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DNA Strand Invasion Promoted by *Escherichia coli* RecT Protein*

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The RecT protein of *Escherichia coli* is a DNA-pairing protein required for the RecA-independent recombination events promoted by the RecE pathway. The RecT protein was found to bind to both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) in the absence of Mg^{2+}. In the presence of Mg^{2+}, RecT binding to dsDNA was inhibited drastically, whereas binding to ssDNA was inhibited only to a small extent. RecT promoted the transfer of a single-stranded oligonucleotide into a supercoiled homologous duplex to form a D (displacement)-loop. D-loop formation occurred in the absence of Mg^{2+} and at 1 mM Mg^{2+} but was inhibited by increasing concentrations of Mg^{2+} and did not require a high energy cofactor. Strand transfer was mediated by a RecT-ssDNA nucleoprotein complex reacting with a naked duplex DNA and was prevented by the formation of RecT-dsDNA nucleoprotein complexes. Finally, RecT mediated the formation of joint molecules between a supercoiled DNA and a linear dsDNA substrate with homologous 3′-single-stranded tails. Together these results indicate that RecT is not a helix-destabilizing protein promoting a reannealing reaction but rather is a novel type of pairing protein capable of promoting recombination by a DNA strand invasion mechanism. These results are consistent with the observation that RecE (exonuclease VIII) and RecT can promote RecA-independent double-strand break repair in *E. coli*.

In *Escherichia coli*, the major recombination pathway requires the function of the RecA and RecBCD proteins (for reviews, see Refs. 1 and 2). The recombination and repair deficiencies in *recB recC* mutants can be suppressed by two types of mutations called *sbcA* and *sbcB(sbcC)* (3–5). The *sbcA* mutations map on the cryptic Rac prophage and induce the expression of the RecE and RecT proteins (for review, see Ref. 6). Recombination in *recB recC sbcA* mutants occurs by what is called the RecE pathway (7), which in many ways is similar to the bacteriophage λ Red pathway (6). A distinctive property of the RecE pathway is that it promotes RecA-independent recombination of circular plasmids as well as intramolecular recombination of linearized plasmid DNAs (8–11) and also promotes RecA-independent double strand break repair (DSBR)[1] (12). These types of recombination events have been shown to require functional *recE* and *recT* genes (13, 14).

The *recE* gene product is an ATP-independent exonuclease, also called exonuclease VIII (15). Exonuclease VIII degrades preferentially linear duplex DNA in the 5′ to 3′ direction, yielding 5′-mononucleotides and also degrades single-stranded DNA (ssDNA) at low rates (16). The *recT* gene product was found to bind to ssDNA and to promote the renaturation of complementary ssDNA in an ATP-independent fashion (17). Also, the RecT protein in combination with exonuclease VIII was shown to promote homologous pairing and strand exchange between a circular ssDNA and a linear duplex DNA. In this reaction, exonuclease VIII degraded the linear duplex to expose ssDNA that was then annealed by RecT to a complementary region on the ssDNA circle. Subsequently, RecT promoted heteroduplex extension and partial strand exchange (18). This degradation/reannealing/strand exchange mechanism explains how the presence of RecE and RecT can render some types of recombination RecA-independent (6). However, *in vivo* evidence indicates that the ends of a linear duplex DNA may not be involved directly in the initial pairing event, and it was suggested that internal duplex-duplex initiation events could be promoted by the RecE pathway (11, 19). Also, it has been pointed out that DSBR, which clearly requires a pairing function, cannot occur simply by a degradation and reannealing mechanism (20). This suggests that recombination promoted by the RecE pathway involves pairing events more complex than annealing of ssDNA, possibly similar to those promoted by RecA (6). These pairing events could either involve additional unidentified protein factors or alternatively, they could be the result of a previously unrecognized RecT pairing activity.

In this paper, we investigate further the pairing activities promoted by RecT *in vitro*. We found that RecT can promote the invasion of a supercoiled DNA by either a homologous ssDNA or homologous single-stranded ends of a linear duplex, a DNA substrate similar to that generated by the RecE exonuclease. The joint molecules contain D-loops (displacement loops), supporting the idea that RecT could be involved directly in the initiation of DSBR in the RecE pathway. Our biochemical analysis of the DNA binding properties of RecT and the requirements for strand transfer indicate that RecT belongs to a novel class of DNA-pairing proteins.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Media—E. coli strains Novablue (*endA1 hsdR17*($r_{K12}^{-}m_{K12}^{+}$) *supE44 thi-1 recA1 gyrA96 relA1 lac*[F′ *proA*$^{+}$*B*$^{+}$ *lacI*^{q}*ZΔM15::Tn10*]) and Novablue(DE3), containing a T7 RNA polymerase gene inducible with isopropyl β-D-thiogalactopyranoside, were from Novagen. pRac31 is pBR322 containing a fragment of the Rac prophage which carries a wild-type *recT* gene (13). pUC18 and pUC19 are from the laboratory collection, and pHV2900OB is a pUC18 derivative carrying a 2.27-kilobase pair DNA fragment inserted into the polylinker (21). *E. coli* cells were grown at 37 °C in LB medium (22) supplemented with 0.2% glucose. Ampicillin and kanamycin were

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[1] The abbreviations used are: DSBR, double strand break repair; D-loop, displacement loop; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

added to final concentrations of 100 and 30 μg/ml, respectively. Transformations were carried out as described previously (22).

Enzymes and Proteins—Restriction enzymes, T4 polynucleotide kinase, T4 DNA ligase, DNA polymerase Klenow fragment, and bovine serum albumin were from New England Biolabs. Bacteriophage T7 gene *6* exonuclease and T7 DNA polymerase (Sequenase version 2.0) were from U. S. Biochemical Corp. Proteinase K was from Boehringer Mannheim.

Oligonucleotides—Synthesis of oligonucleotides was performed at the Molecular Biology Core Facility, Dana Farber Cancer Institute. Primers used to amplify RecT were T1 (5′-cgggatccagaaggaatatgcaaatgactaagcaac) and T2 (5′-acgcgtcgacggtgcattacaccgccaggc); regions complementary to the *recT* region are underlined. Oligonucleotides used in DNA binding and D-loop assays were 50 nucleotides long. Oligonucleotide 26 (5′-caccgtcaccgacttgagccatttgggaattagagccagcaaaatcaccag) corresponds to positions 2629–2579 of bacteriophage M13 wild-type. Oligonucleotide 34 (5′-ctatgcggcatcagagcagattgtactgagagtgcaccatatgcggtgtg) corresponds to positions 146–195 of pUC18. Oligonucleotide 35 (5′-cacaccgcatatggtgcactctcagtacaatctgctctgatgccgcatag) is complementary to oligonucleotide 34.

Overexpression and Purification of RecT—A 0.85-kilobase pair DNA fragment containing the *recT* gene was amplified by polymerase chain reaction from pRac31 with primers T1 and T2, and the polymerase chain reaction product was digested with *Bam*HI + *Sal*I and inserted into pET24+ (Novagen) cleaved with the same enzymes. The resulting plasmid, pRDK577, carried a wild-type *recT* gene (verified by DNA sequencing) under the control of the T7 promoter. The RecT-overproducing strain, RDK3294, was obtained by transformation of pRDK577 into Novablue(DE3). RecT synthesis was induced by the addition of isopropyl β-D-thiogalactopyranoside (1 mM final concentration) to a log-phase culture of RDK3294 (OD_{650} = 0.8) and incubation for 4 h. RecT purification was performed as described previously (17). A final step was added in the purification procedure to remove the KPO_4 buffer from the RecT preparation obtained from the hydroxylapatite column. The RecT fraction (28 mg of protein) was loaded at 25 ml/h onto a PBE94 column (20 × 1 cm) equilibrated with buffer A (20 mM Tris, pH 7.5, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 10% w/v glycerol) containing 0.1 M NaCl and eluted with an 80-ml linear gradient of buffer A containing 0.1–1 M NaCl. The fractions containing RecT were pooled, dialyzed against buffer A containing 60% w/v glycerol and 100 mM NaCl, and stored at −20 °C. Analysis by SDS-polyacrylamide gel electrophoresis and Coomassie staining indicated that the final RecT fraction (25 mg of protein) was greater than 95% pure. Protein concentrations were determined by using the Bio-Rad assay kit with bovine serum albumin as a standard.

DNA Substrates—All DNA concentrations are expressed in nucleotide residues. Double-stranded oligonucleotides were obtained by mixing complementary oligonucleotides in equimolar amounts (2 mM) and annealing them by incubation in a water bath at 95 °C for 5 min in buffer R (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl) followed by slow cooling to room temperature over 12 h. The duplex oligonucleotide was purified from unannealed oligonucleotides by high performance liquid chromatography on a GEN-PAC FAX column (Waters, Milford, MA) using a NaCl gradient (25 ml) from 0.3 to 1 M, in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, run at 0.75 ml/min. Under these conditions, only duplex DNA bound to the column and eluted as a single peak at 0.67 M NaCl. Single- or double-stranded oligonucleotides were 5′-end labeled with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase and then purified on a Sephadex G-25 column (Boehringer Mannheim) to remove unincorporated label.

To generate a 5′-resected DNA duplex, pUC19 DNA was cleaved with *Nar*I, and the linear form was purified by electrophoresis through a 5% polyacrylamide gel. Then, the 5′-ends were digested in the buffer recommended by the supplier with 8 units of T7 gene *6* exonuclease/μg of DNA, for 90 s at 30 °C. Under these conditions, an average of 150 bases was removed from each 5′-end.

DNA Binding Assay—Reaction mixtures (30 μl) contained ^{32}P-labeled ssDNA or dsDNA (0.83 and 1.66 μM, respectively) in buffer T (20 mM Tris-HCl, pH 7.5, 100 μg/ml bovine serum albumin, and 0.5 mM dithiothreitol). The concentrations of NaCl, $MgCl_2$, and RecT are indicated in the figure legends. The reaction mixtures were incubated 20 min at 37 °C and filtered by means of a double-filter system (23) through KOH-treated nitrocellulose (24) and DEAE-cellulose membranes (Schleicher & Schuell). For stability analysis, the RecT-DNA complexes were preformed in 240-μl volumes containing 25 mM NaCl, as described above. A 75-fold molar excess of unlabeled ss- or dsDNA was added to the preformed complex, and aliquots (20 μl) were taken at various time points, filtered, and rinsed with 100 μl of binding buffer at 4 °C, as described (23). Data were quantified with a PhosphorImager (Molecular Dynamics). All experiments were repeated at least three times.

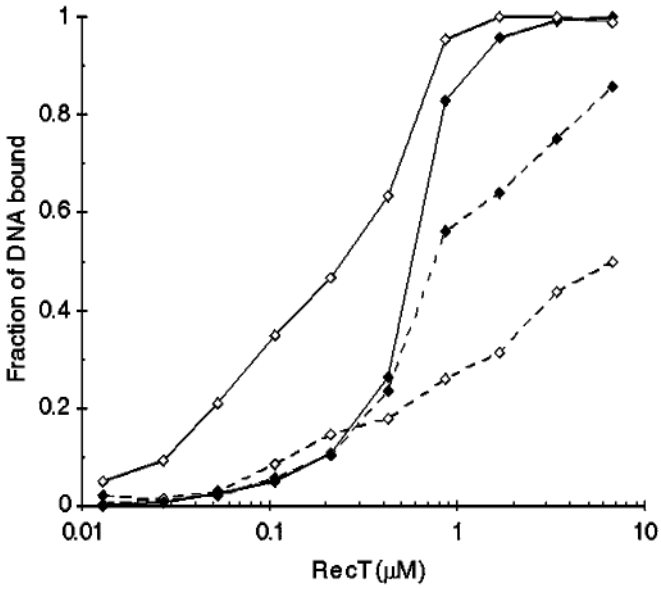

FIG. 1. **Effect of Mg^{2+} on RecT binding to ssDNA and dsDNA.** Binding reactions (30 μl) were performed for 20 min at 37 °C in buffer T containing 0.83 μM ssDNA (♦) and 1.66 μM dsDNA (◇) and the indicated concentrations of RecT. Complex formation was measured by filter binding as described under "Experimental Procedures." All reactions contained 25 mM NaCl and either no $MgCl_2$ (*solid line*) or 5 mM $MgCl_2$ (*broken line*). The plotted data represent the average of at least three independent experiments.

D-loop Formation Assay—Reaction mixtures (30 μl) contained 0.83 μM ^{32}P-labeled single-stranded oligonucleotide, 25 mM NaCl, 20 mM Tris-HCl, pH 7.5, 100 μg/ml bovine serum albumin, 0.5 mM dithiothreitol, and RecT as indicated in figure legends. Mixtures were incubated for 20 min at 25 °C, and supercoiled DNA was added at the concentrations indicated in individual experiments. Mixtures were then incubated for an additional 30 min at 37 °C, and the reactions were stopped by the addition of 0.5 M EDTA, pH 8.0, 10% SDS, and 10 mg/ml proteinase K to the final concentrations of 50 mM, 0.2%, and 550 μg/ml, respectively. Incubation at 37 °C was continued for 20 min, and the mixtures were supplemented with 5 μl of tracking dye (0.25% bromphenol blue, 0.25% xylene cyanol, and 60% glycerol) and loaded on a 0.9% agarose gel in TAE buffer (40 mM Tris acetate, pH 8.0, 2 mM EDTA). Electrophoresis was performed for 150 min at 5 volts/cm at 25 °C. The gel was soaked in 300 ml of water containing 1 μg/ml ethidium bromide for 20 min, photographed, and soaked in 300 ml of 7% trichloroacetic acid for 30 min at 4 °C, neutralized in 200 mM Tris-HCl, pH 8.2, for 20 min, and dried onto Whatman 3MM paper for 3 h at 60 °C. Radiolabeled DNA species were then quantified using a PhosphorImager. Conditions for the reactions using DNA substrates resected with T7 gene *6* exonuclease were the same except that 19 μM resected pUC19 DNA (0.11 pmol) and 40 μM supercoiled pHV2900OB (0.12 pmol) were used as substrates. Electrophoresis was carried out in a 0.7% agarose gel for 16 h at 1.6 volts/cm at 25 °C.

RESULTS

RecT Binds to ssDNA and dsDNA—To examine the DNA binding properties of RecT, nitrocellulose filter binding assays were carried out with single- and double-stranded oligonucleotides, in buffer T containing 25 mM NaCl and no $MgCl_2$. RecT was capable of binding to ssDNA and dsDNA (Fig. 1), and for both DNAs, binding was maximal after 5 min (data not shown). Surprisingly, RecT exhibited a greater affinity for dsDNA than for ssDNA because half-maximum binding was observed at a protein/DNA ratio of 1 RecT monomer/3.3 base pairs and 1 RecT monomer/1.6 bases, respectively. To investigate the effect of Mg^{2+}, binding reactions were repeated in the same buffer containing 5 mM $MgCl_2$ (Fig. 1). RecT binding to dsDNA was reduced dramatically (half-maximum binding at 8 RecT mono-

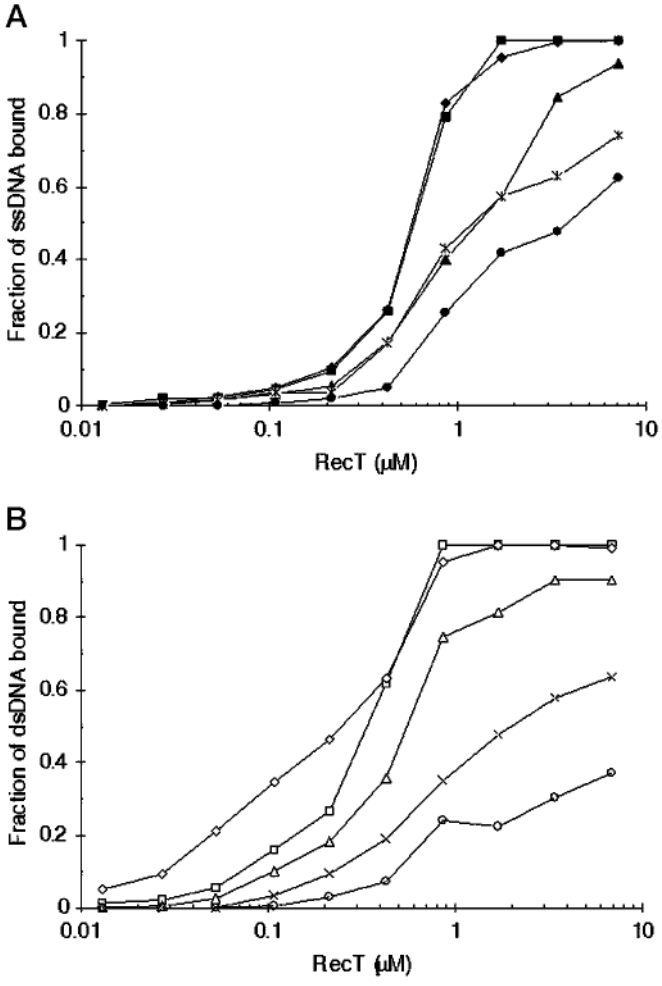

FIG. 2. **Effect of salt concentration on RecT binding to ssDNA and dsDNA.** Reactions were carried out in the absence of Mg^{2+}, as described in the legend to Fig. 1. The concentrations of NaCl in the binding buffer were were 0 mM (■, □), 25 mM (◆, ◇), 50 mM (▲, △), 100 mM (*, ×), and 150 mM (●, ○). *Panel A*, binding to ssDNA (*closed symbols*). *Panel B*, binding to dsDNA (*open symbols*). The plotted data represent the average of at least three independent experiments.

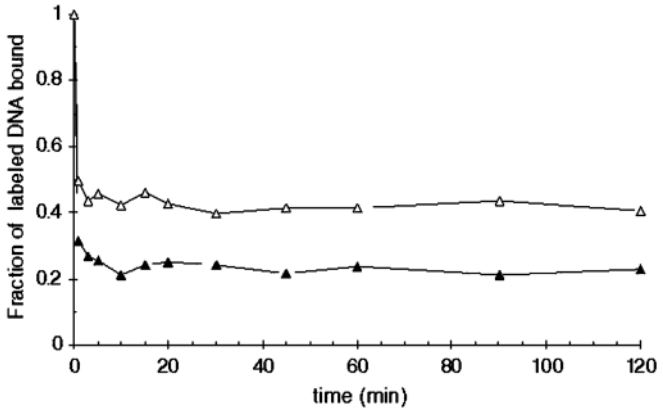

FIG. 3. **Decay of RecT-ssDNA and RecT-dsDNA complexes is biphasic.** Binding was performed in 240-μl reactions containing buffer T, 25 mM NaCl, 0.83 μM ^{32}P-labeled ssDNA (*closed symbols*) or 1.66 μM ^{32}P-labeled dsDNA (*open symbols*), and 3.3 μM RecT (for ssDNA) or 1 μM RecT (for dsDNA). After incubation for 30 min at 37 °C, a 75-fold molar excess of the respective unlabeled DNA was added to the reaction to prevent subsequent binding of RecT to the labeled DNA. At various time points aliquots were drawn, and the amount of DNA remaining bound to RecT was measured by filter binding. The fraction of bound DNA is normalized to a control reaction in which no unlabeled DNA was added. The background level of binding to labeled DNA, determined by adding the 75-fold excess of unlabeled DNA at the onset of the reaction, was 3.7 and 5.5% for ssDNA and dsDNA, respectively. The half-lives of the stable complexes, extrapolated from these data, were about 7 h for ssDNA and dsDNA.

mers/base pair), whereas binding to ssDNA was much less affected (half-maximum binding at 1 RecT monomer/1.2 bases).

The effect of salt concentration on RecT binding was also investigated. Binding isotherms for ssDNA and dsDNA were determined at NaCl concentrations of 0, 25, 50, 100, and 150 mM (Fig. 2). RecT-ssDNA complex formation was optimum between 0 and 25 mM NaCl, and binding was decreased at NaCl concentrations ranging from 50 to 150 mM. Interestingly, at RecT concentrations of less than 0.5 μM, RecT-dsDNA complex formation displayed a different salt sensitivity. As the NaCl concentration was increased, binding to dsDNA was found to be enhanced by the presence of 25 mM NaCl, but then binding was decreased at NaCl concentrations ranging from 50 to 150 mM. These results suggest that RecT could have different modes of binding to ssDNA and dsDNA.

To gain more insight into the nature of the RecT-DNA complexes, RecT-ssDNA and RecT-dsDNA complexes were formed in buffer T containing 25 mM NaCl. These complexes were then challenged with a 75-fold molar excess of unlabeled ss- or dsDNA, and their decay was monitored over time (Fig. 3). Strikingly, both complexes exhibited a biphasic decay, indicating that they were composed of two types of complexes: an unstable complex with a half-life of less than 1 min and a very stable complex with a half-life of greater than 120 min. The RecT-dsDNA complex contained a greater proportion (45%) of the stable form compared with the RecT-ssDNA complex (22%).

RecT Promotes D-loop Formation—The results presented above revealed that RecT has a greater affinity and forms more stable complexes with dsDNA than with ssDNA. To test if RecT could promote a pairing reaction involving a fully duplex DNA, we monitored the invasion of a supercoiled DNA by a ^{32}P-labeled ssDNA (50 nucleotides). The RecT-ssDNA complexes were preformed separately and then mixed with the supercoiled DNA substrate. The joint molecules, which are D-loops, were detected as the comigration of radioactivity with the supercoiled plasmid DNA. The kinetics of joint molecules formation is presented in Fig. 4. After about 30 min, the reaction reached a plateau corresponding to 33% of the supercoiled DNA being converted into D-loops. A slight increase in D-loop formation was observed reproducibly at 120 min (Fig. 4*B*), indicating that the reaction had not yet reached completion. Similar levels of D-loop formation were obtained with oligonucleotides homologous to several different loci in the supercoiled plasmid, indicating that D-loop formation is not specific for a particular DNA sequence (data not shown).

The requirements for D-loop formation are summarized in Table I. D-loop formation was strictly dependent on the presence of RecT. D-loops were not detected when heterologous ssDNA was substituted for homologous ssDNA or when linear pUC18 DNA was substituted for supercoiled pUC18 DNA in the reaction. These results indicate that joint molecule formation requires homologous pairing and is stabilized by supercoiling, consistent with the known properties of D-loops (25). D-loop formation was optimum at 25 mM NaCl and decreased at salt concentrations higher than 50 mM. The presence of 3 mM EDTA did not affect D-loop formation, showing that Mg^{2+} ions are not required. D-loop formation occurred in the presence of 1 and 2.5 mM $MgCl_2$ but was inhibited almost completely by the addition of 5 mM $MgCl_2$. Interestingly, both RecT binding to dsDNA and joint molecule formation were inhibited by the presence of Mg^{2+} (see Fig. 1) and stimulated by the addition of 25 mM NaCl (see Fig. 2). This correlation between D-loop for-

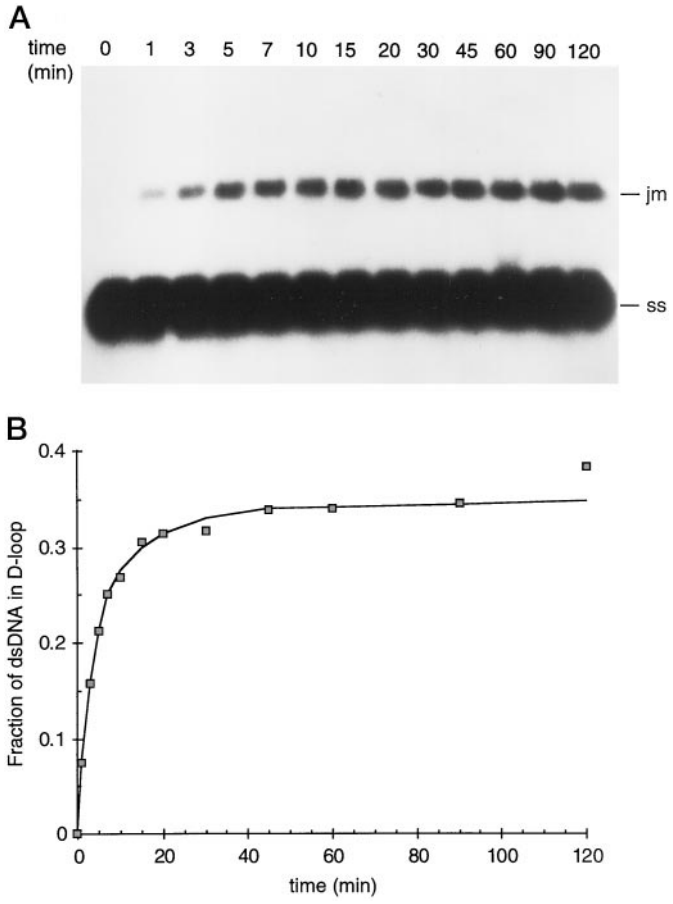

FIG. 4. **RecT promotes D-loop formation.** *Panel A*, reaction mixtures (30 μl) containing ^{32}P-labeled ssDNA (0.83 μM) in buffer T with 25 mM NaCl were preincubated with 0.33 μM RecT. Supercoiled pUC18 DNA (30 μM) was added and the reaction incubated at 37 °C for the indicated times. The reaction products were deproteinized and resolved in a 0.9% agarose gel. *jm*, joint molecules; *ss*, ssDNA. *Panel B*, graphical representation of the results of three independent kinetics experiments.

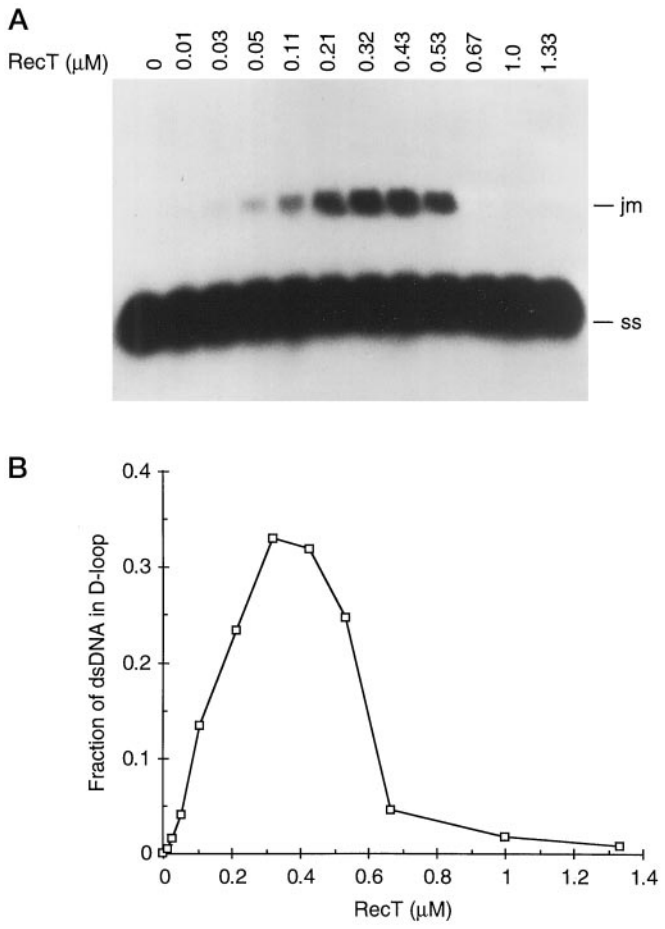

FIG. 5. **D-loop formation as a function of RecT concentration.** *Panel A*, the indicated RecT concentrations were preincubated with 0.83 μM ^{32}P-labeled ssDNA in buffer T containing 25 mM NaCl, for 20 min at 25 °C. Supercoiled pUC18 DNA (30 μM) was added and the reaction mixture (30 μl) incubated for another 30 min at 37 °C. The reaction products were deproteinized and separated by electrophoresis in a 0.9% agarose gel. *jm*, joint molecules; *ss*, ssDNA. *Panel B*, graphical representation of the results of three independent experiments.

mation and RecT binding to dsDNA suggests a potential role for RecT-dsDNA complexes in the pairing reaction.

Under the reaction conditions described above, D-loop formation occurs in the presence of two different types of RecT-ssDNA complexes, the stable and unstable complexes observed in Fig. 3. Although the role of these different complexes is not known, it is noteworthy that the amount of stable RecT-ssDNA complex formed under our experimental conditions (22%) corresponds to the level of RecT-ssDNA complex at which D-loop formation is optimal (20–26%, see below), suggesting that the stable form of RecT-ssDNA complexes could possibly be the active species involved in pairing. To test this hypothesis, RecT-ssDNA complexes were formed with amounts of RecT ranging from 0 to 1.0 μM, under the conditions used for the D-loop assay, and then incubated in the presence of a 64-fold excess of heterologous oligonucleotide for 40 min to compete away the majority of the unstable RecT-ssDNA complexes and only a small portion of the stable RecT-ssDNA complexes. The resulting complexes were then assayed for their activity in D-loop formation assays. The results showed that from 32 to 100% of the D-loop formation activity was retained under these conditions, depending on the initial amount of RecT present (data not shown). These results demonstrate that the stable RecT-ssDNA complexes are active in promoting D-loop formation, and, if the unstable complexes have any activity at all, it must be significantly less that that of the stable RecT-ssDNA complexes.

dsDNA-RecT Complexes Cannot Pair with ssDNA—In the experiments presented above, formation of joint molecules was obtained when ssDNA-RecT complexes were preformed before the addition of dsDNA, consistent with the idea that the RecT-ssDNA filament promoted pairing (26). To investigate further the role of the dsDNA-RecT complexes in joint molecule formation, dsDNA was preincubated with RecT, and the dsDNA-RecT complex was then mixed with uncoated ssDNA. Under these conditions, D-loops were not detected even after 60 min of incubation (data not shown). These results indicate that the RecT-dsDNA complex is incapable of promoting the pairing reaction. However, when RecT was added to a mixture of uncoated ss- and dsDNA substrates, weak but significant D-loop formation (1% of the dsDNA converted) was observed (data not shown). This suggests that RecT can promote pairing of ss- and dsDNA substrates but that the formation of RecT-dsDNA complex inhibits the pairing reaction.

To test this possibility further, the effect of RecT concentration on D-loop formation was examined. Varying amounts of RecT were preincubated with the ssDNA, and the reaction was initiated by adding dsDNA to the mixture. The results are presented Fig. 5. The amount of joint molecules formed increased with increasing concentrations of RecT and reached an optimum at between 0.3 and 0.43 μM RecT, corresponding to a ratio of about 1 RecT monomer/2.5 bases of ssDNA. This optimal pairing activity occurred at subsaturating concentrations

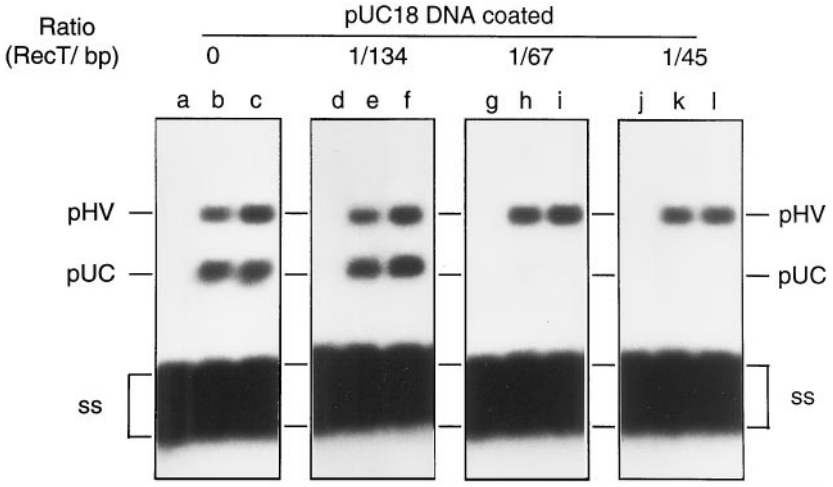

FIG. 6. **RecT-dsDNA complexes inhibit pairing.** Supercoiled pUC18 DNA was precoated with varying amounts of RecT, in 7.5 μl of buffer T + 25 mM NaCl for 20 min at 25 °C, to give the indicated ratios of RecT monomer/base pairs. The RecT-ssDNA filaments were formed with 0.83 μM ^{32}P-labeled ssDNA and either 0 μM (*lanes a*, *d*, *g*, and *j*), 0.17 μM (*lanes b*, *e*, *h*, *k*), or 0.33 μM RecT (*lanes c*, *f*, *i*, *l*), as described under "Experimental Procedures." The reactions (30 μl, final volume) were initiated by adding precoated pUC18 (15 μM) and uncoated pHV2900OB dsDNA (28 μM) and incubated for 30 min at 37 °C. The reaction products were deproteinized and separated by electrophoresis in a 0.9% agarose gel. The joint molecules, which comigrate with the supercoiled molecules, are indicated. *ss*, ssDNA; *pUC*, supercoiled pUC18; *pHV*, supercoiled pHV2900OB

of RecT, where 20–26% of the ssDNA is bound (see Fig. 1). At RecT concentrations exceeding 0.43 μM, D-loop formation diminished sharply, indicating that pairing was inhibited by an excess of RecT. One possible explanation for this inhibition is that in excess of the optimum concentration, enough RecT would be available to bind to the dsDNA substrate and inhibit the pairing reaction.

To test directly whether RecT-dsDNA complexes inhibit the pairing reaction, preformed RecT-ssDNA filaments were mixed with RecT-dsDNA complexes that were made separately in the presence of different concentrations of RecT. In the same reaction, an uncoated dsDNA, pHV2900OB, which is also homologous to the ssDNA and distinguishable from pUC18 by size, was included. The quantities of D-loops formed with the RecT-coated dsDNA and the uncoated dsDNA substrates were compared. As shown in Fig. 6, the capacity to form joint molecules was decreased for the RecT-coated dsDNA but remained unaffected for the uncoated dsDNA present in the same reaction. This inhibition of the pairing reaction occurred at a ratio of 1 RecT monomer/67 base pairs, which is about 70 times below saturation level (from Fig. 1, saturation occurred at about 1 RecT monomer/base pair), suggesting that the inhibition is not caused by complete coating of the dsDNA. This experiment also confirms that RecT-dsDNA complexes are unable to pair with uncoated ssDNA (Fig. 6, *lanes d*, *g*, and *j*).

RecT Mediates the Strand Transfer of the Single-stranded Ends of a DNA Duplex—We have shown that RecT has a higher affinity for dsDNA than for ssDNA and that the RecT-dsDNA complexes inhibited pairing. Together, these findings raised the question of whether RecT-ssDNA complexes competent for strand transfer could be formed at the single-stranded ends of a duplex DNA that has been resected by an exonuclease. Such a resected duplex is assumed to be the DNA substrate to initiate recombination in the RecE pathway (6). To test this idea, pUC19 DNA was linearized by *Nar*I and treated briefly with an exonuclease that degrades the 5′-strands. This linear duplex DNA with 3′-single-stranded tails (3′-ssDNA) was then preincubated with varying amounts of RecT, and supercoiled pHV2900OB was added to initiate the reaction. The formation of joint molecules (Fig. 7) increased with the amount of RecT and reached an optimum at 0.67 μM RecT, where 8% of the supercoiled DNA was trapped in joint molecules. Increasing

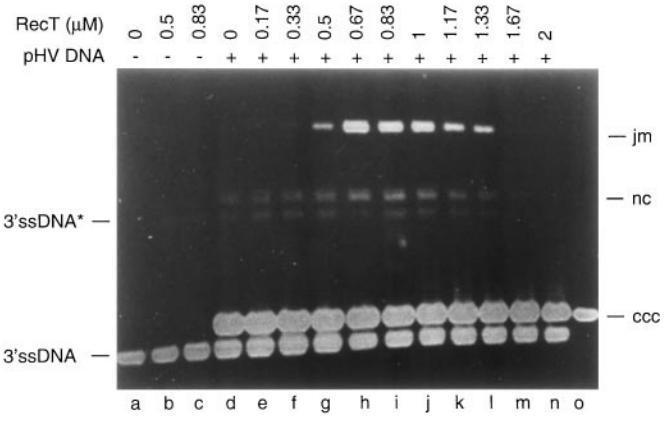

FIG. 7. **Strand transfer with a 5′-resected DNA duplex mediated by RecT.** pUC19 DNA was linearized by *Nar*I and partially degraded with T7 gene *6* exonuclease, as described under "Experimental Procedures," to generate a resected duplex carrying 3′-single-stranded tails about 150 bases long, designated *3′ssDNA*. 3′-ssDNA (19 μM) was incubated with the indicated amounts of RecT for 20 min at 25 °C. Supercoiled pHV2900OB dsDNA (40 μM) was added to initiate the reactions (*lanes d–n*) or was omitted in controls (*lanes a–c*). The reaction mixtures were incubated 50 min at 37 °C, deproteinized, and the products resolved by electrophoresis in a 0.7% agarose gel and revealed by ethidium bromide staining. Unreacted supercoiled pHV2900OB dsDNA is in *lane o*. The slow migrating band in 3′-ssDNA, denoted *3′ssDNA**, was always present after exonuclease degradation and disappeared after 15 min at 70 °C (data not shown), indicating that it is the result of sticky end artifacts. *jm*, joint molecules; *nc*, nicked circular duplex; *ccc*, supercoiled pHV2900OB.

the RecT concentration further resulted in a decrease in joint molecule formation. The formation of joint molecules was not observed if the exonuclease treatment of the linear pUC19 DNA was omitted (data not shown), consistent with the known requirement of RecT for ssDNA ends (18). As a control, a DNA substrate having 29 base pairs of heterology at each single-stranded end was generated by cleaving pUC19 with *Xba*I, at the center of the polylinker region (which is not present in pHV2900OB (21)), followed by limited degradation of the 5′-strands with T7 gene *6* exonuclease. No joint molecule formation was observed with this DNA substrate (data not shown), indicating that homologous single-stranded tails are required

for the formation of D-loops. These results show that RecT can promote pairing of a supercoiled DNA with a DNA duplex having homologous single-stranded tails, indicating that RecT filaments can form on those single-stranded tails. Interestingly, strand transfer occurred at subsaturating concentrations of RecT (1 RecT monomer/14 base pairs), where one would predict that most of the RecT protein would be bound to dsDNA (see Fig. 1).

DISCUSSION

To investigate the pairing reactions promoted by RecT, we examined the interactions between RecT and DNA, using a nitrocellulose filter binding assay. We found that RecT bound to ssDNA as well as to dsDNA but with an apparent higher affinity for dsDNA than for ssDNA. Binding to dsDNA was inhibited by the addition of Mg^{2+}. Previous filter binding studies only revealed RecT binding to ssDNA, and previous electron microscopy studies revealed RecT-ssDNA helical nucleoprotein filaments but only sparse internal binding of RecT with dsDNA (17, 26). These differences can be explained by the fact that the previous binding studies were carried out in the presence of high salt and high Mg^{2+}, conditions which strongly inhibited binding of RecT to dsDNA (see Figs. 1 and 2). Interestingly, RecT binding to dsDNA was enhanced by the addition of 25 mM NaCl, whereas binding to ssDNA was not. Analysis of the decay of RecT-ssDNA and RecT-dsDNA complexes revealed that RecT displayed at least two modes of binding to DNA. Each complex was composed of two forms, an unstable complex with a half-life < 1 min, and a very stable complex with a half-life $>$ 120 min. Under our experimental conditions, more of the bound DNA was of the stable form with dsDNA (45%) than with ssDNA (22%). Although the role of these different complexes is not known, the results of competition experiments suggest that the stable form of the RecT-ssDNA complexes is the most active species involved in pairing and could possibly be the only active species.

A striking feature of RecT binding to DNA is its sensitivity to Mg^{2+}. The addition of 5 mM $MgCl_2$ to the reaction drastically reduced the binding of RecT to dsDNA and, to a much smaller extent, its binding to ssDNA. This observation explains why previous binding studies, carried out in the presence of 13 mM $MgCl_2$, did not detect RecT binding to dsDNA (17). Electron microscopy studies revealed that in the presence of $MgCl_2$, RecT monomers assemble into doughnut-shaped oligomers containing 7 or 8 monomers and rod-like structures but that in the absence of $MgCl_2$, no oligomer was formed (26). Together these results suggest that optimum binding to DNA is achieved when RecT is only in a monomer form, suggesting in turn that RecT oligomers might not be proficient for binding, especially to dsDNA.

The results presented in this study show that RecT protein can efficiently transfer a ssDNA into a homologous duplex DNA *in vitro*, leading to the formation of a joint molecule containing a D-loop. Strand transfer did not require any high energy cofactor; it occurred at 0, 1, and 2.5 mM $MgCl_2$ and was inhibited completely by the presence of 5 mM $MgCl_2$. Optimum strand transfer was achieved when RecT was preincubated with ssDNA at a ratio of 1 RecT monomer/2.5 bases of ssDNA and subsequently reacted with uncoated dsDNA, indicating that pairing is mediated by a RecT-ssDNA complex. RecT-dsDNA complexes were unable to participate in pairing with uncoated ssDNA or with RecT-ssDNA complexes. However, both strand transfer and RecT binding to dsDNA displayed the same sensitivity to salt and Mg^{2+} concentrations, suggesting that the capacity of RecT to bind dsDNA likely plays some role in strand transfer. In contrast, RecT binding to dsDNA was not required for the annealing of complementary ssDNA because this reaction occurred efficiently in the presence of high $MgCl_2$ concentrations (17, 18).[2] Taken together, these results suggest that RecT promotes joint molecule formation by a mechanism that is different from annealing. Finally, RecT can mediate D-loop formation between a supercoiled dsDNA and a linear dsDNA having homologous 3′-single-stranded tails, indicating that, despite its higher affinity for dsDNA, RecT is able to polymerize on the single-stranded tails, making them invasive. Indeed, this type of binding was observed previously by electron microscopy under conditions in which RecT would not bind to dsDNA (26).

Our understanding of homologous pairing reactions comes mostly from the extensive studies of the *E. coli* RecA protein (for a review, see Ref. 27) and similar proteins such as the bacteriophage T4 UvsX protein (28), *Saccharomyces cerevisiae* Rad51 (29), and human Rad51 (30). This class of homologous pairing proteins requires a high energy cofactor and Mg^{2+} to form a nucleoprotein filament on the ssDNA which then promotes synapsis with the homologous duplex partner. RecT protein also forms nucleoprotein filaments with ssDNA which then catalyze the strand transfer reaction. However, unlike RecA, RecT performs strand transfer without ATP and in the absence of Mg^{2+} as well as at low concentrations of Mg^{2+}. Other ATP-independent DNA-pairing proteins have been shown to promote pairing and strand exchange *in vitro*, such as the bacteriophage T7 gene 2.5 protein (31), *E. coli* RecO protein (32), and *S. cerevisiae* Sep1 protein (33, 34). However, in all cases, the addition of Mg^{2+} was required for the reaction. This feature of RecT, which could be related to its higher affinity for dsDNA than for ssDNA, suggests that RecT differs from these other DNA strand transfer proteins. Alternately, the inhibition of strand transfer by high Mg^{2+} concentrations could be similar to the situation with RecA protein where presynaptic filament formation occurs more efficiently at low concentrations of Mg^{2+} compared with high concentrations of Mg^{2+}, presumably because of the inhibitory effect of secondary structure in ssDNA at high Mg^{2+} concentrations (35). It also suggests that the level of unbound Mg^{2+} in the cell could regulate the RecT-promoted strand transfer reactions. Unfortunately, no accurate measurement of free Mg^{2+} levels is currently available (36 and references therein), although they are generally thought to be at low levels in the range on 1 mM where RecT promotes D-loop formation.

Genetic evidence indicates that RecT is required for DSBR in *recBC sbcA* strains (14). This RecT-promoted DSBR is independent of RecA function and occurs by a conservative mechanism (12, 37). Here, we provide *in vitro* evidence that RecT can promote the invasion of a DNA duplex by a ssDNA to form a D-loop, which is the predicted initiation step in DSBR models (38, 39). Therefore, our results indicate that RecT can initiate recombination not only by promoting DNA-reannealing and heteroduplex exchange (18) but also by a DNA strand invasion mechanism, thus providing an explanation for the RecA independence of DSBR in *recBC sbcA* strains. Double strand breaks have been shown to induce recombination-dependent DNA replication in *recBC sbcA* strains where the RecE pathway of recombination is activated (40). In this background, recombination-dependent DNA replication was shown to depend on a functional RecE pathway and partially on RecA function. It was suggested that the residual recombination-dependent DNA replication activity observed in the absence of RecA could be attributed to the pairing activity of RecT (40). Our finding that *in vitro*, RecT promotes efficient D-loop formation with DNA substrates similar to those that the RecE exonuclease

[2] P. Noirot, unpublished results.

would generate strongly supports this interpretation. Taken together, these results suggest that RecT can promote D-loop formation *in vivo*. The biological importance of this process is underlined by mounting evidence suggesting that D-loop formation can be used not only to repair double-strand breaks but also to generate active replication forks (for review, see Ref. 41).

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