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RecBCD-dependent joint molecule formation promoted by the *Escherichia coli* RecA and SSB proteins

(single-stranded DNA-binding protein/genetic recombination/homologous pairing)

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ABSTRACT **We describe the formation of homologously paired joint molecules in an *in vitro* reaction that is dependent on the concerted actions of purified RecA and RecBCD proteins and is stimulated by single-stranded DNA-binding protein (SSB). RecBCD enzyme initiates the process by unwinding the linear double-stranded DNA to produce single-stranded DNA, which is trapped by SSB and RecA. RecA uses this single-stranded DNA to catalyze the invasion of a supercoiled double-stranded DNA molecule, forming a homologously paired joint molecule. At low RecBCD enzyme concentrations, the rate-limiting step is the unwinding of duplex DNA by RecBCD, whereas at higher RecBCD concentrations, the rate-limiting step is RecA-catalyzed strand invasion. The behavior of mutant RecA proteins in this *in vitro* reaction parallels their *in vivo* phenotypes, suggesting that this reaction may define biochemical steps that occur during homologous recombination by the RecBCD pathway *in vivo*.**

The RecA, RecBCD, and SSB (single-stranded DNA-binding) proteins of *Escherichia coli* are key components of the RecBCD pathway of genetic recombination, which is the major pathway for recombination during conjugation and transduction. Mutations in the *recA* gene reduce recombination by as much as 6 orders of magnitude (1), while mutations in the *recB* or *recC* gene can reduce recombination to as low as 0.1% of the wild-type level (2, 3). In addition, mutations in the *ssb* gene reduce recombination by a factor of 5 in this pathway (4). Although the genetic studies have identified the need for these proteins in the RecBCD pathway of recombination, the biochemical basis of their coordinated action remains uncertain.

RecA catalyzes the renaturation of complementary single-stranded DNA (ssDNA) molecules as well as the pairing and exchange of DNA strands between ssDNA and homologous double-stranded DNA (dsDNA) (5, 6). This latter activity is intuitively a good model for the action of RecA during recombination *in vivo*, although some direct involvement of its renaturation activity has not been ruled out.

RecBCD is a multifunctional enzyme with a variety of activities: ssDNA- and dsDNA-dependent ATPase activities, ssDNA and dsDNA nuclease activities, specific cleavage at χ sites, and dsDNA helicase activity (7–9). Genetic evidence suggests that RecBCD enzyme functions, at least early in recombination (i.e., prior to RecA protein action; refs. 10–13), thus supporting a role in initiation of DNA strand exchange for RecBCD. A specific model describing the involvement of both RecA and RecBCD in recombination was proposed by Smith *et al.* (14). A key premise of this model is that RecBCD can unwind dsDNA to produce a ssDNA substrate that, in turn, can be efficiently used by RecA and SSB for synapsis.

We previously described a reaction in which heteroduplex DNA formation between ssDNA and homologous linear dsDNA required both the dsDNA-unwinding activity of RecBCD and the ssDNA-renaturation activity of RecA (15). In this paper, we expand upon the previous *in vitro* studies to include DNA substrates that are more representative of the substrates encountered *in vivo* (i.e., homologous linear dsDNA and supercoiled dsDNA) and that require the joint molecule formation activity of RecA protein. RecA alone is unable to catalyze pairing between two dsDNA molecules unless one of them contains ssDNA in a region of homology (16). Based on our previous observations (15), we proposed that RecA-dependent homologous pairing between linear dsDNA and supercoiled DNA could occur in the presence of RecBCD enzyme. This reaction would require RecBCD helicase action to form ssDNA from the linear dsDNA; RecA and SSB could then use this ssDNA to catalyze DNA strand invasion of the supercoiled DNA. In this paper, we describe the properties of such a reaction. A preliminary account of this work has been published (17).

EXPERIMENTAL PROCEDURES

Protein and DNA Isolation. Replicative form M13 mp7 dsDNA [7.2 kilobases (kb)] was isolated (18) and was linearized with *Eco*RI restriction endonuclease (15). Heterologous DNA was either pBR322 (4.3 kb) or pBEU41 (22 kb) (15, 19).

RecA protein was purified as described (15, 20). Mutant RecA proteins are all from this laboratory. RecBCD enzyme was purified as described (21). The specific activity of the preparation used was 9.4×10^4 nuclease units/mg of protein (22) and 2.1×10^4 helicase units/mg of protein (21), except for the preparation used for the experiments in Fig. 2, which had a specific activity of 5.4×10^4 nuclease units/mg and 1.1×10^4 helicase units/mg. SSB protein was purified as described (15, 23).

Assays. The standard reaction mixture consisted of 25 mM Tris acetate (pH 7.5), 1 mM dithiothreitol, 1 mM ATP, 8 mM magnesium acetate, 1.5 mM phospho*enol*pyruvate, pyruvate kinase (≈4 units/ml), 10 μM linear dsDNA, 5 μM tritium-labeled supercoiled dsDNA, 5 μM RecA, 1 μM SSB, and 5 nM RecBCD (39 helicase units/ml), unless otherwise indicated. Assays were performed at 37°C and were begun with the addition of RecBCD enzyme after preincubation of all other components. Aliquots of the reaction mixture were stopped with 1% SDS to deproteinize the sample and were then placed on ice. When necessary, RecBCD was inactivated after DNA unwinding by phenol/chloroform extraction, followed by ether extraction and use of a Speed Vac to remove residual ether; control DNA treated this way was a substrate for both joint molecule formation and helicase activities. Joint molecule formation was detected either by

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Abbreviations: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

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the nitrocellulose filter assay or by electrophoresis in a 0.7% agarose gel (24, 25).

RESULTS

Formation of Joint Molecules Is Dependent on the Presence of Both RecA and RecBCD. Formation of homologously paired joint molecules can be detected with the nitrocellulose filter assay (25). Fig. 1 shows that joint molecules are formed between M13 linear dsDNA and supercoiled DNA through the combined actions of RecA, RecBCD, and SSB. The extent of RecABCD-dependent joint molecule formation reaches a maximum in about 2 min, with 80% (± 20%) of the supercoiled DNA involved in joint molecules, followed by a slow decrease that reflects the dissociation of joint molecules.

Formation of joint molecules is dependent on the presence of both RecA and RecBCD and on DNA sequence homology; when either protein is omitted, or when nonhomologous linear dsDNA (Fig. 1) is substituted for the homologous linear dsDNA, no detectable joint molecules are formed (Fig. 1). When circular dsDNA is substituted for the linear dsDNA molecule, no joint molecules are detected (data not shown), further supporting the requirement for a RecBCD enzyme activity in this reaction, since RecBCD enzyme cannot initiate unwinding or nuclease activity on dsDNA molecules that lack an end (26). When RecBCD is allowed to act on the DNA substrates for up to 3 min in the absence of both RecA and SSB and then is inactivated by phenol extraction, no joint molecules are detected upon subsequent addition of RecA and SSB (Fig. 1). Three minutes is sufficient time for RecBCD to translocate through the DNA and to completely unwind it if SSB protein (or phage T4-coded gene *32* protein) is present (21); however, in the absence of a ssDNA-binding protein, the unwound ssDNA reanneals behind RecBCD enzyme (9). Thus RecBCD, alone, cannot produce ssDNA that can be subsequently utilized by RecA and SSB to produce joint molecules. On the other hand, when RecBCD is allowed to act on the DNA substrates in the presence of SSB for 3 min and then RecA is added, joint molecule formation is delayed (Fig. 1); this is consistent with the demonstrated delay in the time course of joint molecule formation that occurred when SSB was bound to ssDNA before addition of RecA (27). Thus, the simultaneous action of these three proteins is necessary for optimal joint molecule formation.

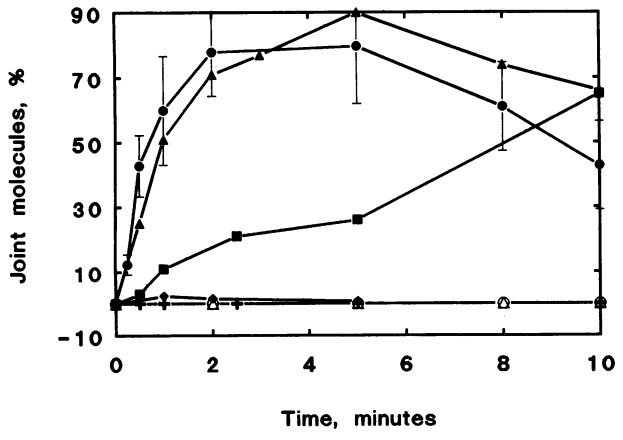

FIG. 1. Formation of joint molecules by the combined actions of RecA, RecBCD, and SSB. ●, Standard assay conditions; ○, without RecA; △, without RecBCD; ◆, standard reaction with nonhomologous linear pBEU41 dsDNA replacing linear M13 dsDNA; ▲, reaction in the absence of RecBCD using heat-denatured linear dsDNA (which had been incubated 2 min with RecBCD prior to heat denaturation); +, RecBCD and linear dsDNA incubated for 3 min and then deproteinized, with subsequent addition of RecA and SSB at time zero; ■, RecBCD, SSB, and dsDNA incubated for 3 min, followed by addition of RecA at time zero.

The initial rate of joint molecule formation in the absence of RecBCD enzyme but using heat-denatured rather than intact linear dsDNA is not reproducibly different (within experimental error) from that observed when ssDNA is created by RecBCD (Fig. 1). This implies that RecA is able to use the ssDNA created by RecBCD helicase activity for strand invasion at least as efficiently as it uses ssDNA created by heat denaturation.

Joint molecules can also be detected by the agarose gel assay as species that migrate more slowly than the linear dsDNA (Fig. 2; lanes 4–9); no joint molecules are detected when heterologous supercoiled pBR322 DNA is used (lanes 1–3). Under these conditions, the yield of joint molecules formed, both in the RecABCD-dependent reaction and in the RecA protein-dependent reaction using heat-denatured linear dsDNA (data not shown), is 10–20% of that observed in the nitrocellulose filter assay; this suggests that most of the joint molecules detected in the nitrocellulose filter assay are not sufficiently stable to survive electrophoresis through an agarose gel. Autoradiography of the gel shown in Fig. 2 confirms the presence of DNA derived from the 5′-end-labeled linear dsDNA in the joint molecule species (data not shown). The joint molecules comprise a heterogeneous distribution upon which are superimposed two discrete species (labeled JM). The heterogeneous population of joint molecules results from the invasion of ssDNA fragments created by the nonspecific nuclease activity of RecBCD during dsDNA unwinding. At 30 sec, ≈10% of the ssDNA produced is full-length or nearly full-length (5–7.2 kb), with the remainder being evenly distributed in length down to 1–2 kb; after 5 min, the ssDNA length is less than 2 kb due to the ssDNA endo- and exonuclease activity of RecBCD (data not shown). In addition to nonspecific cleavage, RecBCD cleaves specifically at the χ site present in M13 DNA [at position 4943; (28)]. The upper discrete band in the gel corresponds to a joint molecule in which a full-length single strand of M13 DNA has invaded the supercoiled M13 DNA, whereas the lower band is an analogous joint molecule that contains a 5.9-kb ssDNA fragment that resulted from strand cleavage by RecBCD at the χ site (D.A.D. and S.C.K., unpublished work). Production of both discrete joint molecule species relative to the heterogeneous species is enhanced at lower RecBCD concentrations, for reasons that will be detailed elsewhere.

Rate of RecABCD-Dependent Joint Molecule Formation Saturates with Increasing RecA Concentration. As shown in Fig. 3, the rate of RecABCD-dependent joint molecule for-

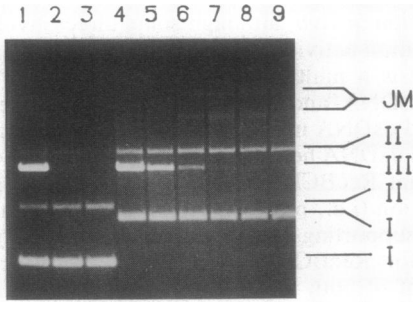

FIG. 2. Analysis of the reaction products by agarose gel electrophoresis. Standard assay conditions were modified to use 40 μM linear M13 dsDNA (form III), 20 μM supercoiled dsDNA (form I), 20 μM RecA, 4 μM SSB, and 0.76 nM RecBCD (2.8 helicase units/ml). Lanes 1–3, form I pBR322 DNA (nonhomologous) at 0, 2.5, and 8 min; lanes 4–9, form I M13 DNA at 0, 0.5, 1, 2.5, 4, and 8 min. The bands corresponding to joint molecule (JM) species are indicated, as well as contaminating nicked circular dsDNA (form II).

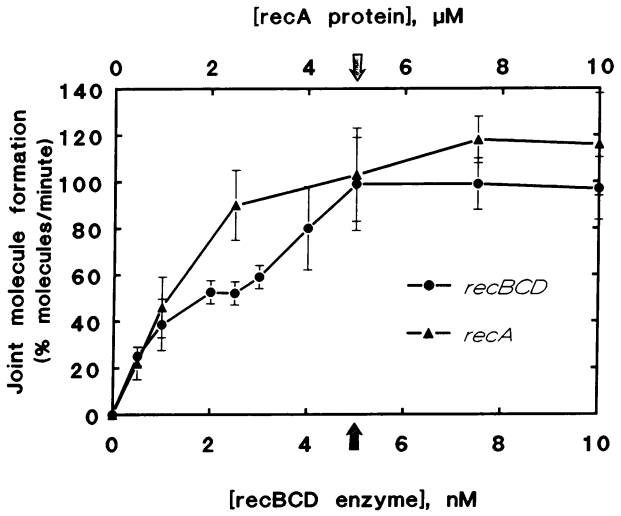

FIG. 3. Protein-concentration dependence of RecABCD-dependent joint molecule formation. ▲, RecA; ●, recBCD. Standard assay conditions, except for protein concentrations, were used. Arrows indicate the concentrations used under standard assay conditions.

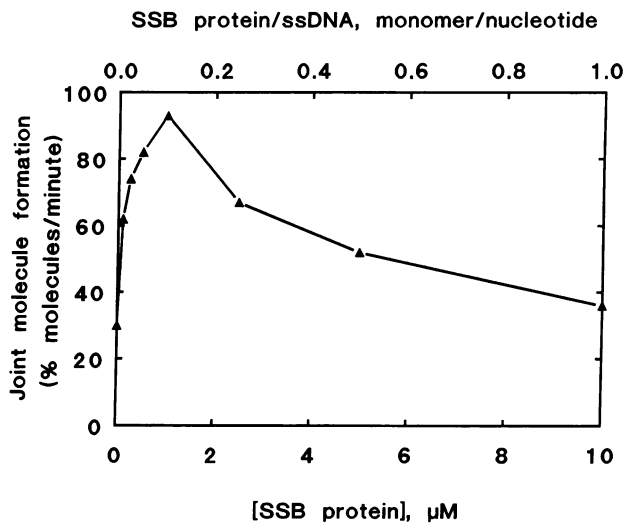

FIG. 4. Effect of SSB on RecABCD-dependent joint molecule formation. The nitrocellulose filter assay was used, except as noted. In the absence of SSB, joint molecule formation could not be assayed reliably by the nitrocellulose filter assay due to retention of the labeled supercoiled DNA when nonhomologous linear dsDNA was used (data not shown). Consequently, the agarose gel assay was used for the 0 μM point and the observed rate was normalized to that obtained at 1 μM SSB; the uncertainty is ± 15%/min.

mation increases with RecA concentration up to a point. The maximum ssDNA concentration possible upon unwinding of the linear dsDNA is 10 μM; based on this number, intersection of the initial data points with the saturation value occurs at an observed stoichiometry of 6 ± 1.5 nucleotides of ssDNA per RecA monomer. When the concentration of linear dsDNA is lowered to 5 μM, the apparent stoichiometry is unaltered (5 ± 1.5 nucleotides of potential ssDNA per RecA monomer; data not shown). When the concentration of supercoiled DNA is increased 4-fold to 20 μM (with the linear dsDNA remaining at 10 μM), the concentration of RecA required for saturation is unaltered (6 ± 1 nucleotides of potential ssDNA per RecA monomer). These results are consistent with need for RecA in this reaction being defined by the ratio of RecA to unwound DNA.

Rate of RecABCD-Dependent Joint Molecule Formation Saturates with Increasing RecBCD Enzyme Concentration. The maximum rate of DNA unwinding by RecBCD is obtained when both ends of the linear dsDNA are saturated ($K_m \approx 1$ nM) with functional enzyme (21). The helicase activity of this preparation of RecBCD saturates at 3.8 RecBCD enzyme molecules per dsDNA end (data not shown), corresponding to a RecBCD concentration of 5.2 nM for the 10 μM (nucleotides) linear M13 dsDNA used here. The effect of RecBCD concentration on the rate of RecABCD-dependent joint molecule formation is shown in Fig. 3. At subsaturating concentrations of RecBCD, the rate increases with enzyme concentration. Saturation of the initial rate occurs at a RecBCD concentration of 4.7 ± 0.5 nM; this demonstrates that the action of recBCD enzyme is rate-limiting for the pairing reaction at subsaturating recBCD enzyme concentrations. The standard enzyme concentrations used for the reactions in Fig. 1 (5 μM RecA and 5 nM RecBCD) are just saturating for both enzymes.

Effect of SSB on the Initial Rate of RecABCD-Dependent Joint Molecule Formation. The rate of RecA-dependent joint molecule formation between circular ssDNA and linear dsDNA is stimulated by SSB when present at SSB (monomer)/ssDNA (nucleotide) molar ratios between 0.075 and 0.14, but is inhibited at higher ratios (27). Fig. 4 demonstrates that stimulation of RecABCD-dependent joint molecule formation by SSB occurs at SSB/ssDNA ratios up to 0.1; above this ratio, the reaction is inhibited. Thus, SSB protein has equivalent effects on both reactions.

Effects of Salt and ATP Concentrations on RecABCD-Dependent Joint Molecule Formation. To further confirm that RecABCD-dependent joint molecule formation is similar to that observed in the well-characterized RecA-dependent reaction between ssDNA and linear dsDNA, the effects of NaCl, Mg^{2+}, and ATP concentration were examined. The sensitivity of RecABCD-dependent joint molecule formation to salt concentration almost exactly parallels that of RecA-dependent joint molecule formation (Table 1), except at 2 and 4 mM $Mg(OAc)_2$. The explanation for this difference is unknown but may reflect an inability of RecA to compete kinetically with SSB for ssDNA on the time scale of these experiments. As indicated in Table 1, a lag of 1–2 min is observed in the pairing reaction at 4 and 6 mM $Mg(OAc)_2$; this

Table 1. Effect of mono- and divalent salt concentration on RecA- and RecBCD-dependent activities

Salt conc., mM	% activity		
	RecBCD helicase*	RecA-dependent JM formation†	RecABCD-dependent JM formation‡
NaCl			
0	100	100	100
50	100	50	65
100	95	20	19§
200	40	5	<1
$Mg(OAc)_2$			
1	100	<1	2
2	114	9	1
4	91	29	5§
6	95	42	24§
8	91	100	100
10	95	ND	92

JM, joint molecule; ND, not determined.
*Data from ref. 21. Maximum activity (100%) is that observed at 0 mM NaCl or at 1 mM $Mg(OAc)_2$.
†Data from refs. 21 and 29. Maximum activity is that observed at 0 mM NaCl or at 8 mM $Mg(OAc)_2$.
‡Data from this work; standard assay conditions were used, except for added salt. Maximum activity is that observed at 0 mM NaCl or at 8 mM $Mg(OAc)_2$.
§Rate after a lag of 1–2 min.

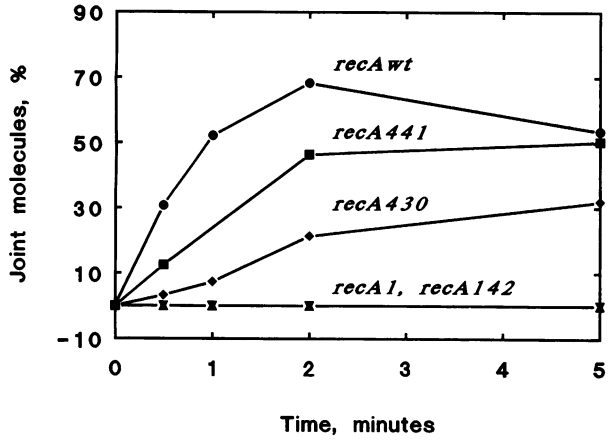

FIG. 5. Effect of mutant RecA proteins on RecABCD-dependent joint molecule formation. Standard assay conditions were used. ●, Wild-type (wt) RecA; ■, RecA441; ◆, RecA430; ▼, RecA1; ▲, RecA142.

lag dissappears at 8 mM. Following this lag, joint molecules are formed at the reported rate for the next 5–10 min; the lag at 2 mM $Mg(OAc)_2$ may be longer than the 5-min assay time used. The ATP concentration dependence of joint molecule formation is sigmoidal (half-maximal at 150 ± 10 μM; data not shown), which is a characteristic of RecA activity.

Ability of Mutant RecA Proteins to Catalyze RecABCD-Dependent Joint Molecule Formation Parallels Their *in Vivo* Phenotype. To establish whether a parallel exists between behavior *in vivo* and the function of RecA in RecABCD-dependent joint molecule formation, we examined the activities of several mutant RecA proteins. RecA1 and RecA142 proteins are defective in both genetic recombination (1, 30) and DNA strand exchange *in vitro* (31, 32); RecA430 protein has reduced (≈40%) recombination function (33) and *in vitro* joint molecule formation activity (≈50%) relative to wild-type protein (34); and RecA441 protein is proficient in both recombination (35) and joint molecule formation *in vitro* (29). In agreement, the RecA1 and RecA142 proteins show no measurable RecBCD-dependent joint molecule formation (Fig. 5), whereas the initial rate of joint molecule formation by the RecA430 and RecA441 proteins is approximately 30% and 65%, respectively, of that observed for wild-type protein.

DISCUSSION

The RecBCD pathway of genetic recombination requires functional RecA, RecBCD, and SSB proteins. Using purified protein components and DNA substrates that are likely to reflect those involved in the RecBCD pathway of recombination, we can demonstrate the formation of homologously paired joint molecules. This reaction shows that RecBCD can promote the initiation of RecA-dependent pairing *in vitro*, and is therefore consistent with data (10–13) and models (14) indicating that RecBCD acts early in genetic recombination (i.e., before exchange of DNA strands).

A Model for RecABCD-Dependent Joint Molecule Formation Catalyzed by RecA and RecBCD. Our data are consistent with the following series of molecular events (Fig. 6). RecBCD begins the process by binding to the end of the linear dsDNA (step a) and initiating unwinding (step b). Due to both nonspecific nuclease activity and specific cutting activity at the χ site, the majority of the ssDNA produced is not full-length. SSB and RecA bind to and trap the ssDNA produced by RecBCD (steps c and f); in the absence of these proteins, no free ssDNA is produced. Presumably, complete unwinding of the dsDNA is not required [although, under these conditions, RecBCD can unwind ≈30 kb from each dsDNA end (L.J.R., A. K. Eggleston, and S.C.K., unpublished data) at a rate of 930 base pairs per sec per functional RecBCD molecule (21)]; the formation of short segments of ssDNA, coated with SSB and RecA, by the combined helicase and nuclease activities of RecBCD may be sufficient. RecA then homologously pairs the ssDNA with the supercoiled DNA (steps d and g) and catalyzes DNA strand invasion (steps e and h).

Unwinding of dsDNA by RecBCD Is Required. The major role of RecBCD in this *in vitro* reaction is to initiate events by unwinding the dsDNA. The formation of joint molecules does not occur in the absence of RecBCD and is completely dependent on the presence of a linear dsDNA substrate. At concentrations of RecBCD that are subsaturating for helicase activity, the rate of joint molecule formation is directly proportional to the amount of RecBCD present, suggesting that the initiation of joint molecule formation by dsDNA unwinding is rate-limiting under such conditions.

Trapping of ssDNA Produced by the Unwinding Activity of RecBCD Is Required. As the dsDNA is being unwound by RecBCD enzyme, a ssDNA-binding protein (i.e., RecA or SSB) must trap the ssDNA, which would otherwise renature behind the enzyme (36, 37). In the absence of a ssDNA-binding protein, RecBCD enzyme alone cannot produce ssDNA that can be productively utilized by the separate action of RecA and SSB (Fig. 1, +). The simultaneous presence of both RecA and RecBCD is required for efficient joint molecule formation, suggesting that their coordinated function is important.

When present together, both SSB and RecA must be binding to and trapping the ssDNA produced by RecBCD-catalyzed unwinding. When only SSB is present during dsDNA unwinding (producing SSB-coated ssDNA), a time lag is observed in the pairing reaction (Fig. 1, ■); prebinding of SSB to ssDNA results in a lag phase for all RecA-dependent activities that coincides with the displacement of SSB from the ssDNA (27, 29, 34, 38). This displacement step becomes slower and less efficient at lower Mg^{2+} concentra-

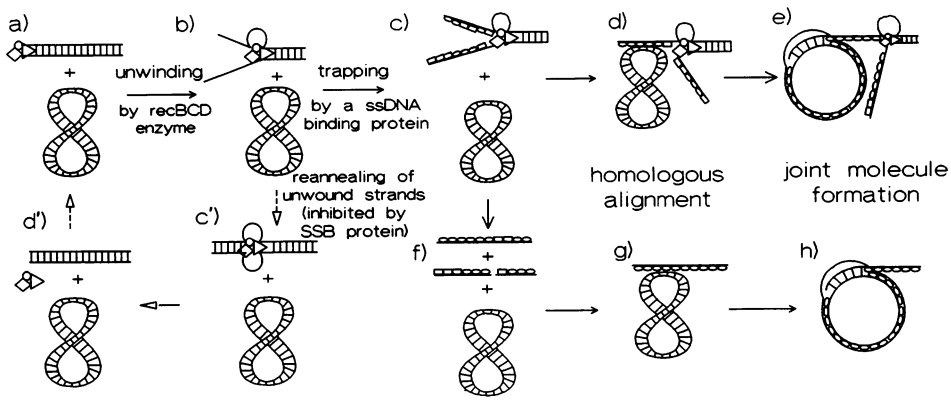

FIG. 6. Model for joint molecule formation catalyzed by both RecBCD and RecA. Small ovals, RecA; small rectangles, SSB; diamond/triangle/circle, RecBCD. See text for explanation.

tions, which may contribute to the lag observed in RecABCD-dependent joint molecule formation at higher NaCl or lower Mg^{2+} concentrations; the low rate of joint molecule formation at 2 and 4 mM Mg^{2+} may be a consequence of the poor SSB-displacement ability of RecA at low Mg^{2+} concentrations. The inhibitory effect of SSB at higher concentrations is also consistent with the displacement scenario.

Joint Molecule Formation by RecA Is Required. The behavior of RecA in RecABCD-dependent joint molecule formation must be similar to that required in RecA-dependent joint molecule formation, by the following criteria: (*i*) optimal joint molecule formation is dependent on the presence of a nearly saturating amount of RecA; (*ii*) joint molecule formation is stimulated at low molar ratios of SSB to ssDNA (≤ 0.1) but is inhibited at higher ratios (ref. 27); (*iii*) the activities of the mutant RecA follow the same trends in both reactions (39); (*iv*) the NaCl and $Mg(OAc)_2$ concentration dependences are, for the most part, parallel; and (*v*) the ATP concentration dependence of joint molecule formation is similarly sigmoidal.

We have described two biochemical pathways for homologous pairing *in vitro* that are dependent on RecBCD helicase activity for initiation. The first is a reaction between circular ssDNA and linear dsDNA that is dependent on the renaturation activity of RecA (15), while the second, a reaction between linear dsDNA and supercoiled dsDNA, is dependent on RecA-catalyzed strand-invasion activity; in both reactions, the unwinding of linear dsDNA by RecBCD is essential. The reaction between linear dsDNA and supercoiled dsDNA should be representative of the situation encountered in the RecBCD pathway of recombination (i.e., during conjugation and transduction). In this pathway, the joint molecule formation activity of RecA is used to produce homologously paired DNA molecules. In addition, DNA hetroduplex formation via RecA-dependent renaturation of ssDNA produced may provide an alternative mechanism for the repair of UV-induced DNA damage (15). Thus, both the strand-invasion and DNA-renaturation activities of RecA may be significant *in vivo*, depending upon cellular conditions.

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