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Homologous pairing and strand exchange promoted by the *Escherichia coli* RecT protein

(genetic recombination/RecE pathway/strand transfer)

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ABSTRACT **RecT protein of *Escherichia coli* promotes the formation of joint molecules between homologous linear double-stranded M13mp19 replicative-form bacteriophage DNA and circular single-stranded M13mp19 DNA in the presence of exonuclease VIII, the *recE* gene product. The joint molecules were formed by a mechanism involving the pairing of the complementary strand of the linear double-stranded DNA substrate with the circular single-stranded DNA substrate coupled with the displacement of the noncomplementary strand. When the homologous linear double-stranded DNA substrate had homologous 3′ or 5′ single-stranded tails, then RecT promoted homologous pairing and strand exchange in the absence of exonuclease VIII. Histone H1 could substitute for RecT protein; however, joint molecules formed in the presence of histone H1 did not undergo strand exchange. These results indicate that under the reaction conditions used, the observed strand exchange reaction is promoted by RecT and is not the result of spontaneous branch migration. These results are consistent with the observation that expression of RecE (exonuclease VIII) and RecT substitutes for RecA in some recombination reactions in *E. coli*.**

Alternative pathways for recombination in *Escherichia coli* were discovered by the analysis of mutations that suppress mutations in the *recB* and *recC* genes (1, 2). Recombination occurs by the RecE pathway in *recB recC sbcA* mutants (3, 4). *sbcA* mutations map on a λ-like cryptic prophage, called Rac, and induce the expression of *recE* and *recT* (1, 2, 4–9). The RecE pathway is complex and the gene products required depend on the substrates studied (3, 6, 10–13). Conjugational recombination in *E. coli recB recC sbcA* strains requires *recA*, *recE*, and probably all of the genes of the RecF pathway (5, 6, 13–16). Recombination of circular plasmids requires *recE* and a subset of the RecF-pathway genes but does not require *recA* (refs. 11–13 and 17; N.-W. Chi and R.D.K., unpublished results). Recombination of linear dimer plasmid DNA and recombination of bacteriophage λ *red* mutants requires *recE* but does not require *recA* or other RecF-pathway genes (refs. 5, 6, 13, 18, and 19; N.-W. Chi and R.D.K., unpublished results).

At least two proteins encoded by the *recE* region are required for RecE-pathway recombination (8, 9). Originally, the *recE* region was thought to encode only one protein, exonuclease VIII (exoVIII) (5, 6, 20). DNA sequence analysis has shown this region contains two overlapping open reading frames: *recE*, which encodes exoVIII, and *recT* (8, 9). Both exoVIII and RecT are required for recombination and repair promoted by the RecE pathway (3, 5, 6, 9, 21).

The RecE pathway appears similar to the λ Red pathway (3, 5, 22). The *recE* region is able to substitute for two λ-encoded proteins, λ exonuclease (λ exo) encoded by the *red α* gene and β-protein encoded by the *red β* gene, which are required for λ recombination (5, 6, 23, 24). The following evidence supports this. (*i*) There are similarities between the distribution of exchanges among replicated phage and similarities between the distribution among unreplicated phage during λ recombination by the RecE pathway and the λ Red pathway (3, 5, 22). (*ii*) λ phage mutants which lack the *red* genes revert to Rec$^+$ by substitution of DNA from Rac encoding *recE* and *recT* (23, 24). (*iii*) *sbcA* mutations, which induce expression of the *recE* coding region, complement λ *red* mutations (5, 6). (*iv*) exoVIII is similar to λ exo (23, 25–27). (*v*) RecT is similar to λ β-protein (8, 28, 29).

RecA is the only homologous-pairing protein known in *E. coli*. Expression of *recE* and *recT* allows some recombination events to occur independently of *recA*, which suggests that other homologous-pairing proteins exist in *E. coli*. The ability of RecT to renature DNA like RecA and λ β-protein implicates it as a candidate (8). Consistent with this is the observation that λ β-protein and RecT substitute for RecA under some conditions (ref. 30; I. Berger and A. Cohen, personal communication). Eukaryotes contain homologous-pairing proteins—such as Sep1, HPP1, and RRP1—which are different from RecA (31–37). These proteins have intrinsic or associated exonuclease activities and promote the pairing of linear double-stranded DNA (dsDNA) and homologous circular single-stranded DNA (ssDNA). They promote pairing reactions by digesting the ends of the linear dsDNA to expose a homologous single-stranded tail, annealing the single-stranded tail to the circular ssDNA molecule, and then promoting strand exchange (37) (Fig. 1). The association of an exonuclease such as exoVIII or λ exo with a protein that can promote renaturation like RecT or λ β-protein in bacterial recombination systems is reminiscent of these proteins. This has led us to test whether RecE (exoVIII) and RecT proteins promote strand exchange reactions.

MATERIALS AND METHODS

Materials. [*methyl*-^{3}H]Thymidine was from DuPont. Sepharose 2B was from Pharmacia/LKB.

Enzymes and Proteins. *Sma* I was from New England Biolabs. *E. coli* exonuclease III and bacteriophage T7 gene *6* exonuclease were from United States Biochemical. RecT was purified as described (8) and exoVIII was purified by a method similar to a described method (38). Both protein preparations were >98% pure as determined by NaDodSO$_4$/PAGE. Histone H1 was from both Calbiochem and Boehringer Mannheim. Proteinase K was from Boehringer Mannheim. Protein

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Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; exoVIII, exonuclease VIII; λ exo, λ exonuclease; RF, replicative form.

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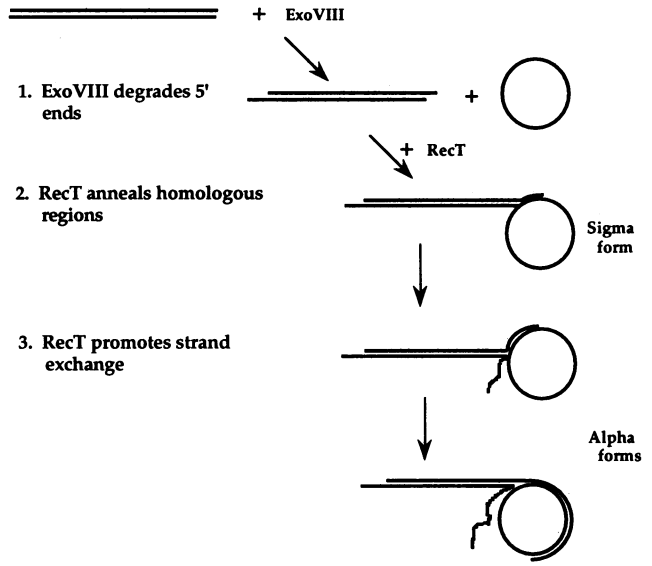

FIG. 1. Illustration of homologous pairing and strand exchange of linear dsDNA and homologous circular ssDNA promoted by exonuclease-associated homologous-pairing proteins. The first step of the reaction is the partial degradation of the linear dsDNA by an exonuclease activity to produce a single-stranded tail. The single-stranded tail is annealed to the homologous single-stranded circle to yield a sigma-form intermediate. Protein-promoted branch migration then produces alpha-form intermediates characteristic of a strand exchange reaction. In this illustration, roles are indicated for exoVIII (RecE) and RecT that are consistent with the results presented in this paper.

concentrations were determined by the Bradford method (Bio-Rad).

DNA Substrates. Unlabeled and ^{3}H-labeled M13mp19 replicative-form (RF) (9000 cpm/nmol) and viral (3800 cpm/nmol) DNAs were prepared as described (39). ϕX174 RF and viral DNAs were from New England Biolabs. Resected linear dsDNA substrates were prepared as follows. *Sma* I-digested ^{3}H-labeled M13mp19 RF DNA was digested with T7 gene *6* exonuclease (242 μg of DNA and 800 units of enzyme in 150 μl) or exonuclease III (5 μg of DNA and 80 units of enzyme in 150 μl) in 50 mM Tris, pH 8.0/5 mM $MgCl_2$/300 mM KCl/5 mM 2-mercaptoethanol. The reaction mixture with T7 gene *6* exonuclease was incubated for 1 min at 25°C, and the mixture with exonuclease III was incubated for 4 min at 23°C and followed by 2 min at 37°C. Each reaction mixture was extracted once with a 1:1 mixture of phenol and chloroform/isoamyl alcohol (24:1, vol/vol), and then the DNA was precipitated with ethanol and suspended in 10 mM Tris, pH 8.0/1 mM EDTA. Degradation averaged 80 nt and 230 nt released per DNA end for exonuclease III and gene *6* exonuclease, respectively.

Strand Exchange Reactions. Standard strand exchange reaction mixtures (30 μl) contained 20 mM Tris (pH 7.0), 0.3 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 200 ng of *Sma* I-digested M13mp19 RF DNA, 100 ng of M13mp19 ssDNA, and exoVIII and recT as indicated. Although the order of addition was not significant, exoVIII was added to each reaction mixture first, with immediate mixing and then RecT was added in all experiments reported here. After incubation at 37°C, each reaction was terminated by the addition of 0.5 M EDTA (pH 8.0), 1% $NaDodSO_4$, and 2% proteinase K to final concentrations of 50 mM, 0.1%, and 530 μg/ml, respectively. Incubation at 37°C was continued for 10 min, 2 μl of 0.25% bromophenol blue/0.25% xylene cyanol/40% sucrose was added, and the samples were fractionated by electrophoresis through an 0.8% agarose gel run in 40 mM Tris acetate, pH 7.9/1 mM EDTA with ethidium bromide at 0.5 μg/ml. Joint molecules were visualized by UV fluorescence and quantitated by densitometry of photographic negatives. Conditions for the reactions using DNA substrates resected with either exonuclease III or T7 gene *6* exonuclease were the same except that exoVIII was omitted from the reactions. In some experiments, histone H1 was substituted for RecT.

Electron Microscopy. Samples for electron microscopy (90 μl) were prepared under standard strand exchange conditions except that all the reactions were terminated by the addition of 0.5 M EDTA (pH 8.0) and 2% proteinase K to final concentrations of 50 mM and 530 μg/ml, respectively, and incubation was continued for 10 min at 37°C. This final reaction mixture (≈130 μl) was then chromatographed on a 300- to 400-μl column of Sepharose 2B equilibrated and run in 10 mM Tris, pH 8.0/1 mM EDTA. The DNA-containing fractions were pooled. In some experiments, samples were prepared using 1- to 1.5-ml Sepharose 2B columns. These DNA samples were analyzed by electron microscopy (40).

RESULTS

exoVIII and RecT Promote Strand Exchange. RecT and exoVIII promote the formation of joint molecules from homologous linear double-stranded and circular single-stranded M13mp19 DNA substrates in Tris buffer at pH 7.0 containing 0.3 mM $MgCl_2$. Under these conditions, the exonuclease activity of exoVIII is reduced compared with previously described reaction conditions (ref. 27; unpublished results). A time course of joint-molecule formation is shown in Fig. 2. The joint molecules appeared as a discrete band and a more slowly migrating heterogeneous mixture of DNA which migrated behind the linear dsDNA species in an agarose gel (Fig. 2). The reaction was linear for 10 min. A maximum of 50–60 nt per minute were degraded per duplex end (data not shown). The maximum extent of joint-molecule formation observed was never more than 40–50%. Titration experiments (Fig. 3) demonstrated that ≈1 tetramer of RecT per 13 nt of ssDNA and ≈1.5 exoVIII tetramers per end of linear dsDNA were required for optimal formation of joint molecules. These values were calculated from the amounts of each protein required for maximal activity (Fig. 3*A*), molec-

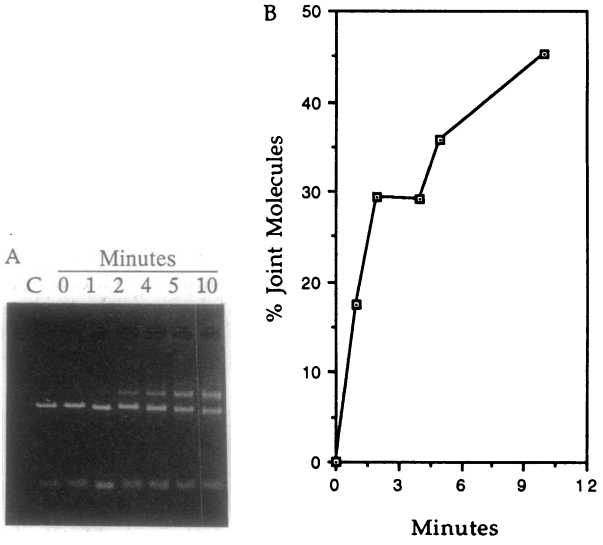

FIG. 2. Time course of joint-molecule formation promoted by exoVIII and RecT. Strand exchange reaction mixtures (30 μl) (see *Materials and Methods*) containing 3 μg of RecT, 62.5 ng of exoVIII, 100 ng of single-stranded M13mp19 viral DNA and 200 ng of *Sma* I-digested M13mp19 dsDNA were incubated at 37°C for the indicated times and then analyzed by agarose gel electrophoresis. (*A*) Analysis of a typical strand exchange reaction. Lane C (control) contained unreacted substrate DNAs. All other lanes are labeled with the number of minutes that each complete reaction mixture was incubated at 37°C. (*B*) Quantification of the gel presented in *A*.

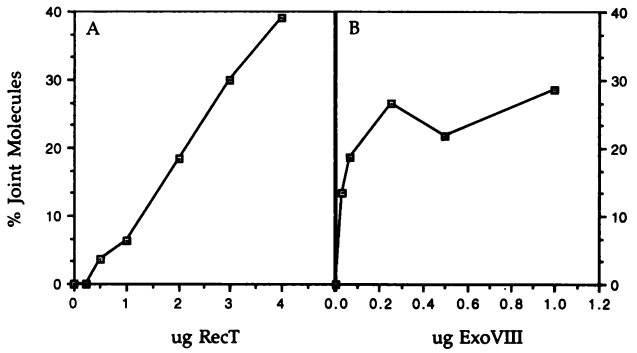

FIG. 3. Effect of the concentration of RecT and exoVIII on joint-molecule formation. Standard 30-μl strand exchange reaction mixtures were incubated at 37°C for 10 min. (*A*) Mixtures containing 62.5 ng of exoVIII and the indicated amount of RecT. (*B*) Mixtures containing 3 μg of RecT and the indicated amount of exoVIII.

ular weights of 140,000 and 33,500 for the exoVIII and RecT polypeptides, and the observation that each protein is a tetramer in solution (unpublished results).

Joint-molecule formation was found to require $MgCl_2$, exoVIII, RecT, and both linear double-stranded M13 DNA and circular single-stranded M13 DNA substrates (Table 1). Joint molecules were not formed when nonhomologous linear double-stranded M13 DNA and circular single-stranded φX174 DNA were used as substrates.

Electron Microscopic Analysis of Joint Molecules Formed by exoVIII and RecT. Two types of joint molecules, sigma forms and alpha forms, were observed by electron microscopy (Fig. 4). Each sigma form appeared to consist of a circular ssDNA molecule with an attached linear dsDNA tail that was the length of M13mp19 RF DNA. Each alpha form consisted of a partially double-stranded circle with both a double-stranded branch and a single-stranded branch attached to the circle at the same end of the double-stranded segment of the circle. The formation of both types of joint DNA molecules required exoVIII and RecT (Table 2). pRac3 exonuclease, a truncated form of exoVIII, substituted for exoVIII, consistent with previous genetic studies (4, 7). Early during the reaction time course, sigma forms were the predominant type of joint molecule. At later times, the proportion of sigma forms decreased and the proportion of alpha forms increased, consistent with the idea that sigma forms were precursors to the alpha forms. The observation of significant levels of alpha forms is consistent with the idea that the majority of the joint molecules are formed by a strand exchange mechanism as illustrated in Fig. 1. Circular products were not observed,

Table 1. Requirements for strand exchange reactions

Reaction mixture	% joint molecules
Complete	25
− $MgCl_2$	<1
− 2-mercaptoethanol	32
− RecT	<1
− ExoVIII	<1
− RecT and exoVIII	<1
− linearized M13mp19 dsDNA	<1
− M13mp19 viral ssDNA	<1
− M13mp19 viral ssDNA + φX177 viral DNA	<1

Strand exchange reactions were carried out in 30 μl as described in Fig. 2 except that the reaction mixtures were incubated for 10 min at 37°C. Additions and omissions are as indicated.

indicating that the strand exchange reaction did not go to completion under the reaction conditions used.

Joint molecules were also formed when an optimal amount of histone H1, a protein known to promote annealing of homologous ssDNA molecules (41), was substituted for RecT (Fig. 5). However, the extent of the reaction was always significantly less. Analysis of the joint molecules formed in the presence of histone H1 indicated that essentially all of the joint molecules were sigma molecules and that strand exchange to yield alpha forms did not occur at significant rates. Because the reaction mixtures with histone H1 were deproteinized and processed exactly as those containing RecT, this control indicates that strand exchange promoted by RecT is not the simple consequence of annealing of the circular ssDNA with linear dsDNA that had been partially degraded by exoVIII. This suggests that RecT actively promotes strand exchange under the reaction conditions used and that the observed reaction is not due to spontaneous branch migration that occurs after deproteinization of the joint molecules or during mounting of the samples for electron microscopy.

Role of exoVIII and RecT During Strand Exchange. One possible mechanism for the formation of joint molecules is that exoVIII partially degrades the linear dsDNA substrate to expose a homologous single-stranded tail; RecT anneals this tail to the circular ssDNA and then promotes strand exchange. To test this, we examined the ability of RecT to promote joint-molecule formation in the absence of exoVIII. When the linear dsDNA was predigested with either T7 gene *6* exonuclease or *E. coli* exonuclease III, RecT promoted the formation of joint molecules in the absence of exoVIII (Table 3). Both sigma forms and alpha forms were observed, indi-

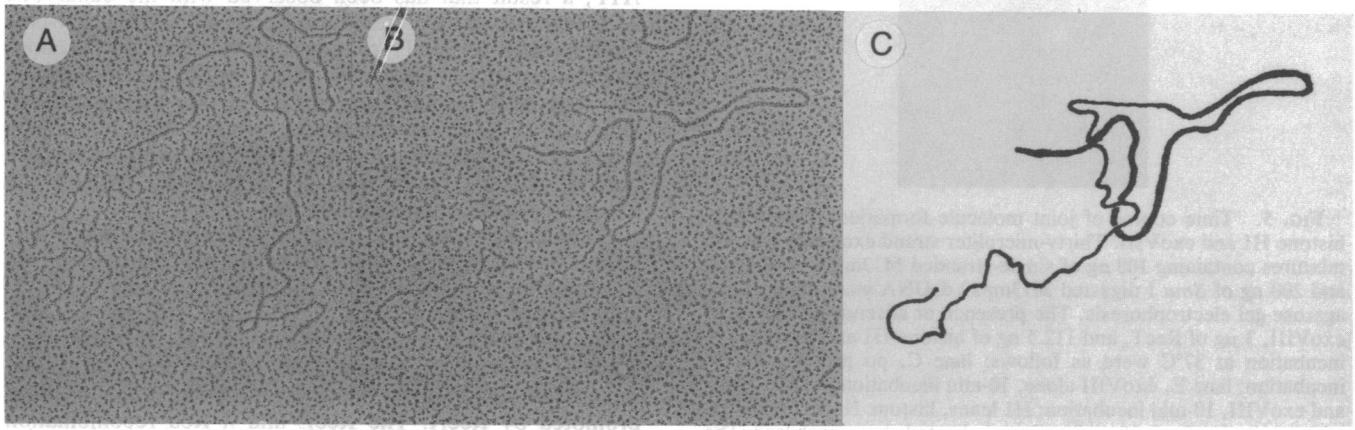

FIG. 4. Electron microscopic analysis of the structure of joint molecules. Ninety-microliter strand exchange reaction mixtures were incubated at 37°C for 10 min. The reaction products were then purified and analyzed by electron microscopy. (*A*) Representative sigma molecule. (*B*) Representative alpha molecule. (*C*) Illustration of the alpha molecule presented in *B*.

Table 2. Electron microscopic characterization of joint molecules formed in strand exchange reactions

Reaction component(s)	Time, min	N	% linear dsDNA	% joint molecules	
				Sigma	Alpha
No protein	10	553	99.0	0.5	0.5
RecT	10	229	100	<0.4	<0.4
RecT and exoVIII	0	119	99.2	<0.8	0.8
	2	449	92.7	5.8	1.5
	5	570	80.7	14.6	4.7
	10	424	71.7	12.2	16.0
	30	57	68.4	1.8	29.8
RecT and pRac3 exo	10	389	85.6	6.7	7.7
exoVIII	10	216	98.1	<0.5	1.9
Histone H1 and exoVIII	10	541	94	5.2	0.7

Strand exchange reaction mixtures (90 μl) containing 600 ng of linear M13mp19 dsDNA and 300 ng of circular M13mp19 viral ssDNA were incubated for the indicated times at 37°C, as described in Fig. 2. The presence or absence of 9 μg of RecT, 1.88 μg of exoVIII, 1.88 μg of pRac3 exo, and 0.150 μg of histone H1 was as indicated. All DNA molecules containing double-stranded regions were scored. *N*, no. of molecules analyzed.

cating that RecT by itself could promote both annealing of homologous single-stranded tails to the circular ssDNA to yield sigma forms and subsequent strand exchange to yield alpha forms. In control experiments where histone H1 was substituted for RecT, all of the joint molecules formed were sigma forms. The observation that alpha forms could be produced by RecT independent of the polarity of digestion of the linear dsDNA substrate indicates that RecT, like Sep1 and Dpa1 (37, 42), can promote strand exchange in either of the two possible polarities.

DISCUSSION

Genetic evidence suggests that exoVIII (RecE) and RecT are required for genetic recombination, that they substitute for RecA under some conditions, and that they are similar to some eukaryotic homologous-pairing proteins. Under conditions in which the exonuclease activity of exoVIII is reduced,

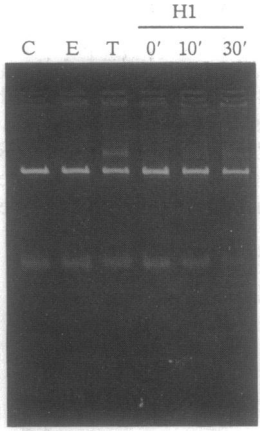

FIG. 5. Time course of joint molecule formation promoted by histone H1 and exoVIII. Thirty-microliter strand exchange reaction mixtures containing 100 ng of single-stranded M13mp19 viral DNA and 200 ng of *Sma* I digested M13mp19 dsDNA were analyzed by agarose gel electrophoresis. The presence or absence of 62.5 ng of exoVIII, 3 μg of RecT, and 112.5 ng of histone H1 and the times of incubation at 37°C were as follows: lane C, no protein, 10-min incubation; lane E, exoVIII alone, 10-min incubation; lane T, RecT and exoVIII, 10-min incubation; H1 lanes, histone H1 and exoVIII, with incubation for 0, 10, or 30 min as indicated above each lane. The amount of histone H1 used was the amount determined to give maximal joint-molecule formation. No joint molecules were observed with histone H1 alone.

Table 3. Electron microscopic characterization of joint molecules using resected DNA

Reaction component	Substrate	N	% linear dsDNA	% joint molecules	
				Sigma	Alpha
No protein	5′ resected	222	99.0	<0.5	<0.5
RecT	5′ resected	448	83.2	6.3	10.5
RecT	3′ resected	475	83.8	3.6	12.6
Histone H1	5′ resected	271	83.0	17.0	<0.4

Strand exchange reaction mixtures (90 μl) were incubated for 10 min at 37°C as described in Table 2 except that 5′- or 3′-resected linear dsDNA was substituted for the blunt-ended linear dsDNA substrate. The presence or absence of 9 μg of RecT or 0.150 μg of histone H1 was as indicated. All DNA molecules containing double-stranded regions were scored. *N*, no. of molecules analyzed.

exoVIII and RecT promote homologous pairing of linear dsDNA and homologous circular ssDNA. The mechanism of the reaction involves limited digestion of the linear dsDNA by exoVIII to expose a homologous single-stranded tail, annealing of this tail to the single-stranded circle by RecT in an exoVIII independent step, and subsequent strand exchange promoted by RecT (Fig. 1). This mechanism is similar to the mechanism proposed for proteins such as Sep1, Dpa1, HPP1, and RRP1 (36, 37, 42, 43). This mechanism is different from the mechanism of the RecA-family proteins, which initiate pairing through the formation of paranemic joints that lead to subsequent strand exchange (44–46). Regardless of the exact mechanism of pairing by exoVIII and RecT, the observation that these proteins are required for RecE recombination (5, 6, 9, 13, 19) provides a possible explanation for how recombination can be *recA*-independent (5, 11–13, 17, 19, 47) when exoVIII and RecT are expressed.

Formation of sigma-form DNA molecules with annealing agents such as histone H1 (Table 3; Fig. 5) or spermidine (data not shown) did not lead to strand exchange. Such control reactions indicate that strand exchange promoted by RecT is not due to spontaneous branch migration that occurs after deproteinization of the joint molecules or during mounting of the samples for electron microscopy. The formation of heteroduplex joints as long as ≈6 kb by RecT in 10 min suggests that RecT promotes directional branch migration at rates as high as 10 bases per second, similar to other homologous-pairing proteins (34, 44, 45, 48). Much like some of the eukaryotic homologous-pairing proteins (37, 42), the polarity of RecT-promoted strand exchange was determined by the polarity of the single-stranded end of the linear dsDNA which was complementary to the circular ssDNA. Strand exchange did not require hydrolysis of a high-energy cofactor such as ATP, a result that has been observed with the eukaryotic homologous-pairing proteins and RecA (49, 50). Exactly how proteins such as RecT promote directional strand exchange reactions is unclear. However, work by Sikorav and Church (51) suggested that such proteins could alter the structure of DNA so as to lower the energy of activation for branch migration. This property, possibly combined with more stable binding of RecT to the final products, could promote the extensive strand exchange observed.

Genetic, physical, and biochemical analyses have suggested that the mechanisms of recombination mediated by the RecE pathway and the λ Red pathway are similar (3, 5, 22) and involve exonucleolytic degradation and reannealing, unlike reactions promoted by RecA (44–46, 52). However, there is evidence to suggest that both RecE and λ Red recombination also involve pairing reactions similar to those promoted by RecA. The RecE and λ Red recombination pathways promote double-strand-break repair, which is unlikely to occur by a degradation and reannealing mechanism (53, 54). Analysis of linear dimer plasmid recombination

promoted by the RecE pathway was also consistent with the involvement of other types of pairing reactions, like those promoted by RecA (19, 47). These studies suggest that the RecE recombination pathway involves other types of homologous-pairing reactions besides exonucleolytic degradation and reannealing. Our work demonstrates that a combination of exoVIII and RecT can promote pairing reactions that are more complex than simple degradation and reannealing of DNA. However, it is not clear whether these proteins promote the types of pairing reactions the mechanistic studies of RecE recombination have postulated to occur.

Much of our understanding of homologous-pairing reactions in recombination comes from analysis of the *E. coli* RecA and bacteriophage T4 UvsX proteins. These homologous-pairing proteins appear to initiate pairing through the formation of paranemic joints (44–46, 55). The pairing reactions promoted by exoVIII and RecT are different in that they appear to require the action of an exonuclease to initiate stable pairing. exoVIII and RecT are representative members of a second class of homologous-pairing proteins. Other potential members of this class include the λ exo and β-protein and also T7 gene *6* exonuclease and gene *2.5* protein (28, 29, 56–59). Only the T7 gene *2.5* protein is known to promote strand exchange reactions (S. Tabor, personal communication). However, β-protein can substitute for RecA in some recombination reactions, suggesting that it might promote strand exchange reactions (30). Also included in this class of proteins are the eukaryotic homologous-pairing proteins containing intrinsic or associated exonuclease activity (31, 36, 37). In addition, pairing activity like that associated with RecT is also associated with the *Saccharomyces cerevisiae* DPA1 protein, the herpes simplex virus type 1 DNA-binding protein ICP8, and a DNA-binding protein encoded by vaccinia virus (42, 60, 61). An associated exonuclease has not been identified for these proteins. A major question regarding this second class of homologous-pairing proteins concerns their biological relevance. Of these proteins, exoVIII and RecT are required for recombination and can replace RecA (refs. 6, 9, 11–13, and 19; A. Cohen, personal communication), and *sep1* mutants have multiple recombination defects during meiosis (ref. 62; D. Tishkoff and R.K.D., unpublished results). λ exonuclease and β-protein and T7 gene *6* exonuclease and gene *2.5* protein are important for recombination (58, 59, 63, 64). It is not known whether *Drosophila* Rrp1, human HPP1, *S. cerevisiae* DPA1, herpes simplex virus ICP8, and the vaccinia protein function in recombination.

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