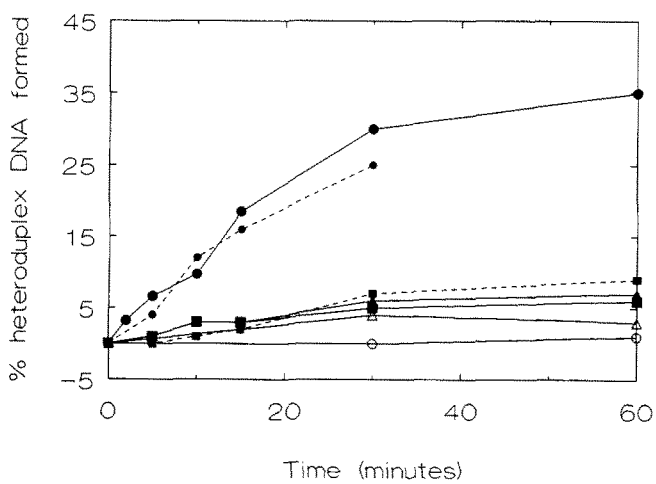

FIG. 1. **The formation of heteroduplex DNA by the combined actions of recA and recBCD proteins.** *Circles*, standard reaction containing 10 μM linear dsDNA, 5 μM circular ssDNA, 5 μM recA protein, and 13.9 nM recBCD enzyme; *triangles*, standard reaction without recA protein; *squares*, standard reaction without recBCD enzyme; *open circles*, standard reaction with circular dsDNA (M13 RF) instead of linear dsDNA; *open triangles*, standard reaction with linear BEU plasmid dsDNA instead of linear M13 dsDNA. The *solid* and *dashed lines* represent data at 25 and 37 °C, respectively.

for recA protein-dependent renaturation (Bryant and Lehman, 1985). The simplest interpretation of both the agarose gel and the S1 nuclease results is that recBCD enzyme is unwinding the dsDNA to form a substrate (ssDNA) to which recA protein can bind. RecA protein can then pair and renature the complementary strands of DNA,[4] forming a DNA network which does not migrate into an agarose gel.

We also investigated the effect of recBCD enzyme on recA protein-dependent formation of heteroduplex DNA at 37 °C, under conditions where recA protein alone can catalyze limited heteroduplex DNA formation. As shown in Fig. 1, the rate of heteroduplex DNA formation in the absence of recBCD enzyme is only slightly above background levels. Under these conditions, recBCD enzyme stimulates the rate of this reaction by 5-fold over that observed with recA protein alone (Fig. 1). Since recBCD enzyme will completely unwind the dsDNA in approximately 30 s at 37 °C (not shown) and heteroduplex DNA formation is slow under these conditions, the heteroduplex DNA formed is probably due mainly to recBCD-dependent stimulation of the renaturation activity of recA protein rather than to stimulation of joint molecule formation[5] (see below).

The Unwinding of dsDNA by recBCD Protein Is Not the Rate-limiting Step in Heteroduplex DNA Formation—The amount of recBCD enzyme used in these reactions is sufficient to saturate only about 60% of the dsDNA ends present (with respect to helicase activity, see Roman and Kowalczykowski, 1989; not shown). Thus, if the rate of dsDNA unwinding by recBCD enzyme was rate-limiting in heteroduplex DNA formation, then the rate of heteroduplex DNA formation would be dependent on recBCD enzyme concentration. An increase in the amount of recBCD enzyme, however, has a relatively small effect on the rate of heteroduplex DNA formation. When the concentration of recBCD enzyme is doubled, the concomitant rate increase is less than 20%. A 10-fold increase in recBCD enzyme concentration (from 2 to 20 nM) results in an increase in the rate of unwinding of less than 2-fold (36.0–62.5 nM/min). Thus, the helicase activity of recBCD enzyme is not rate-limiting in this reaction. This is consistent with the observation that, under these conditions (*i.e.* at 25 °C), recBCD enzyme will unwind all of the dsDNA present in under 2 min, yet complete heteroduplex DNA formation requires more than 30 min.

The Rate of Heteroduplex DNA Formation Is Dependent on the Absolute Concentration of recA Protein—To determine the function of recA protein in heteroduplex DNA formation, we examined the dependence of this reaction on recA protein concentration (Fig. 2, *squares*). The rate of heteroduplex DNA formation as a function of recA protein concentration exhibits a broad optimum with a midpoint of 3 μM. At the optimal recA protein concentration (3 μM), the molar ratio of protein to total DNA (in nucleotides) is 1 to 5. In contrast, the optimal ratio for recA protein-dependent renaturation activity occurs at a protein to nucleotide ratio of 1 to 30 (Bryant and Lehman, 1985; McEntee, 1985).

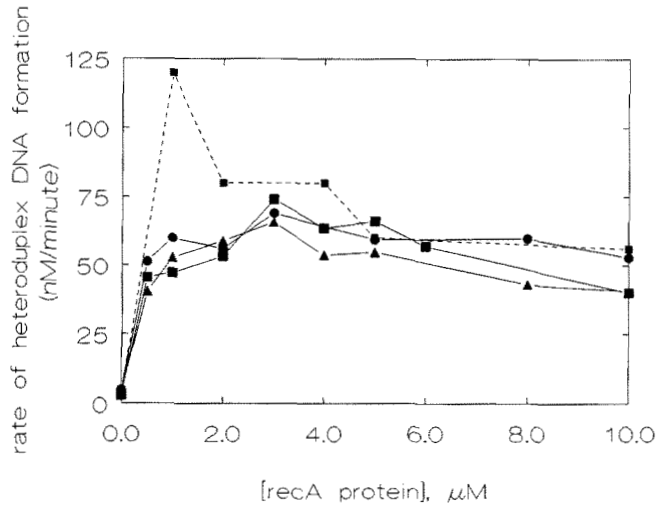

FIG. 2. **RecA protein concentration dependence of heteroduplex DNA formation.** *Squares*, reactions contained 10 μM dsDNA and 5 μM ssDNA; *circles*, reactions contained 20 μM dsDNA and 5 μM ssDNA; *triangles*, reactions contained 10 μM dsDNA and 10 μM ssDNA. All reactions contained 13.9 nM recBCD enzyme and were performed in standard reaction buffer. The *solid* and *dashed lines* represent data at 25 and 37 °C, respectively.

To determine whether the optimal amount of recA protein required for heteroduplex DNA formation is a function of the amount of ssDNA present (as would be the case in recA protein-dependent renaturation), the recA protein concentration was varied in reactions containing twice the amount of either ssDNA (10 μM) or dsDNA (20 μM) as those described above. As shown in Fig. 2, the optimal recA protein concentration required for either reaction remains unchanged at 3 μM. Since the optimal recA protein concentration does not change with either the ssDNA or dsDNA concentration, it appears that the absolute concentration of recA protein present is more critical than the ratio of protein to DNA. Thus, the role of recA protein in recBCD-dependent heteroduplex DNA formation must be more than to simply catalyze the renaturation of ssDNA.

Fig. 2 also shows the dependence of heteroduplex DNA formation on recA protein concentration at 37 °C. Note that the concentration of recA protein required to achieve the maximum rate of heteroduplex DNA formation has been shifted to 1 μM, indicating that the property of recA protein responsible for the dependence of this reaction on absolute recA protein concentration is sensitive to temperature.

Properties of recA Protein-catalyzed Renaturation—Since the buffer and DNA substrates used here differ from those used to study recA protein-catalyzed renaturation, we examined recA protein-dependent DNA renaturation under our experimental conditions. The initial rates of renaturation of circular ssDNA and heat-denatured linear dsDNA as a function of recA protein concentration are shown in Fig. 3. The optimal rate of renaturation, using either our buffer or that described by Bryant and Lehman (1985), is observed at 0.5 μM recA protein, corresponding to a stoichiometry of recA protein to total nucleotides present of 1 to 30. This is the same stoichiometry observed by Bryant and Lehman (1985) using complementary ssDNA substrates. This result eliminates the possibility that the anomalous optimal ratio of protein to DNA observed for heteroduplex DNA formation is

[4] The final extent of heteroduplex DNA formation in this reaction is comparable to that observed in the renaturation of denatured dsDNA by recA protein. In the renaturation studies by Bryant and Lehman (1985), recA protein was able to renature approximately 75% of denatured dsDNA. Similar studies by McEntee (1985) showed that the final extent of renaturation by recA protein is approximately 60% of the input DNA. For our DNA substrates, only half the extent of renaturation observed by other investigators would be expected since our system contains an equal concentration of both duplex and ssDNA molecules. When the duplex is unwound, there is an equal probability that a ssDNA molecule will reanneal with an unlabeled partner as with the labeled ssDNA. The final extent of reaction in our system is approximately 30%, one-half the amount observed in renaturation studies using equal concentrations of ssDNA.

[5] To permit unambiguous mechanistic analysis of the reaction described in this paper, most experiments were conducted at 25 °C, where recA protein-dependent joint molecule formation is prevented.

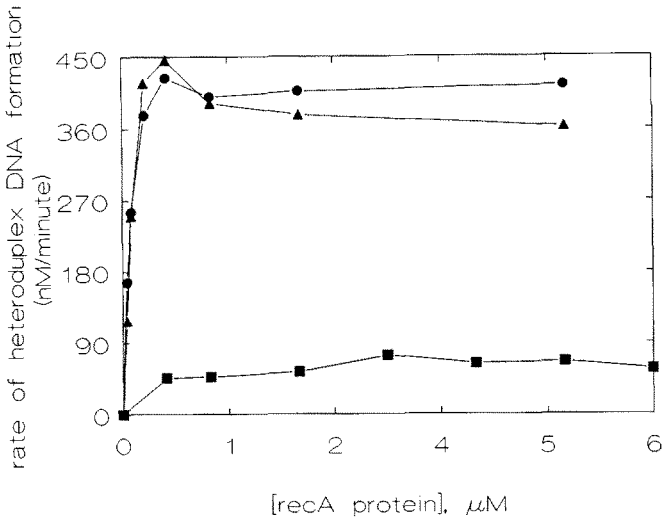

FIG. 3. **RecA protein concentration dependence of renaturation and heteroduplex DNA formation.** *Circles,* renaturation in the buffer described by Bryant and Lehman (1985) (25 mM Tris-HCl, pH 7.2, 10 mM magnesium chloride, 200 μM ATP, 5% glycerol, and an ATP regenerating system); *triangles,* renaturation in standard buffer (25 mM Tris acetate, pH 7.5, 1 mM each of dithiothreitol, ATP, and magnesium acetate, 2 mM spermidine acetate and an ATP regenerating system); *squares,* heteroduplex DNA formation in standard reaction buffer. Reactions contained 10 μM dsDNA, 5 μM tritium-labeled ssDNA, and 13.9 nM recBCD enzyme, when present. The dsDNA used in the renaturation reactions was heat-denatured prior to use.

merely a result of our assay conditions. Instead, it supports the proposal that recA protein is required for more than just the catalysis of renaturation in heteroduplex DNA formation.

To ensure that the action of recBCD enzyme does not alter the dsDNA such that the rate or extent of recA protein-catalyzed renaturation is affected, recBCD enzyme was allowed to unwind duplex DNA in the absence of recA protein. This mixture was then extracted with phenol to remove recBCD enzyme, and the dsDNA was heat-denatured. Both the rate and extent of recA protein-catalyzed renaturation of this DNA were indistinguishable from those of heat-denatured DNA which had not been pretreated with recBCD enzyme (not shown).

A comparison of the reaction rates obtained for the recA- and recBCD protein-dependent heteroduplex DNA formation *versus* those for recA protein-catalyzed renaturation reveals that, although the final extents of the reaction are identical in both cases (30 ± 5%; not shown), the *rates* of heteroduplex DNA formation are 8–9-fold slower in the reactions containing recBCD enzyme as compared to those using heat-denatured dsDNA (Fig. 3). This suggests that, compared to heat denaturation, the helicase activity of recBCD enzyme is much less effective at providing a ssDNA substrate suitable for subsequent recA protein action. This is true despite the fact that recBCD enzyme unwinds the M13 dsDNA substrate in approximately 2 min in both the presence and absence of recA protein (not shown). Under these conditions, the half-time for recA protein-catalyzed renaturation is approximately 2 min whereas the half-time for heteroduplex DNA formation is approximately 20 min, implying that recA protein is 10-fold less effective at utilizing the transient ssDNA produced by recBCD enzyme unwinding activity than the ssDNA produced by heat denaturation. These results suggest that trapping of the strands of DNA unwound by recBCD enzyme is an important function of recA protein in the heteroduplex DNA formation reactions.

The Ability of Mutant recA Proteins to Catalyze Heteroduplex DNA Formation Parallels Their in Vivo Phenotype—To assess the biological significance of this reaction, we examined the behavior of several mutant recA proteins in recBCD-dependent heteroduplex DNA formation. Those examined were: recA1 protein (Clark and Margulies, 1965) and recA142 protein (Clark, 1973), which are defective in genetic recombination; recA430 protein, which has 80% of wild-type recombination function (Morand *et al.*, 1977); and recA441 protein, which is recombination-proficient (Castellazi *et al.*, 1972).

The initial rates of recBCD-dependent heteroduplex DNA formation are shown as a function of mutant recA protein concentration in Fig. 4. The recA1 and recA142 proteins have no measurable heteroduplex DNA formation activity. RecA430 protein has reduced levels of activity, while recA441 protein is capable of catalyzing heteroduplex DNA formation at a maximal rate 1.4-fold greater than that of wild-type recA protein. Thus, the trends observed for heteroduplex DNA formation *in vitro* are similar to the *in vivo* recombination activities of each mutant recA protein.

To establish whether there is a parallel between the heteroduplex DNA formation reaction and the renaturation activity of the mutant recA proteins, renaturation reactions were performed under the same buffer conditions used in heteroduplex DNA formation. The initial rates of renaturation are shown in Table I. RecA1 and recA142 proteins are totally deficient in renaturation, paralleling their lack of activity in

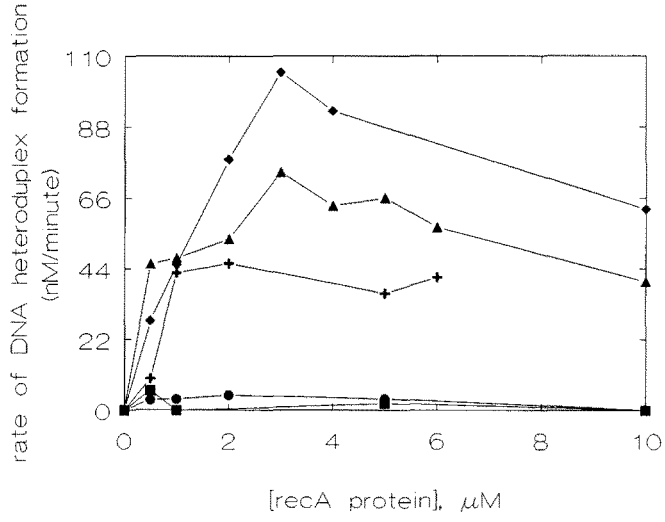

FIG. 4. **Effect of mutant recA proteins on heteroduplex DNA formation.** *Circles,* recA1 protein; *squares,* recA142 protein; *crosses,* recA430 protein; *triangles,* wild-type recA protein; *diamonds,* recA441 protein. Reactions contained 10 μM dsDNA, 5 μM tritium-labeled ssDNA, and 13.9 nM recBCD enzyme and were performed in standard reaction buffer.

TABLE I

Initial rates of renaturation of denatured DNA by mutant recA proteins

Reactions contained 10 μM heat-denatured dsDNA, 5 μM tritium-labeled ssDNA, and 5 μM recA protein. The extent of renaturation at completion was 30 ± 5% for all reactions. Standard buffer conditions were used.

Protein	Rate of renaturation
	nM/min
recAwt	350
recA1	6
recA142	6
recA430	140
recA441	250

the recBCD-dependent heteroduplex DNA formation reaction. The rate of renaturation catalyzed by recA430 protein is approximately 40% of that of wild-type recA protein; this result also parallels the slightly inhibited performance of recA430 protein in the heteroduplex DNA formation reaction. RecA441 protein, which catalyzes heteroduplex DNA formation at a rate 1.4-fold that of wild-type recA protein, renatures DNA with an initial rate that is only 70% of that of wild-type recA protein. These observations again suggest that while renaturation activity is necessary, it is not the sole recA protein-dependent function contributing to the formation of heteroduplex DNA.

The Effect of SSB Protein on the Initial Rate of Heteroduplex DNA Formation—McEntee (1985) showed that *Escherichia coli* SSB protein will greatly inhibit the rate of recA protein-catalyzed renaturation; the rate of renaturation is inhibited by 50% at a concentration of SSB protein that saturates less than 10% of the total DNA. Since our data suggest that one of the roles of recA protein in heteroduplex DNA formation is the renaturation of ssDNA produced by recBCD enzyme, SSB protein should also have an inhibitory effect on heteroduplex DNA formation. On the other hand, SSB protein may enhance heteroduplex DNA formation by assisting recA protein in the trapping of ssDNA produced by recBCD enzyme (Roman and Kowalczykowski, 1989).

The data shown in Table II indicate that, at low concentrations of SSB protein, heteroduplex DNA formation is virtually unaffected. At SSB protein concentrations greater than or equal to 0.5 μM, however, the reaction is abolished. Since SSB protein has no effect on the initial rate of recBCD enzyme helicase activity (Roman and Kowalczykowski, 1989), SSB protein must exert its effect on one of the functions of recA protein in this system.

Tsang *et al.* (1985) described the aggregation of recA protein and ssDNA and suggested that aggregates containing ssDNA and recA protein are intermediates in the pairing of complementary ssDNA prior to renaturation. They observed that recA protein-dependent aggregation of ssDNA is inhibited by SSB protein. Therefore, we examined this aggregation behavior using the same buffer conditions and SSB protein concentrations used in heteroduplex DNA formation (Table II). The concentration of SSB protein required to inhibit aggregation is exactly the same as that required to inhibit heteroduplex DNA formation. Thus, inhibition of aggregation and, therefore, recA protein-catalyzed renaturation of ssDNA by SSB protein appears to be the basis for the inhibitory effect of SSB protein on heteroduplex DNA formation.

ATP Is Required for Heteroduplex DNA Formation—The activities of both recA and recBCD proteins are either dependent on or stimulated by ATP hydrolysis. Therefore, the effect of ATP concentration on both heteroduplex DNA formation and renaturation was examined (Fig. 5). There is little heteroduplex DNA formation in the absence of ATP. The apparent K_m values for ATP in heteroduplex DNA formation and renaturation (if only data to 100 μM are considered) reactions are 22 (±14) and 6 (±4) μM ATP, respectively.

A limited extent of renaturation (about 10% of that in the presence of ATP only) can be catalyzed by recA protein in the absence of ATP using 200 μM ATPγS as the nucleotide cofactor (Bryant and Lehman, 1985). As shown in Table III, the rate of renaturation of denatured DNA in the presence of 500 μM ATPγS (in the absence of ATP) is 7.5% of the renaturation rate observed in the presence of 500 μM ATP (in

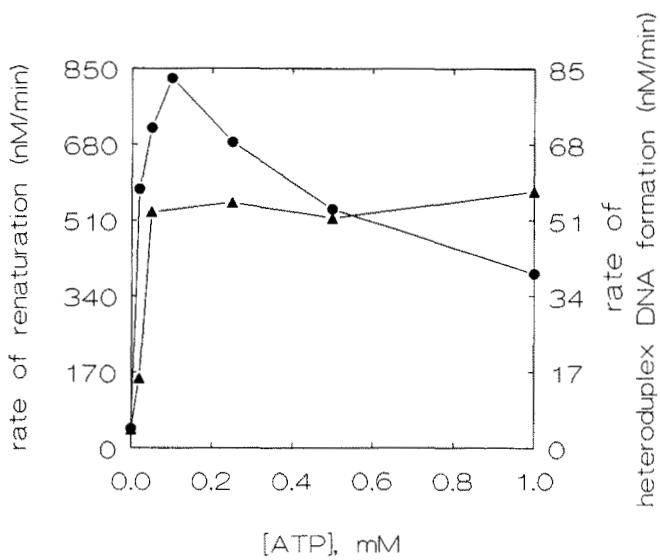

FIG. 5. **Heteroduplex DNA formation and DNA renaturation as a function of ATP concentration.** *Triangles*, heteroduplex DNA formation; *circles*, DNA renaturation. Reactions contained 10 μM dsDNA (heat-denatured for DNA renaturation experiments), 5 μM tritium-labeled ssDNA, and 5 μM recA protein in standard reaction buffer. Heteroduplex DNA formation reactions contained 13.9 nM recBCD enzyme.

TABLE II

Effect of SSB protein on heteroduplex DNA formation and on aggregation of ssDNA by recA protein

Heteroduplex DNA formation reactions contained 5 μM tritium-labeled ssDNA, 10 μM dsDNA, 5 μM recA protein, and 13.9 nM recBCD enzyme. Aggregation reactions contained 15 μM M13 ssDNA and 5 μM recA protein. All reactions were performed in the standard buffer.

SSB protein	Rate of heteroduplex formation	Aggregation[a]
μM	nM/min	
0.0	57	+
0.05	53	ND
0.1	49	+
0.2	41	+
0.5	<1	±[b]
1.0	<1	−
5.0	2	−

[a] +, all ssDNA was present in the pellet; −, all ssDNA was present in the supernatant; ±, ssDNA present in both the pellet and the supernatant; ND, not determined.

[b] The pellet and supernatant contained about 25 and 75%, respectively, of the ssDNA.

TABLE III

Heteroduplex DNA formation as a function of ATP concentration in the presence of 500 μM ATPγS

Reactions contained 10 μM dsDNA, 5 μM tritium-labeled ssDNA, and 5 μM recA protein. The heteroduplex DNA formation reactions contained 13.9 nM recBCD enzyme. In the renaturation experiments, the dsDNA was heat-denatured before use. In all the reactions containing ATPγS, a lag of approximately 5 min is observed before the onset of heteroduplex DNA formation. The rates reported are those achieved after the lag. Under our conditions, in the presence of 500 μM ATPγS, the extent of renaturation is about 12%, approximately equal to the amount of heteroduplex DNA formation under the same conditions (10%; not shown).

[ATP]	Rate of renaturation	Rate of heteroduplex formation
mM	nM/min	
0	40	1
0.1		27
0.25	56	34
0.5		51
1.0	51	67

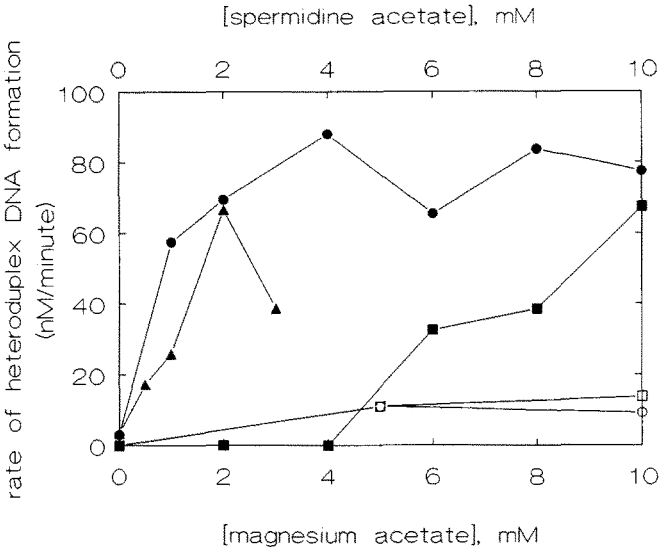

FIG. 6. **Effect of spermidine and magnesium acetate on heteroduplex DNA formation.** *Closed symbols* represent the data obtained in the presence of both recA and recBCD proteins. *Open symbols* represent the data obtained in the presence of only recA protein (no recBCD enzyme was present). *Circles*, vary magnesium acetate in the presence of 2 mM spermidine acetate; *squares*, vary magnesium acetate in the absence of spermidine acetate; *triangles*, vary spermidine acetate in the presence of 1 mM magnesium acetate. Reactions contained 10 μM dsDNA, 5 μM-tritium-labeled ssDNA, 13.9 nM recBCD enzyme, and 5 μM recA protein.

the absence of ATPγS). The nucleotide cofactor ATPγS does not support either the ATPase or helicase activities of recBCD enzyme, nor does it interfere significantly with these activities in the presence of ATP.[6] As ATP concentration is increased, the initial rate of heteroduplex DNA formation increases while the initial rate of renaturation changes little. The fact that renaturation is able to occur with only ATPγS as cofactor while heteroduplex DNA formation (and recBCD enzyme helicase activity) cannot (Table III) underscores the requirement for recBCD enzyme helicase activity in the heteroduplex DNA formation reaction.

Effect of Ions on the Formation of Heteroduplex DNA by recA Protein and recBCD Enzyme—Our buffer contains the polyion spermidine, because both proteins are active under these conditions and the nuclease activity of recBCD enzyme is controlled. (Under our buffer conditions, the ssDNA produced by recBCD enzyme ranges in length from 2 to 7.2 kilobase pairs (not shown).) To determine the optimum, we varied the concentration of spermidine acetate in the heteroduplex DNA formation reaction (Fig. 6, *triangles*). In the absence of spermidine acetate, the rate of heteroduplex DNA formation is only 4.8% of that measured in 2 mM spermidine acetate. At 1 or 3 mM spermidine acetate, the rate of heteroduplex DNA formation is approximately half of that at 2 mM.

Since the magnesium ion has a large effect on both the renaturation (McEntee, 1985; Bryant and Lehman, 1985) and DNA strand exchange activities (Cox and Lehman, 1987; Roman and Kowalczykowski, 1986) of recA protein, we examined the effect of the magnesium ion on heteroduplex DNA formation in the presence and absence of spermidine acetate (Fig. 6). In the presence of 2 mM spermidine acetate (*circles*), there is little or no difference in the rate of heteroduplex DNA formation when the magnesium acetate concentration is greater than 1–2 mM.

In the absence of spermidine acetate, however, no formation of heteroduplex DNA is observed at concentrations of magnesium acetate below 6 mM (Fig. 6, *squares*). Above 6 mM magnesium acetate, the rate of heteroduplex DNA formation increases with increasing magnesium acetate concentration, although a lag of approximately 5 min is observed. At higher magnesium acetate concentrations, the rate of recBCD enzyme-independent heteroduplex DNA formation rises slightly (*open symbols*).

Both magnesium acetate and spermidine acetate affect the aggregation state of recA protein (Roman and Kowalczykowski, 1986; Griffith and Shores, 1985). The magnesium ion concentration has also been shown to exert a large effect on the rate of recA protein association to ssDNA (Chabbert *et al.*, 1987). To determine whether these ions altered the optimal concentration of recA protein required for heteroduplex DNA formation, the recA protein concentration was varied in a buffer containing 10 mM magnesium acetate, either in the presence or in the absence of 2 mM spermidine acetate (not shown). We found that, in the presence of spermidine acetate, there is a very broad optimal concentration of recA protein ranging from 0.5 to 6 μM. In the absence of spermidine, the optimal concentration of recA protein remains the same as that seen in standard buffer (3 μM) (not shown). This result implies that the aggregation state of recA protein, its rate of association with the ssDNA, or both, may be important in determining the optimal concentration of recA protein required for recBCD-dependent heteroduplex DNA formation.

DISCUSSION

We have established an *in vitro* reaction with which to study the formation of heteroduplex DNA promoted by the combined actions of recA and recBCD proteins. This reaction is dependent on the presence of recA protein, recBCD enzyme, and homologous DNA. Heteroduplex DNA formation *in vitro* is dependent on recombination-proficient recA protein since this reaction does not occur when mutant recA proteins defective in genetic recombination *in vivo* are substituted for wild-type protein.

This model system was established as a first step in reconstituting an *in vitro* recombination reaction using purified components of the recBCD pathway for homologous recombination and, more specifically, to determine whether recBCD enzyme could promote the initiation of recA protein-dependent heteroduplex DNA formation *in vitro*. Our data are consistent with the following series of molecular events (see Fig. 7). First, recBCD enzyme unwinds the dsDNA, creating a substrate which can potentially be used by recA protein in a pairing reaction. Secondly, recA protein must trap this ssDNA and prevent it from renaturing with its original complementary strand. Finally, recA protein homologously pairs and reanneals two different, complementary strands of DNA, forming heteroduplex DNA. The rationale for this order of events is presented below.

Unwinding of dsDNA by recBCD Enzyme Is Required—The first step and the major role of recBCD enzyme in the formation of heteroduplex DNA is to initiate the reaction by unwinding the dsDNA. Heteroduplex DNA formation does not occur in the absence of either recBCD enzyme or ATP and is dependent on the presence of a linear dsDNA substrate; such a dsDNA substrate and ATP are required for recBCD enzyme to initiate unwinding (Taylor and Smith, 1985). Studies in the presence of ATPγS also support the role of recBCD enzyme in this process. ATPγS neither supports nor completely inhibits the ATP hydrolysis or DNA helicase activities of recBCD enzyme (in the presence of ATP; not shown). That heteroduplex DNA formation does not occur in the presence

[6] L. J. Roman and S. C. Kowalczykowski, unpublished observation.

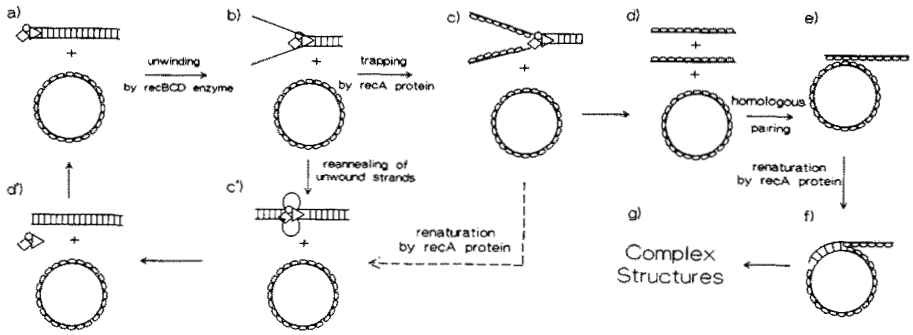

FIG. 7. **Model for heteroduplex DNA formation catalyzed by recBCD and recA proteins.** The symbols shown are: *ovals*, recA protein; *diamond/triangle/circle*, recBCD enzyme. For the sake of simplicity, the single strands of DNA formed by recBCD unwinding are shown as full-length; in actuality, the nuclease activity of recBCD enzyme will cause some nicking of the ssDNA.

of ATPγS (without ATP), even though recA protein is capable of limited DNA renaturation, implies that recBCD enzyme helicase activity is necessary.

Renaturation of ssDNA by recA Protein Is Required—After the dsDNA is unwound by recBCD enzyme, recA protein plays its role in the formation of heteroduplex DNA. The most logical role for recA protein is the renaturation of the substrates produced by recBCD enzyme. The effect of SSB protein on the reaction is consistent with this conclusion. Subsaturating amounts of SSB protein (relative to the concentration of ssDNA) inhibit both recA protein-catalyzed renaturation (McEntee, 1985) and heteroduplex DNA formation. This inhibition of heteroduplex DNA formation by SSB protein occurs at concentrations of SSB protein which parallel the inhibition of recA-dependent ssDNA aggregation, a presumed intermediate step in the pairing of complementary strands prior to their renaturation (Tsang *et al.*, 1985).

The behavior of the mutant recA proteins also supports the role of recA protein-dependent renaturation in heteroduplex DNA formation. RecA1 and recA142 proteins are unable to catalyze renaturation *in vitro*; they are also deficient in the recBCD-dependent heteroduplex DNA formation reaction. The rate of recA430 protein-catalyzed renaturation is decreased *in vitro*; its ability to catalyze heteroduplex DNA formation *in vitro* is also depressed. Thus, the activities of these mutant recA proteins follow the same trends in both the renaturation and heteroduplex DNA formation reactions *in vitro*.

RecA Protein-dependent Trapping of ssDNA Produced by recBCD Enzyme Is Required before DNA Renaturation—Although catalysis of DNA renaturation appears to be a major role for recA protein in heteroduplex DNA formation, the recA protein concentration dependence of heteroduplex DNA formation does not follow that of the renaturation reaction but, rather, is independent of the DNA concentration present. The recA protein concentration optimum for heteroduplex DNA formation appears to be 3 μM at 25 °C, whether the total amount of DNA (in nucleotides) is 15, 20, or 25 μM. This implies that an intrinsic property of free recA protein is important to heteroduplex DNA formation. A possible explanation for this phenomenon is that a recA protein concentration-dependent polymerization or association process is required for trapping of the unwound strands of DNA (see below).

A Model for Heteroduplex DNA Formation Catalyzed by recA and recBCD Enzymes—Fig. 7 depicts a model for the reaction steps which must be occurring during the formation of heteroduplex DNA in our system. The substrates present initially are linear dsDNA and circular ssDNA. RecBCD enzyme begins the process by binding to the end of the linear dsDNA (*a*) and initiating unwinding (*b*). This unwinding is rapid, occurring at 165 base pairs unwound/s/functional recBCD enzyme (not shown). The nuclease activity of recBCD enzyme is also partially active under these conditions, and the ssDNA produced by recBCD enzyme ranges in size from full-length (7.2 kilobase pairs) to about 2.0 kilobase pairs (as measured by agarose gel electrophoresis; not shown). Free recA protein binds to and traps this ssDNA produced by recBCD enzyme (*c–d*), pairs complementary strands (*e*), and begins to renature them (*f*). Extensive renaturation results in the entanglement of these DNA molecules, culminating in complex DNA structures which fail to enter an agarose gel (*g*). One of the recA protein-dependent steps (either *c*, *d*, *e*, or *f*) represents the slow step of heteroduplex DNA formation which occurs over a 30-min time span (see below). This model can, in principle, be generalized to include other proteins (*i.e.* SSB protein for the trapping step (*c* and *d*)), or other DNA substrates (*i.e.* two dsDNA molecules) by substituting the DNA strand invasion activity of recA protein for its renaturation activity (*steps e–f*).[7]

The Nature of the Rate-limiting Step in Heteroduplex DNA Formation—RecBCD enzyme helicase activity is not the rate-limiting step in heteroduplex DNA formation, as supported by the following observations: 1) the rate of unwinding of dsDNA by recBCD enzyme is much faster than that of heteroduplex DNA formation; 2) the rate of heteroduplex DNA formation is not linearly proportional to recBCD enzyme activity; and 3) the ATP concentration dependence of the rate-limiting step in heteroduplex DNA formation (K_m = 22 ± 14 μM ATP) more closely parallels that of recA protein-dependent renaturation (K_m = 6 ± 4 μM ATP) than that of recBCD enzyme-catalyzed unwinding (K_m = 130 μM ATP; Roman and Kowalczykowski, 1989).

Since the unwinding of the dsDNA by recBCD enzyme is not the rate-limiting step in heteroduplex DNA formation, which of the recA protein-catalyzed steps, trapping or renaturation, is? The following observations suggest that renaturation is not rate-limiting in heteroduplex DNA formation; therefore, trapping of the ssDNA produced by recBCD enzyme unwinding must be. First, the rate of renaturation of substrates which have been produced by heat denaturation occurs on a 10-fold faster time scale than the renaturation of identical DNA substrates produced by recBCD enzyme unwinding activity (Fig. 3). Secondly, unlike renaturation, the heteroduplex DNA formation reaction is not inhibited at high ATP concentrations. Thirdly, in the presence of both ATPγS and ATP, the rate of renaturation is reduced, whereas heteroduplex DNA formation proceeds normally. Finally, recA441 protein renatures DNA at a rate that is only 70% of that of wild-type recA protein, yet recA441 protein can catalyze het-

[7] L. J. Roman and S. C. Kowalczykowski, manuscript submitted for publication.

eroduplex DNA formation with an initial rate that is 1.4 times faster than that of wild-type recA protein. Since the rate of renaturation can be reduced greatly (>90%) without inhibiting the rate of heteroduplex DNA formation, renaturation cannot be the rate-limiting step and, therefore, trapping must be.

There are two explanations for the apparently slow trapping of unwound ssDNA by recA protein; either the trapping by recA protein is intrinsically inefficient or the trapping is actually very efficient, but recA protein rapidly reanneals the ssDNA tails formed by recBCD enzyme. The net effect of either possibility is that recBCD enzyme would need to unwind a particular DNA molecule many times before heteroduplex DNA could be formed. We favor the explanation that trapping by recA protein is poor (Fig. 7, *c'-d'*), which is consistent with the following data.

First, the rate of association of recA protein with ssDNA is slow (Chabbert *et al.*, 1987). The postulate that poor trapping is due to the slow association of recA protein with ssDNA is supported by three observations: 1) the optimal concentration of recA protein for heteroduplex DNA formation is shifted to less than 1 μM in buffer conditions which promote aggregation of recA protein (10 mM magnesium acetate and 2 mM spermidine acetate); 2) recA441 protein, which may associate with ssDNA much faster than wild-type recA protein (Lavery and Kowalczykowski, 1988), exhibits the fastest rate of heteroduplex DNA formation (1.4-fold faster than wild-type protein), although its rate of renaturation is 30% lower; and 3) less recA protein is required for optimal heteroduplex DNA formation at 37 °C (Fig. 2) where the association rate of recA protein onto DNA is faster than it is at 25 °C.

Second, the absolute concentration of recA protein, rather than the ratio of recA protein to DNA (as would be expected for renaturation) is important. The requirement for an optimal recA protein concentration could then be explained by two competing effects: at low recA protein concentration, the rate of polymerization onto ssDNA is too slow and trapping becomes less efficient; at high recA protein concentration, trapping becomes very efficient but renaturation is inhibited (Bryant and Lehman, 1985).

Finally, if recA protein were immediately trapping and reannealing the ssDNA created by recBCD enzyme, then a rapid activation of recA protein ssDNA-dependent ATPase activity would be observed. We find that a recA protein-dependent steady-state rate of ATP hydrolysis is not achieved until an amount of time sufficient for recBCD enzyme to have passed through the dsDNA approximately four times has elapsed (not shown). These results are consistent with the poor trapping explanation, although some limited reannealing of the newly formed ssDNA is probably occurring.

The observation that the formation of heteroduplex DNA by mutant recA proteins correlates with their *in vivo* recombination abilities is significant. This correlation implies that either renaturation activity or some other biochemical function which parallels renaturation activity is essential to both the *in vitro* and *in vivo* reactions. In either case, this reaction must reflect properties of recA protein which are likely to be important *in vivo*. Further definition of these specific functions will require detailed examination of more complex reactions, such as those involving two dsDNA molecules,[7] as well as of the behavior of mutant recA and recBCD proteins in these *in vitro* reactions.

How relevant is this reaction to recombination *in vivo*? These studies demonstrate that given appropriate DNA substrates recA and recBCD proteins can function together to generate heteroduplex DNA *in vitro*. Presumably, when presented with analogous DNA molecules *in vivo*, these proteins will catalyze a similar reaction. There are conditions, such as UV-induced damage, which result in extensive formation of free ssDNA and breakage of dsDNA. Since production of SSB protein is not induced until a few hours after DNA-damaging treatment (Brandsma *et al.*, 1983; Moreau, 1987), the ratio of ssDNA to SSB protein will initially be very large. Under these conditions, recA protein renaturation activity may not be fully inhibited by SSB protein, thus allowing recA protein-promoted renaturation between ssDNA produced by DNA damage and ssDNA produced by recBCD enzyme unwinding to occur.

The reaction we have described is interesting in that it is the first report of heteroduplex DNA formation dependent on both recA and recBCD proteins, and it demonstrates that recBCD enzyme can function in the initiation of heteroduplex DNA formation *in vitro*. It is consistent with genetic evidence which implies that recBCD enzyme acts early in recombination and with the Smith *et al.* (1984) model of recombination via the recBCD pathway. They propose that recBCD enzyme initiates recombination by unwinding dsDNA to form ssDNA, a substrate which is suitable for subsequent recA protein activity. This *in vitro* system will serve an important function for understanding the biochemical mechanism of the recA- and recBCD-dependent heteroduplex DNA formation reaction in much the same way the identical DNA substrates have served to elucidate the recA protein-catalyzed DNA strand exchange reaction.

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REFERENCES

Brandsma, J. A., Bosch, D., Backendorf, C., and van de Putte, P. (1983) *Nature* **305**, 243–245

Bryant, F. R., and Lehman, I. R. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 297–301

Bryant, F. R., and Lehman, I. R. (1986) *J. Biol. Chem.* **261**, 12988–12993

Castellazzi, M., George, S., and Buttin, G. (1972) *Mol. Gen. Genet.* **119**, 139–152

Chabbert, M., Cazenave, C., and Hélène, C. (1987) *Biochemistry* **26**, 2218–2225

Clark, A. J. (1973) *Annu. Rev. Genet.* **7**, 67–86

Clark, A. J., and Margulies, A. D. (1965) *Proc. Natl. Acad. Sci. U. S. A.* **53**, 451–459

Cox, M. M., and Lehman, I. R. (1987) *Annu. Rev. Biochem.* **56**, 229–262

Dykstra, C. C., Prasher, D., and Kushner, S. R. (1984) *J. Bacteriol.* **157**, 21–27

Eichler, D. C., and Lehman, I. R. (1977) *J. Biol. Chem.* **252**, 499–503

Griffith, J., and Shores, C. G. (1985) *Biochemistry* **24**, 158–162

Kowalczykowski, S. C. (1987) *Trends Biochem. Sci.* **12**, 141–145

Kowalczykowski, S. C., and Roman, L. J. (1989) *UCLA Symp. Mol. Cell Biol. New Ser.*, in press

Lavery, P. E., and Kowalczykowski, S. C. (1988) *J. Mol. Biol.* **203**, 861–872

Mackay, V., and Linn, S. (1976) *J. Biol. Chem.* **251**, 3716–3719

McEntee, K. (1985) *Biochemistry* **24**, 4345–4351

Messing, J. (1983) *Methods Enzymol.* **101**, 20–78

Morand, P., Blanco, M., and Devoret, R. (1977) *J. Bacteriol.* **131**, 572–582

Moreau, P. L. (1987) *J. Mol. Biol.* **194**, 621–634

Muskavitch, K. M. T., and Linn, S. (1982) *J. Biol. Chem.* **257**, 2641–2648

Porter, R. D., McLaughlin, T., and Low, B. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 1043–1046

Radding, C. M. (1982) *Annu. Rev. Genet.* **16,** 405–437
Roman, L. J., and Kowalczykowski, S. C. (1986) *Biochemistry* **25,** 7375–7385
Roman, L. J., and Kowalczykowski, S. C. (1989) *Biochemistry* **28,** 2863–2873
Rosamond, J., Telander, K. M., and Linn, S. (1979) *J. Biol. Chem.* **254,** 8646–8652
Ruyechan, W. T., and Wetmur, J. G. (1975) *Biochemistry* **14,** 5529–5534
Smith, G. R. (1988) *Microbiol. Rev.* **52,** 1–28
Smith, G. R., Amundsen, S. K., Chaudhury, A. M., Cheng, K. C., Ponticelli, A. S., Roberts, C. M., Schultz, D. W., and Taylor, A. F. (1984) *Cold Spring Harbor Symp. Quant. Biol.* **49,** 485–495
Taylor, A. F. (1988) in *Genetic Recombination* (Kucherlapati, R., and Smith, G. R., eds) pp. 231–263, American Society for Microbiology, Washington, D. C.
Taylor, A. F., and Smith, G. R. (1980) *Cell* **22,** 447–457
Taylor, A. F., and Smith, G. R. (1985) *J. Mol. Biol.* **185,** 431–443
Telander-Muskavitch, K., and Linn, S. (1981) in *The Enzymes* (Boyer, P. D., ed) Vol. 14, pp. 233–250, Academic Press, New York
Tsang, S. S., Chow, S. A., and Radding, C. M. (1985) *Biochemistry* **24,** 3226–3232
Uhlin, B. E., Schweikart, V., and Clark, A. J. (1983) *Gene (Amst.)* **22,** 255–265
Weinstock, G. M., McEntee, K., and Lehman, I. R. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 126–130
Yancey, S. D., and Porter, R. D. (1985) *J. Bacteriol.* **162,** 29–34