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Role of the *Escherichia coli* Recombination Hotspot, χ, in RecABCD-dependent Homologous Pairing*

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Genetic recombination occurring in wild type *Escherichia coli* is stimulated at DNA sequences known as χ sites, 5′-GCTGGTGG-3′. *In vitro*, homologous pairing between duplex DNA substrates dependent upon the RecA, RecBCD, and SSB proteins is stimulated by the presence of a χ sequence in the donor linear double-stranded DNA. We show that this stimulation is due to two factors: 1) the enhanced production of χ-specific single-stranded DNA fragments and 2) their preferential use in the RecA protein-promoted pairing step. Furthermore, under conditions of limiting Mg^{2+} concentration, joint molecule formation does not occur, even though DNA unwinding and χ-specific single-stranded DNA fragment production are observed. Also, under these conditions, χ-specific fragments derived from both the upstream and downstream regions of the DNA strand containing χ and from cleavage of the non-χ-containing DNA strand are detected. Finally, the behavior of mutant RecBCD enzymes (RecBC*D and RecBCD‡) in this *in vitro* reaction is shown to parallel their *in vivo* phenotypes with respect to χ stimulation of recombination. Thus we suggest that, in addition to its ability to regulate the degradative activities of RecBCD enzyme, χ itself may be a preferred site for initiation of homologous pairing in this concerted process.

Homologous recombination in *Escherichia coli* occurs primarily through the RecBCD pathway of generalized recombination (for reviews see Smith (1988, 1989), Clark and Sandler (1994), and Kowalczykowski, *et al.* (1994)). Genetic analysis demonstrates that recombination occurring through this pathway is dependent on the RecA, RecBCD, and single-stranded DNA binding (SSB)[1] proteins (Clark and Margulies, 1965; Glassberg *et al.*, 1979; Emmerson and Howard-Flanders, 1967; Howard-Flanders and Theriot, 1966). Mutations in the *recA* gene decrease levels of recombination by as much as 6 orders of magnitude (Clark and Margulies, 1965), mutations in the *recB* or *recC* genes reduce recombination by 99% (Emmerson and Howard-Flanders, 1967; Howard-Flanders and Theriot, 1966), and defects in the *ssb* gene can reduce recombination by 80% (Glassberg *et al.*, 1979). In addition to these protein components, recombination in the RecBCD pathway is enhanced by recombination hotspots, called χ sites (5′-GCTGGTGG-3′), which increase the frequency of genetic exchange 5- to 10-fold in their vicinity (Lam *et al.*, 1974; McMilin *et al.*, 1974; Smith *et al.*, 1981; Smith, 1983; Stahl *et al.*, 1975).

The biochemical roles of RecA and SSB proteins in recombination reactions *in vitro* are well established (for reviews, see Kowalczykowski (1991a, 1991b), Radding (1991), West (1992), Cox (1993), and Kowalczykowski and Eggleston, 1994). RecA protein promotes both the exchange of DNA strands between complementary single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) and the renaturation of homologous ssDNA. The ability of RecA protein to exchange complementary DNA strands is stimulated by SSB protein *in vitro*, and studies using mutant RecA proteins suggest that the process of DNA strand exchange is likely to represent recombinational events occurring *in vivo* (see Kowalczykowski (1991a) and Kowalczykowski *et al.* (1994)).

The RecBCD enzyme (exonuclease V) is multifunctional and consists of three nonidentical subunits, the RecB, RecC, and RecD polypeptides. *In vitro*, it has the following biochemical activities: DNA-dependent ATPase, ssDNA and dsDNA exonuclease, ssDNA endonuclease, and ATP-dependent DNA helicase (for reviews, see Taylor (1988), Smith (1990), Kowalczykowski (1994), and Kowalczykowski *et al.* (1994)). In addition to its nonspecific nuclease activities, RecBCD enzyme can generate single-stranded DNA fragments whose 3′-end terminates 4–6 nucleotides to the 3′-side of the χ recombination hotspot (Ponticelli *et al.*, 1985; Taylor *et al.*, 1985) (see Smith (1994)). This specific interaction is orientation-dependent, since RecBCD enzyme must approach from the 3′-side of the χ sequence for recognition to occur (Taylor *et al.*, 1985).

The ability of χ to stimulate recombination in a polar fashion stems from the fact that the χ site is a regulatory DNA sequence, which acts to attenuate the nonspecific nuclease activity, but not the helicase activity, of the RecBCD enzyme; this results in the creation and preservation of a ssDNA fragment containing the χ sequence (Dixon and Kowalczykowski, 1991; Dixon and Kowalczykowski, 1993) (see Kowalczykowski (1994)). This attenuation of nuclease activity is a result of the loss or functional inactivation of the RecD subunit through interaction with χ (Dixon, *et al.*, 1994).

Certain mutations in the *recB*, *recC*, or *recD* genes yield altered RecBCD enzymes that display differential effects with regard to χ-stimulation of recombination. The *recC** class of mutations was isolated as pseudorevertants of a presumed missense mutation in the *recC* gene (Schultz *et al.*, 1983). While being moderately recombination-proficient and maintaining wild type levels of dsDNA exonuclease activity, the RecBC*D enzyme is, however, unable to stimulate recombination at χ sites (Schultz *et al.*, 1983). The *recBCD*‡* mutations appear to be nonsense mutations that map primarily to the *recD* gene and

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[1] The abbreviations used are: SSB, single-stranded DNA-binding; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

thus are devoid of a full-length *recD* gene product (Amundsen *et al.*, 1986; Chaudhury and Smith, 1984). These mutants are similar to wild type cells in that they are fully viable and are recombination-proficient; in fact, they display elevated levels of conjugal, plasmid, and phage λ recombination (Chaudhury and Smith, 1984; Lovett *et al.*, 1988; Thaler *et al.*, 1989). However, despite the elevated recombination levels, the *recD*‡ mutants do not display χ-stimulation of recombination (Chaudhury and Smith, 1984). The most noticeable difference between double dagger mutants and wild type RecBCD enzyme is that the mutant RecBCD‡ enzymes are devoid of any detectable nuclease activities *in vivo* and in cell extracts (Amundsen *et al.*, 1986; Chaudhury and Smith, 1984; Taylor, 1988), but they retain helicase activity (Rinken *et al.*, 1992).

Previously, the formation of joint molecules *in vitro* between dsDNA substrates was shown to depend on the RecA, RecBCD, and SSB proteins in a coordinated process referred to as the "RecABCD-dependent reaction" (Roman *et al.*, 1991). Formation of joint molecules requires creation of ssDNA through unwinding of linear dsDNA by RecBCD enzyme, trapping of the ssDNA strands by RecA and SSB proteins, and DNA strand invasion of homologous supercoiled DNA molecule promoted by RecA protein (Kowalczykowski and Roman, 1990). χ enhances the RecABCD-dependent reaction *in vitro* with a polarity identical to that expected from *in vivo* observations (Dixon and Kowalczykowski, 1991). The role of χ was to down-regulate the destructive nuclease activity of RecBCD enzyme, while allowing the recombination-promoting helicase activity to persist.

In this report, we expand on our previous findings to demonstrate that χ-dependent joint molecule formation occurs more rapidly and at a higher frequency than χ-independent pairing events. At reduced magnesium ion concentrations, the formation of χ-specific ssDNA fragments is enhanced, and fragments derived from both the 5′- and 3′-sides of χ are detected; however, under these conditions, homologous pairing is limited by the inability of the RecA protein to promote DNA strand invasion. Furthermore, mutant RecBCD enzymes that do not recognize χ *in vivo* fail to stimulate χ-dependent joint molecule formation to the same extent as the wild type RecBCD enzyme does.

MATERIALS AND METHODS

Enzymes—The various RecBCD enzymes were purified as described (Roman and Kowalczykowski, 1989a), and their protein concentrations were determined using an extinction coefficient of 4.0×10^5 M^{-1} cm^{-1} at 280 nm (Roman and Kowalczykowski, 1989a). The specific activity of the wild type enzyme preparation was 5.4×10^4 nuclease units/mg of protein or 1.1×10^4 helicase units/mg of protein. The RecBC*D enzyme was purified from strain V193 that bears a chromosomal deletion of *recBCD* and contains a plasmid with the *recC1003* allele (Schultz *et al.*, 1983). The purity of this preparation was estimated to be >95% as judged by SDS-polyacrylamide gel electrophoresis; the *recC1003* mutation does not appear to affect the yield of the RecC subunit, since all three subunits appear in approximately stoichiometric amounts (data not shown). The specific activity of this enzyme preparation was 1.1×10^5 nuclease units/mg of protein or 1.9×10^4 helicase units/mg of protein. The RecBCD‡ enzyme was purified from strain V410 that bears a chromosomal deletion of *recBCD* and contains a plasmid with the *recD1011* allele (Amundsen *et al.*, 1986; Chaudhury and Smith, 1984). The purity of this preparation was estimated to be >95% as judged by SDS-polyacrylamide gel electrophoresis; the RecB and RecC subunits are present in stoichiometric amounts by visual inspection, and the RecD subunit is undetectable by silver staining. The specific activity of the enzyme preparation was 6.0×10^2 nuclease units/mg of protein or 7.2×10^2 helicase units/mg of protein. Strains V186 (wild type), V193 (*recC*1003*), and V410 (*recD‡1011*) were provided by A. F. Taylor, S. K. Amundsen, and G. R. Smith (Hutchinson Cancer Research Center, Seattle, WA). Nuclease units and helicase units were measured as described by Eichler and Lehman (1977) and Roman and Kowalczykowski (1989a), respectively.

RecA protein was purified using a procedure based on spermidine precipitation (Griffith and Shores, 1985).[2] Protein concentration was determined using an extinction coefficient of 2.7×10^4 M^{-1} cm^{-1} at 280 nm.

SSB protein was isolated from strain RLM727 and purified according to LeBowitz (1985). Protein concentration was determined using an extinction coefficient of 3.0×10^4 M^{-1} cm^{-1} at 280 nm (Ruyechan and Wetmur, 1975).

All restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs, Pharmacia LKB, or Life Technologies, Inc. The enzymes were used as described by Sambrook *et al.* (1989) or as indicated by the specific vendor.

DNA Substrates—The plasmids pBR322 χ° (wild type), pBR322 χ⁺F225 (Smith *et al.*, 1981), pBR322 χ⁺H (Dixon and Kowalczykowski, 1991), and pBR322 χ⁺FH (Dixon and Kowalczykowski, 1993) were prepared from strains S819, S818, SKDD003, and SKDD004, respectively. All plasmid DNA was purified by CsCl density gradient centrifugation (Sambrook *et al.*, 1989). The molar concentration of dsDNA in nucleotides was determined by using an extinction coefficient of 6290 M^{-1} cm^{-1} at 260 nm. Plasmid DNAs were linearized with appropriate restriction enzymes and radioactively end-labeled either at the 5′-end by sequential reaction with calf intestinal phosphatase and then with T4 polynucleotide kinase and [χ-^{32}P]ATP (ICN Pharmaceuticals, Inc.) or at the 3′-end using the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP (ICN Pharmaceuticals, Inc.). Supercoiled plasmid pBR322 χ° was uniformly labeled with ^{3}H by Sss I (C*pG) methylase (New England Biolabs) and *S*-[*methyl*-^{3}H]adenosylmethionine (DuPont NEN) according to a method provided by New England Biolabs. Subsequent purification of radiolabeled DNA was accomplished by passage over an Elutip-d column (Schleicher and Schuell) and precipitation with ethanol.

Reaction Conditions—The standard RecABCD reaction mixture consisted of 25 mM Tris acetate (pH 7.5), 8 mM magnesium acetate, 5 mM ATP, 1 mM dithiothreitol, 1 mM phosphoenolpyruvate, approximately 4 units/ml pyruvate kinase, 10 μM nucleotides linear dsDNA (2.3 nM dsDNA ends), 5 μM nucleotides supercoiled pBR322 χ° DNA, 5 μM RecA protein, 1 μM SSB protein, and 0.025 molecules of functional wild type or mutant RecBCD enzyme per linear dsDNA end. The concentration of functional enzyme was determined by titration of helicase activity (Roman and Kowalczykowski, 1989a), and the results are shown in Table II (the respective total amount of each RecBCD enzyme is indicated in the figure legends). Assays were performed at 37 °C and were begun with the addition of RecBCD enzyme after preincubation of all other components for 1 min.

Joint Molecule Formation Assays—The detection of RecBCD enzyme-dependent joint molecule formation was accomplished using either a nitrocellulose filter binding assay (Roman *et al.*, 1991) or an agarose gel electrophoresis assay (Dixon and Kowalczykowski, 1991). For the nitrocellulose filter binding assay, standard RecABCD reaction conditions were used with 5 μM ^{3}H-labeled supercoiled pBR322 χ° DNA as the recipient DNA molecule. At the indicated times, aliquots of the reaction mixture were stopped with 1% SDS to deproteinize the sample and placed on ice. 10 volumes of D-loop buffer (2 M NaCl, 150 mM sodium citrate) were added, and the mixture was immediately filtered through a nitrocellulose disc (HAWP 025; Millipore Corp.) that was presoaked in D-loop buffer. The filter was washed with 3 ml of D-loop buffer, dried, and counted in 10 ml of nonaqueous scintillation fluid (RPI). The values are reported as a percentage of the total input supercoiled DNA contained within a reaction and are the average of experiments repeated at least three times.

For the agarose gel assay, standard RecABCD reaction conditions were used, except all DNA and protein components were increased by a factor of 4 unless otherwise indicated, and the donor linear dsDNA was radiolabeled at the 5′-end. Aliquots of the reaction mixture (40 μl) were taken at the indicated time points and were added to 10 μl of stop buffer (0.1 M EDTA, 2.5% SDS, 40% glycerol, 0.125% bromphenol blue, and 0.125 xylene cyanol) to deproteinize the sample. Electrophoresis was performed for 10 h at 2.1 V/cm using 0.75% agarose gels in TAE buffer (40 mM Tris acetate (pH 8.0), 2 mM EDTA). The gels were dried, and autoradiography was at −20 °C with Kodak XAR-5 film and using an intensifying screen. The autoradiogram was analyzed using a Bio Image system (Millipore). The reported values are expressed as the percentage of the total input linear dsDNA. The χ-dependent and χ-independent joint molecules are defined as containing either the ssDNA fragment downstream of the χ sequence or a full-length ssDNA strand, respectively, as the invasive ssDNA strand (Dixon and Kowalczykowski, 1991).

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

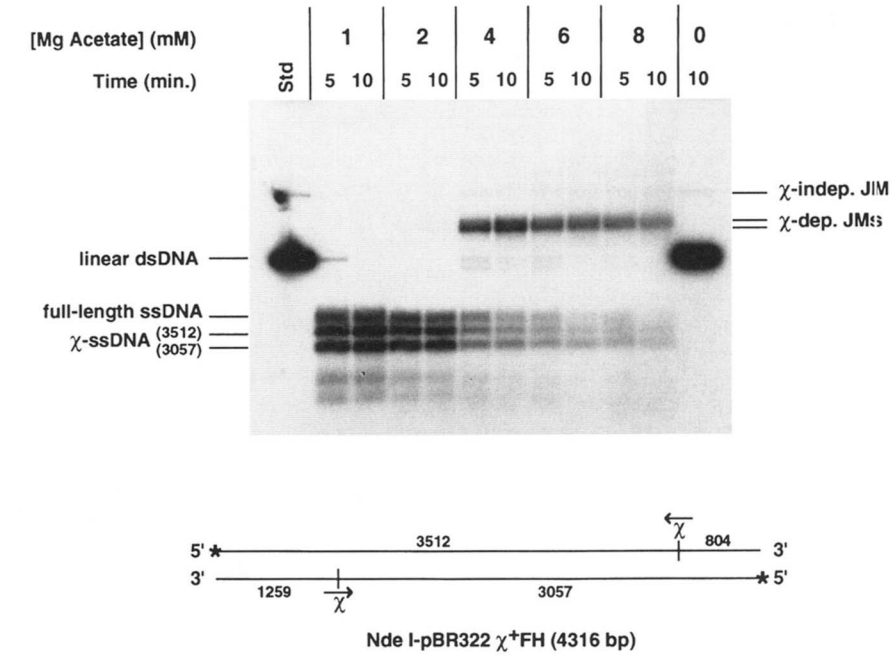

FIG. 1. **RecABCD-dependent joint molecule formation at various concentrations of Mg^{2+}.** RecABCD-dependent reactions were performed using standard RecABCD-reaction conditions at the indicated concentrations of magnesium acetate (labeled *[Mg Acetate] (mM)*). The ATP concentration was 1 mM, and DNA and protein concentrations were at the standard levels (*i.e.* they were not increased 4-fold). The linear dsDNA substrate *Nde*I-pBR322 χ^+FH was radioactively labeled at the 5′-ends. Reactions were initiated with the addition of 0.31 nM wild type RecBCD enzyme (0.025 functional enzyme/dsDNA end). All values reported are a percentage of the total input linear dsDNA contained in a reaction (*Std lane*). χ-dependent joint molecules (*χ-dep. JMs*) containing either the 3512- or 3057-nucleotide χ-specific ssDNA fragment (*χ-ssDNA*) are indicated as a doublet; the χ-independent joint molecule (*χ-indep. JM*) contains a full-length ssDNA strand. A control reaction containing no Mg^{2+} is shown in the *far right lane*.

DNA Unwinding and χ-Specific ssDNA Formation Assays—Standard conditions for the DNA unwinding and χ-specific ssDNA formation assays consisted of 25 mM Tris acetate (pH 7.5), 8 mM magnesium acetate, 1 mM ATP, 1 mM dithiothreitol, 1 mM phosphoenolpyruvate, approximately 4 units/ml pyruvate kinase, 10 μM nucleotides linear dsDNA (2.3 nM dsDNA ends), 2 μM SSB protein, and RecBCD enzyme. The linear dsDNA, either lacking χ sites (χ°) or containing χ sites (χ^+) was radioactively labeled at either the 5′- or 3′-end as indicated. The presence of SSB protein facilitates the unwinding reaction by trapping the unwound DNA strands and inhibits the post-unwinding degradation of ssDNA by RecBCD enzyme (MacKay and Linn, 1976; Roman and Kowalczykowski, 1989a). Control experiments confirmed that the amount of post-unwinding degradation of ssDNA is < 2% over the reaction times examined here (data not shown). Assays were performed at 37 °C and were initiated with the addition of ATP and pyruvate kinase after preincubation of all other components for 1 min. Reactions were performed and treated identically to those described for the agarose gel assay for joint molecule formation, except that electrophoresis was for 15 h using 1.2% agarose gels at 1.7 V/cm in TAE buffer. The reported values are expressed as a percentage of the total input linear dsDNA.

Fluorescent Helicase Assay—The helicase activity of wild type and mutant RecBCD enzymes was also assayed by measuring the quenching of the intrinsic protein fluorescence of SSB protein upon binding to ssDNA (Roman and Kowalczykowski, 1989a) under standard DNA unwinding reaction conditions. The decrease in fluorescence was monitored using a Shimadzu RF-5000 spectrofluorophotometer, and the data were analyzed as described previously (Roman and Kowalczykowski, 1989a; Roman *et al.*, 1992).

RESULTS

The Effect of Mg^{2+} Concentration on χ-Dependent Stimulation of Joint Molecule Formation—The formation of homologously paired joint molecules, dependent on the coordinated actions of RecA, RecBCD, and SSB proteins (called the RecABCD-dependent reaction), was originally demonstrated and characterized using a nitrocellulose filter binding assay (Roman *et al.*, 1991). The reaction was dependent on Mg^{2+} concentration, as expected for RecA protein-promoted pairing reactions, but the role of χ under these different conditions was not examined. Since *in vitro* reaction conditions also have a dramatic effect on the activities of RecBCD enzyme (for example, the ratio of Mg^{2+} concentration to ATP concentration, *i.e.* the free Mg^{2+} concentration, dictates the level of dsDNA exonuclease activity yet leaves the helicase activity relatively unaltered (Eichler and Lehman, 1977; Eggleston and Kowalczykowski, 1993a)) and since dsDNA unwinding and nucleolytic action are crucial to χ-specific events, we examined the effect of Mg^{2+} concentration on the ability of RecA, RecBCD, and SSB proteins to promote χ-dependent joint molecule formation. Fig. 1 illustrates the effect that Mg^{2+} concentration has on χ-dependent joint molecule formation using χ^+FH dsDNA as the donor DNA. Since recognition of χ by RecBCD enzyme occurs only 25–30% of the time, unwinding and cleavage of χ^+FH dsDNA can produce not only discrete sized, *χ-dependent* ssDNA fragments that derive from strand cleavage in the vicinity of the χ sequence but also random-sized, nonspecific cleavage products; furthermore, in addition to the discrete sized χ-dependent species expected, a discrete sized, *χ-independent* ssDNA product that corresponds to full-length ssDNA strand can be produced (Dixon and Kowalczykowski, 1991, 1993). The invasion of homologous supercoiled DNA by either of these discrete sized ssDNA species results in formation of χ-dependent or χ-independent joint molecules, respectively. At 1–2 mM Mg^{2+}, joint molecule formation is undetectable, even though dsDNA unwinding and χ recognition occur; at 1 mM Mg^{2+}, 19 ± 2% and 20 ± 2% of the input dsDNA is converted to either full-length or χ-dependent ssDNA fragments, respectively. A distinct increase in joint molecule formation is seen at 4 mM Mg^{2+}, with 19 ± 2% of the input DNA used to form χ-dependent joint molecules; similar values are observed at Mg^{2+} concentrations of 6 and 8 mM. Since DNA unwinding and χ-fragment production are not reduced at low Mg^{2+} concentrations (see below) and since RecA protein is known to typically require at least 4 mM Mg^{2+} for joint molecule formation (Cox and Lehman, 1982; Roman and Kowalczykowski, 1986; Roman *et al.*, 1991), the observed Mg^{2+} concentration dependence of the RecABCD reaction must reflect a Mg^{2+} requirement of RecA protein. Moreover, the rate of joint molecule formation was optimal when the ratio of protein to linear dsDNA was about 1 monomer/9 (± 2) nucleotides for both RecA and SSB proteins when each was present at its optimal concentration (data not shown); these values are similar to those reported previously for χ-independent RecABCD-dependent pairing (Roman *et al.*, 1991).

The data in Fig. 1 also highlight a common feature of χ-stimulated, RecABCD-dependent pairing reactions. Typically, the

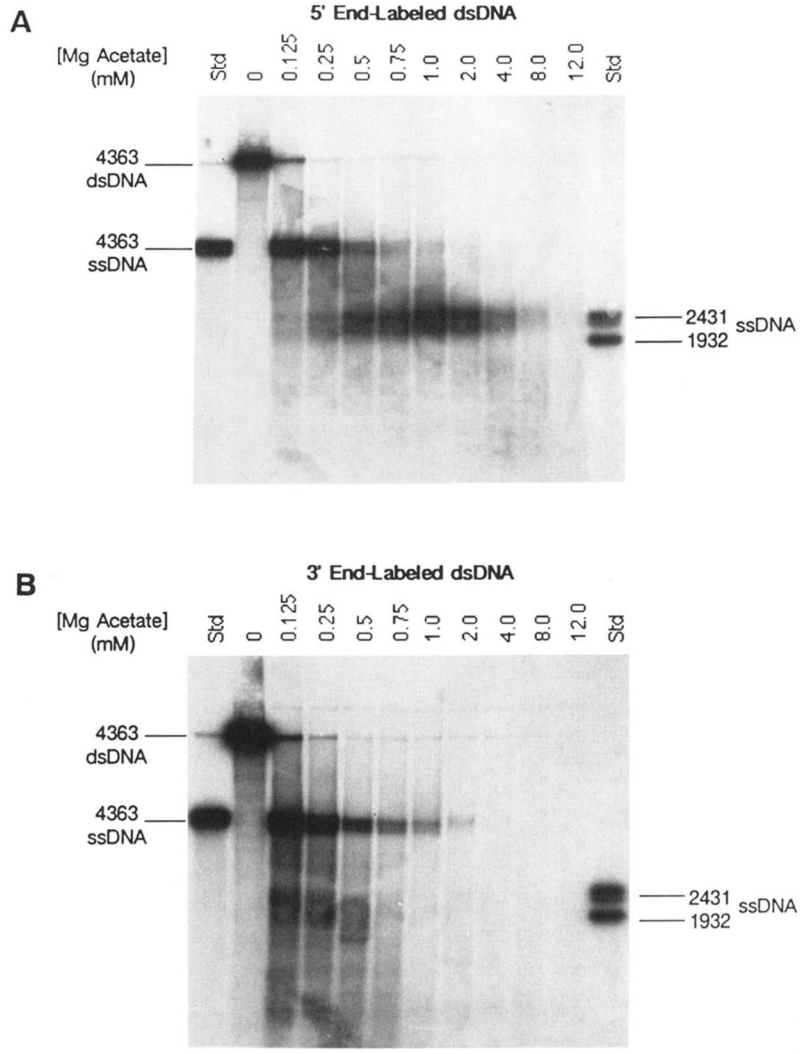

FIG. 2. **Unwinding and degradation of χ° dsDNA at various concentrations of Mg^{2+}.** Unwinding reactions were carried out under standard DNA unwinding reaction conditions. The dsDNA substrate was pBR322 dsDNA (4363 base pairs) linearized with the restriction enzyme *Eco*RI and radioactively end-labeled at either the 5′- (*A*) or 3′-ends (*B*). The concentration of magnesium acetate used in each reaction is indicated. The reactions contained 15 nM RecBCD enzyme (1.2 functional RecBCD enzyme molecules/dsDNA end), and the time for each unwinding reaction is 1 min. The main ssDNA species produced at low Mg^{2+} concentrations has a size similar to that of a full-length ssDNA standard (*Std*) of 4363 nucleotides (*A* and *B*, *4363 ssDNA*); whereas at high Mg^{2+} concentrations, the size of the major ssDNA species produced is approximately one-half the length of a DNA strand as compared with the ssDNA standards (2431 and 1932 ssDNA).

extent of χ-dependent joint molecule formation reaches a maximum at about 5 min, with 16 ± 5% (based on multiple replicate experiments) of the input linear χ-containing dsDNA (after conversion to ssDNA) used in the formation of this joint molecule species; in contrast, χ-independent joint molecule formation (using either χ^+ or χ° dsDNA) achieved a maximum of at most 5 and 3% of the input χ^+ and χ° (linear duplex) DNA, respectively (data not shown). Significantly, the limited amount of χ-independent joint molecules formed (compared to the amount of χ-dependent joint molecules) is not due to the lack of full-length ssDNA generated in these reactions, since approximately 10–20% of the input dsDNA is converted to full-length ssDNA (all of the dsDNA is unwound, and the remainder of the ssDNA produced is shorter than full-length), but is not used to form stable joint molecules. This suggests that χ enhances overall formation of joint molecules because χ-specific fragments are used preferentially for pairing.

Interestingly, at 1 and 2 mM Mg^{2+} concentrations, two novel discrete ssDNA species are present. Their sizes (approximately 800 and 1250 nucleotides) are consistent with ssDNA fragments expected from cutting the DNA strand opposite the χ sequence (*i.e.* the strand containing the complement of χ) in the vicinity of χ. Studies to be reported elsewhere[3] confirm these fragments to be both χ-specific and derived from the non-χ-containing strand. Similar observations regarding cleavage of the non-χ-containing strand near χ have been noted by Taylor and Smith.[4]

The Frequency of DNA Cleavage during DNA Unwinding Is Sensitive to Mg^{2+} Concentration—Previously, we showed that RecBCD enzyme degrades dsDNA asymmetrically during DNA unwinding, with the 3′-terminal strand being degraded more extensively than the 5′-terminal strand (Dixon and Kowalczykowski, 1993). Because of the nuclease attenuation phenomenon elicited by χ, χ-specific fragments were derived from the downstream portion (*i.e.* 5′-end) of the strand that contained the χ sequence (Dixon and Kowalczykowski, 1991, 1993; Kowalczykowski, 1994). Since the nuclease activity of RecBCD enzyme is reduced with decreasing free Mg^{2+} concentration (Eggleston and Kowalczykowski, 1993a) and since the yield of χ-specific fragments is clearly affected by changes in the Mg^{2+} concentration (Fig. 1), we examined more closely the effect of Mg^{2+} concentration on the generation both of intact (full-length) ssDNA and of χ-specific ssDNA fragments using an assay described previously (Dixon and Kowalczykowski, 1993). Briefly, RecBCD enzyme was incubated with dsDNA in the presence of Mg^{2+}; synchronous DNA unwinding and degradation was initiated by addition of the cofactor ATP. Under conditions of high nuclease activity and when the concentration of RecBCD enzyme is saturating with respect to dsDNA ends, ssDNA that is one-half as long as the original dsDNA substrate is produced preferentially from the 5′-terminal ends of the DNA strands (see Dixon and Kowalczykowski (1993)).

[3] D. Anderson and S. C. Kowalczykowski, unpublished observations.

[4] A. F. Taylor and G. R. Smith, submitted for publication.

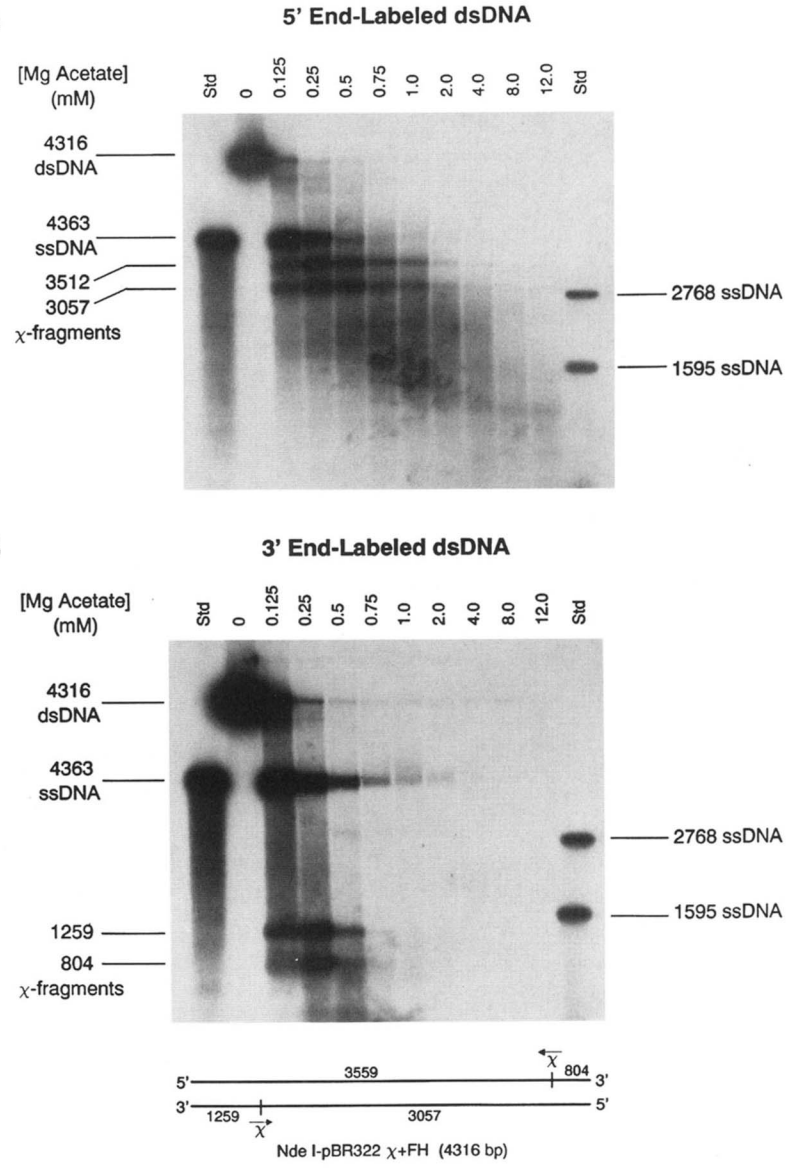

FIG. 3. **Unwinding and degradation of χ^+ dsDNA at various concentrations of Mg^{2+}.** Linear χ-containing dsDNA substrate was created by cutting the plasmid pBR322 χ^+FH (4316 base pairs), which contains two χ sites, with the restriction enzyme *Nde*I. The DNA was labeled at either the 5′-end (*A*) or 3′-end (*B*) with ^{32}P. The full-length ssDNA fragment produced at low Mg^{2+} concentrations is a ssDNA band with a mobility similar to the ssDNA standard (*Std*) of 4363 nucleotides (*4363 ssDNA*). χ-dependent ssDNA fragments (*χ-fragments*) have calculated lengths near the predicted sizes of 3512 and 3057 nucleotides (*A*) and 1259 and 804 nucleotides (*B*) compared with the ssDNA standards of 2768 and 1595 nucleotides.

This pattern of product formation is evident in Fig. 2 at Mg^{2+} concentrations above 1 mM; using 5′-end labeled dsDNA, the predominant product is half-length ssDNA (*panel A*), whereas using 3′-end labeled dsDNA under identical conditions, essentially no discrete length ssDNA is detected, consistent with the asymmetric pattern of degradation described above. In addition, as described previously (Eggleston and Kowalczykowski, 1993a), when the Mg^{2+} concentration is increased, degradation increases, resulting in decreased production of half-length ssDNA. The enhanced degradation of DNA coincides with reaction conditions in which the Mg^{2+} concentrations exceed the ATP concentration (*i.e.* 2.0–12 mM Mg^{2+}) (Eggleston and Kowalczykowski, 1993a).

In contrast, when the Mg^{2+} concentration is decreased (to below 1.0 mM) the decreased frequency of degradation, combined with unwinding, results in the production of ssDNA whose size is full-length (4363 nucleotides; Fig. 2*A*). At the lowest Mg^{2+} concentration examined (0.125 mM), the amount of full-length ssDNA produced is 80 ± 10% of the input dsDNA. When 3′-end-labeled dsDNA is unwound under equivalent conditions (Fig. 2*B*), at low Mg^{2+} concentrations, the major ssDNA product is also full-length, but the yield is somewhat lower (62 ± 8% of the unwound dsDNA is full-length at 0.125 mM Mg^{2+}). When the Mg^{2+} concentration is elevated, the 3′-terminal DNA strand is rapidly degraded. To ensure that the production of full-length ssDNA seen at the low Mg^{2+} ion concentrations is not due to the inhibition of the ability of RecBCD enzyme to bind and unwind from both ends of the dsDNA simultaneously, both dsDNA filter binding and titration of helicase activity control experiments were performed to confirm that the dsDNA ends are saturated with enzyme at the indicated Mg^{2+} ion concentrations (Taylor and Smith, 1985; data not shown). These results demonstrate that the enhanced degradation of the DNA coincides with reaction conditions in which the Mg^{2+} concentration exceeds the ATP concentration (2.0–12.0 mM Mg^{2+}) (Eggleston and Kowalczykowski, 1993a). The simplest interpretation of these results is that degradation is asymmetric, and the frequency of nucleolytic cutting by RecBCD enzyme increases with increased free Mg^{2+} concentration (Dixon and Kowalczykowski, 1993; Eggleston and Kowalczykowski, 1993a; Kowalczykowski, 1994).

χ-Specific ssDNA Fragment Production Is Sensitive to Mg^{2+} Concentration—To determine the consequences of variations in the level of nonspecific nuclease activity on χ-specific ssDNA fragment formation, unwinding reactions similar to those seen in Fig. 2 were performed using the linear dsDNA substrate, χ^+FH, which contains two oppositely oriented χ sites. Fig. 3*A* shows the products of an unwinding reaction using 5′-end-

labeled χ^+FH DNA with saturating amounts of RecBCD enzyme at varying Mg^{2+} concentrations. χ recognition is detected by the appearance of the two downstream 5′-end-labeled χ-dependent ssDNA fragments, approximately 3500 and 3000 nucleotides in length. Optimal production of these χ-dependent fragments occurs at 0.25 mM Mg^{2+}. The yields of 3512 and 3057 nucleotide χ-dependent ssDNA fragments are 24 ± 2% and 29 ± 3% of the input dsDNA, respectively, with 28 ± 3% of the input dsDNA being unwound into full-length ssDNA. At a lower Mg^{2+} concentration (0.125 mM), formation of χ-specific ssDNA is decreased (18 ± 2% and 19 ± 2% for the 3512 and 3057 nucleotide χ-dependent ssDNA fragments, respectively), but production of full-length ssDNA is significantly increased (60 ± 10%). At higher Mg^{2+} concentrations, χ-specific fragments are produced, but heterogeneous ssDNA fragments, with sizes ranging from approximately 3000–800 nucleotides, become more prevalent (Dixon and Kowalczykowski, 1993). Also visible are trace amounts of discrete sized DNA fragments whose sizes are approximately 800 and 1250 nucleotides (see also Fig. 1).

DNA labeled at the 3′-end yields qualitatively similar results as those obtained with 5′-end-labeled DNA (Fig. 3*B*). The optimum Mg^{2+} concentration for the appearance of the upstream χ-dependent ssDNA fragment is 0.25 mM, with 5 ± 1% and 3 ± 1% of input dsDNA being cleaved to create the 1259- and 804-nucleotide upstream χ-dependent fragments, respectively. This optimum is identical to that observed in Fig. 3*A*, but the yield of χ-specific fragments derived from the 3′-side of χ is reduced by 5–10-fold, despite the higher probability of recovering (due to the lower probability of a single random cleavage event) a ssDNA fragment that is at least 5-fold shorter than the downstream fragment. A reduction of χ-specific fragment production can again be seen at the Mg^{2+} concentration of 0.125 mM (3 ± 1% and 2 ± 1% of input dsDNA for the 1259- and 804-nucleotide fragments, respectively). This decrease coincides with a decrease in nonspecific degradation; an increase in full-length ssDNA is observed at 0.125 mM Mg^{2+} compared with 0.25 mM Mg^{2+} ion (37 ± 4% *versus* 14 ± 2% for Mg^{2+} concentrations of 0.125 mM and 0.25 mM, respectively). When elevated Mg^{2+} concentrations are used, the ssDNA containing the 3′-end is degraded into small oligonucleotide fragments (Fig. 3*B*), as demonstrated previously (Dixon and Kowalczykowski, 1993). Ponticelli *et al.* (1985) demonstrated the production of a specific χ-dependent ssDNA fragment derived from the 3′-terminal end at the enzyme's entry site; their reaction conditions employed a low free Mg^{2+} concentration. Our results clearly demonstrate that the formation of χ-dependent ssDNA fragments is sensitive to Mg^{2+} concentration, thus providing an explanation for the seemingly different results reported (Ponticelli *et al.*, 1985; Dixon and Kowalczykowski, 1991, 1993). The yield of χ-specific fragments is obviously governed by a compromise between the reduced overall level of cleavage (at both nonspecific and χ sites) at low Mg^{2+} concentrations *versus* the enhanced DNA cleavage at all sites seen at high Mg^{2+} concentrations; at the higher Mg^{2+} concentrations, χ-specific ssDNA fragments are being produced, but their detection is completely obscured by the higher level of nucleolytic activity displayed by the attenuated enzyme downstream of χ. Regardless of experimental conditions, the DNA strand 3′ at the entry site is always cleaved more often than the 5′-strand, and a productive interaction with χ always results in an attenuation of degradation.

The Ability of Mutant RecBCD Enzymes to Promote χ-stimulated Joint Molecule Formation Parallels Their in Vivo Phenotype—To establish whether there is a parallel between χ hotspot activity seen *in vivo* and χ-dependent stimulation of joint molecule formation *in vitro*, we examined the ability of

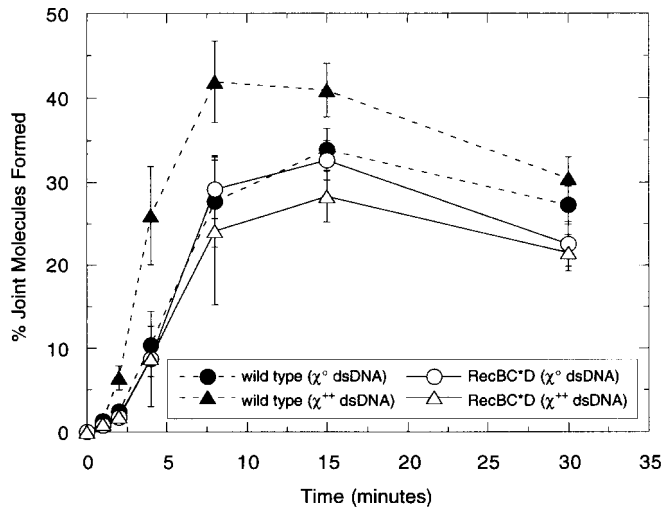

FIG. 4. **Effect of the mutant RecBC*D enzyme on joint molecule formation.** Joint molecule formation was detected by filter binding assays described under "Materials and Methods." Standard RecABCD-dependent reaction conditions containing 10 μM nucleotides (2.3 nM dsDNA ends) of either *Nde*I-pBR322 χ° (χ° *dsDNA*, *circles*) or *Nde*I-pBR322 χ^+FH (χ^{++} *dsDNA*, *triangles*) and 5 μM ^{3}H-labeled supercoiled pBR322 χ° DNA were used (see Fig. 1 for diagrams of DNA substrates). Reactions were initiated by the addition of 0.31 nM RecBCD enzyme (*filled symbols*) or 0.17 nM RecBC*D enzyme (*open symbols*), which is equivalent to 0.025 functional enzymes/dsDNA end for both sets. The percentage of joint molecules formed is based on the total input supercoiled DNA contained in a reaction; the values reported are the average of three experiments, and *error bars* indicate the standard deviation.

mutant RecBCD enzymes to promote joint molecule formation. The mutant proteins examined were the RecBC*D enzyme (Schultz *et al.*, 1983), which is moderately proficient in genetic recombination while maintaining wild type levels of dsDNA exonuclease activity, and the RecBCD‡ enzyme (Amundsen *et al.*, 1986; Chaudhury and Smith, 1984), which is recombination-proficient but devoid of dsDNA exonuclease activity *in vivo*. Both mutant proteins fail to show χ-dependent stimulation of recombination *in vivo*.

Both mutant enzymes were examined in RecABCD-dependent pairing reactions using χ^+FH dsDNA (χ^{++} dsDNA; see Fig. 1) to maximize χ-dependent stimulation of joint molecule formation and using subsaturating concentrations of RecBCD enzyme (0.025 functional RecBCD enzyme molecules/linear dsDNA end). Fig. 4 shows that both the rate and the extent of RecABCD-dependent joint molecule formation by the wild type enzyme are increased due to the presence of χ sites in the linear dsDNA (labeled χ^{++} dsDNA) (see also Eggleston and Kowalczykowski (1993b)); the maximal rate and extent of joint molecule formation are increased approximately 2.3- and 1.2-fold, respectively, compared with the χ° reaction (Table I). The extent of joint molecule formation reaches a maximum in about 8 min for the χ-containing reactions, whereas for the non χ-containing reactions, it reaches a maximum in about 15 min. The slow decrease in joint molecule formation seen at the extended time points reflects the RecA protein-dependent dissociation of joint molecules (Shibata *et al.*, 1982; Roman and Kowalczykowski, 1989a). Both mutant enzymes unwind DNA (data not shown), and equivalent amounts of functional enzyme (based on the apparent stoichiometry derived from DNA helicase activity shown in Table II) were used in experiments. In agreement with both *in vivo* observations and with their capacity to unwind dsDNA, both the RecBC*D enzyme (Fig. 4) and the RecBCD‡ enzyme (Fig. 5) promote RecA protein- and SSB protein-dependent joint molecule formation between lin-

TABLE I
Effect of χ on joint molecule formation by wild-type and mutant RecBCD enzymes

Reactions were performed as described under "Materials and Methods" using standard RecABCD reaction conditions containing recipient ^{3}H-labeled supercoiled pBR322 χ° DNA.

Enzyme	Linear dsDNA[a]	Max. extent of JM Formation[b]	Rate of JM Formation[c]
Wild type	χ°	34	4.3 ± 0.7
Wild type	χ^+	37	7.8 ± 1.6
Wild type	χ^{++}	42	9.8 ± 2.0
RecBC*D	χ°	33	5.1 ± 0.5
RecBC*D	χ^{++}	28	4.3 ± 1.4
RecBCD‡	χ°	38	6.4 ± 1.1
RecBCD‡	χ^{++}	38	5.7 ± 1.0

[a] The indicated linear dsDNA used in each reaction: χ°, *Nde*I-linearized pBR322 (χ°); χ^+, *Eco*RI-linearized pBR322 (χ^+F); χ^{++}, *Nde*I-linearized pBR322 (χ^+FH).

[b] The maximum extent of joint molecule (JM) formation is a percentage of the input supercoiled DNA; see Figs. 4 and 5 for experimental uncertainties.

[c] The maximal rate of joint molecule (JM) formation (% joint molecules formed/min) is based on total input supercoiled DNA.

TABLE II
*Comparison of the helicase activity of wild type RecBCD, RecBC*D, and RecBCD‡ enzymes*

Helicase activity was measured using the fluorescent DNA unwinding assay (Roman and Kowalczykowski, 1989a). Reaction conditions consisted of 25 mM Tris-acetate (pH 7.5), 1 mM magnesium acetate, 1 mM dithiothreitol, 1 mM ATP, 2 mM phosphoenolpyruvate, 16 units/ml pyruvate kinase, 10 μM (nucleotides) *Eco*RI-linearized M13 mp7 linear dsDNA, 2 μM SSB protein, and RecBCD enzyme at 25 °C.

Enzyme	Apparent stoichiometry (per dsDNA end)[a]	Apparent k_{cat}[b]	Corrected k_{cat}[c]
		s^{-1}	s^{-1}
Wild type[d]	5.2 ± 0.3	62 ± 5	322 ± 28
RecBC*D[e]	2.8 ± 0.4	105 ± 17	315 ± 54
RecBCD‡[f]	≥31	≥3	≥93

[a] The stoichiometry is the experimentally observed number of enzyme molecules required to saturate helicase activity at a fixed DNA concentration of 1.4 nM dsDNA ends.

[b] The apparent k_{cat} or apparent turnover number is the experimentally observed rate of unwinding in base pairs/s/total enzyme concentration.

[c] The corrected k_{cat} is the apparent k_{cat} multiplied by the apparent stoichiometry; this is equivalent to dividing the observed plateau value for the saturated rate of unwinding (nM/sec) by the concentration of dsDNA ends (1.4 nM), a calculation that assumes that the maximum observed rate of DNA unwinding is limiting when all of the DNA ends are saturated with functional enzyme. Each calculation yields the same result for the corrected k_{cat} within experimental error.

[d] The kinetic parameters for wild type RecBCD enzyme are the average of two independent data sets.

[e] The kinetic parameters obtained for this mutant enzyme were derived from a single data set.

[f] Due to the low specific activity of the RecBCD‡ enzyme preparation, saturating rates of dsDNA unwinding could not be attained with an experimentally accessible RecBCD‡ concentration. Thus, the observed stoichiometry and apparent k_{cat} are minimal estimates based on highest rate of DNA unwinding (135 nM base pairs/s) that was experimentally attainable; the highest rate obtained was about ⅓ of the plateau value obtained for both wild type and RecBC*D enzymes (400–450 nM base pairs/s).

ear and supercoiled dsDNA. Joint molecule formation was slightly lower for RecBC*D and slightly higher for RecBCD‡, but in neither case was pairing affected by the presence of χ sites. Table I summarizes the extents and rates of joint molecule formation with the mutant enzymes for reactions containing χ^{++} or χ° dsDNA.

The failure of the mutant enzymes to display stimulation by χ in the joint molecule formation assays suggested that they were defective in χ recognition. To assess this possibility more directly, the production of χ-specific ssDNA fragments was assayed (Dixon and Kowalczykowski, 1991). Fig. 6 shows that

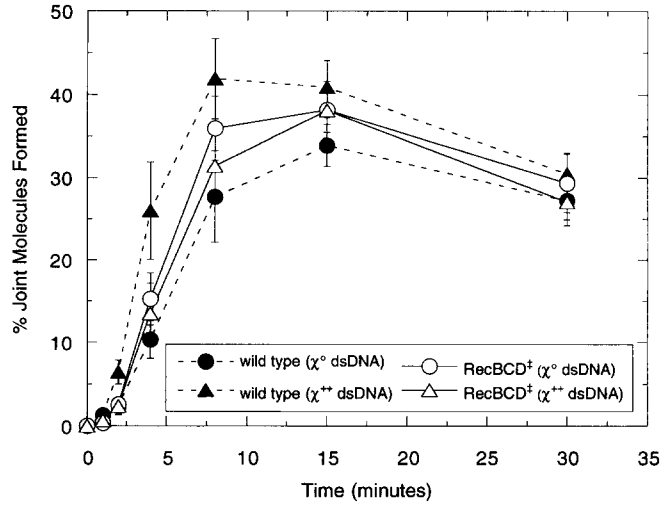

FIG. 5. **Effect of the mutant RecBCD‡ enzyme on joint molecule formation.** Standard RecABCD-dependent filter binding reactions were performed and analyzed in a manner identical to that described in the legend to Fig. 4. Linear dsDNA substrates were *Nde*I-pBR322 χ° (χ° *dsDNA, circles*) and *Nde*I-pBR322 χ^+FH (χ^{++} *dsDNA, triangles*). *Open symbols* represent reactions done in the presence of 1.8 nM RecBCD‡ enzyme. Data with the wild type RecBCD enzyme from Fig. 4 is shown with *closed symbols* for reference. *Error bars* indicate the standard deviation of three experiments.

the RecBC*D enzyme is unable to produce a χ-dependent ssDNA fragment, implying that either χ recognition or attenuation of nuclease activity is defective. The RecBCD‡ enzyme is able to produce a limited amount of χ-dependent ssDNA (about one-third of the amount produced by wild type RecBCD enzyme). The production of full-length ssDNA by the RecBC*D enzyme is approximately equal to that produced by the wild type enzyme; however, the RecBCD‡ enzyme produces approximately 3.5 times more full-length ssDNA than the wild type enzyme. The residual ability of RecBCD‡ enzyme to recognize χ and to degrade dsDNA might arise from the presence of undetectable amounts of full-length RecD subunit that may result from limited suppression of the presumed nonsense mutation in the *recD1011* allele, or it may be an intrinsic property of the truncated RecD polypeptide. Regardless, the findings presented here are consistent with results using crude cell extracts of *recBC*D* and *recBCD‡* mutant strains (Amundsen *et al.*, 1986; Chaudhury and Smith, 1984; Ponticelli *et al.*, 1985; Schultz *et al.*, 1983; Taylor, 1988).

Detection by gel electrophoresis of the joint molecules produced in RecABCD-dependent joint molecule formation using the mutant RecBC*D and RecBCD‡ enzymes is shown in Fig. 7. Reactions were similar to those in Figs. 4 and 5 except that the linear dsDNA contains a single χ sequence (*Eco*RI-pBR322 χ^+F). Wild type enzyme shows the characteristic formation of both χ-independent (due to invasion by full-length ssDNA) and χ-dependent (due to invasion by the ssDNA fragment downstream of χ) joint molecules. In contrast, no discrete joint molecules are produced in reactions with the RecBC*D enzyme at early times; after 10 min, only χ-independent (both full-length and heterogeneous length) joint molecules are detected. In reactions containing the RecBCD‡ enzyme, nearly all of the unwound DNA is full-length and, consequently, the predominant joint molecule produced is derived from invasion by the full-length ssDNA (Fig. 7, *χ-indep. JM*).

DISCUSSION

The formation of homologously paired joint molecules by the concerted action of RecA, RecBCD, and SSB proteins is clearly stimulated by the presence of the recombination hotspot, χ. χ

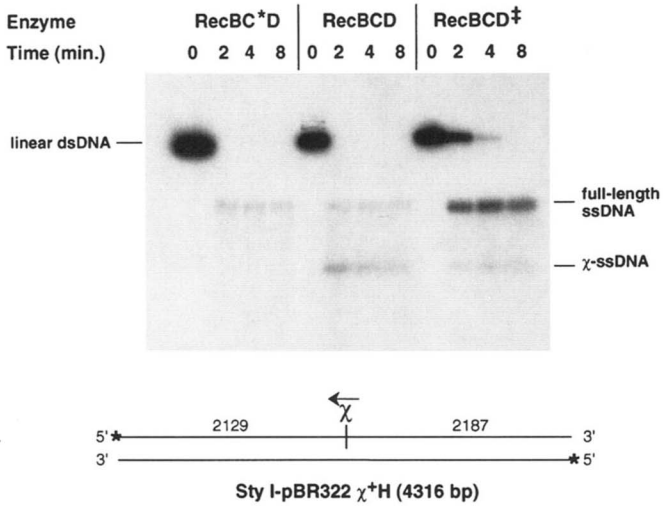

FIG. 6. **Ability of RecBC*D and RecBCD‡ enzymes to recognize χ.** Specific χ-recognition assays contained the standard DNA unwinding reaction mixture; the linear dsDNA *Sty*I-pBR322 χ^+H (*bottom of figure*) was radioactively end-labeled at the 5′-end. Reactions were performed and analyzed in a manner similar to that described in the legend to Fig. 3. Reactions were initiated with 0.31, 0.17, and 1.8 nM wild type RecBCD, RecBC*D, and RecBCD‡ enzymes, respectively (0.025 functional enzyme/dsDNA end based on the observed stoichiometry shown in Table II). The unwound full-length ssDNA (4316 nucleotides) and χ-dependent ssDNA (2129 nucleotides; *χ-ssDNA*) are indicated.

promotes an increase in both the rate and extent of overall joint molecule formation when present in the donor linear dsDNA (Dixon and Kowalczykowski, 1991; Eggleston and Kowalczykowski, 1993b). Here we refine this conclusion by demonstrating that 1) the relative amount of χ-specific joint molecule formation exceeds that expected based solely on the increased level of χ-fragment production that results from attenuation of nuclease activity, suggesting an additional role for χ; 2) the pattern of ssDNA product formation is highly dependent on Mg^{2+} concentration and, for χ^+ DNA, χ-specific ssDNA fragments derived from both the χ-containing and χ-complementary strands are detected; and 3) the *in vitro* behavior of two additional mutant RecBCD enzymes in χ-dependent reactions is consistent with the expectations of the nuclease attenuation model (Dixon and Kowalczykowski, 1991, 1993; Eggleston and Kowalczykowski, 1993a, 1993b; Kowalczykowski, 1994).

A common observation in the χ-dependent RecABCD reactions is the enhanced formation of χ-dependent joint molecules relative to χ-independent joint molecules (*e.g.* see Fig. 1). Since binding of the ssDNA fragments by RecA protein is required for joint molecule formation, the observed stimulation of χ-dependent joint molecule formation can therefore result from an increase in the concentration of χ-specific ssDNA fragments relative to that of the nonspecific fragments as a consequence of nuclease attenuation, from an increased kinetic ability of RecA protein to bind to the χ-containing ssDNA as it is being produced by RecBCD enzyme, or from an increased stability of the RecA protein-χ-specific ssDNA complexes. The first possibility is the simplest explanation for the increased invasion by χ-specific ssDNA fragments. Since χ attenuates the nuclease activity of RecBCD enzyme (Dixon and Kowalczykowski, 1991; Dixon and Kowalczykowski, 1993), the concentration of χ-specific fragments is greater than that of any other specific-length ssDNA fragments produced in the presence or in the absence of χ. This hypothesis, however, does not fully explain the bias displayed for invasion by χ-specific fragments over the full-length linear ssDNA in these reactions, since especially when normalized for the amount produced, formation of χ-dependent joint molecules is 4–5-fold more efficient than formation of χ-independent joint molecules. The kinetic explanation is based on the fact that the 3′-end of ssDNA is more invasive than the 5′-end (Dixon and Kowalczykowski, 1991, Konforti and Davis, 1987, 1990); therefore, a simple explanation for the bias in joint molecule formation is that the 3′-end at χ is formed first, whereas the 3′-end of the complementary (5′-terminal) strand is distal to χ and is liberated as ssDNA later. Immediate binding of RecA protein to the 3′-end at χ permits rapid formation of a presynaptic complex at that end. The outcome of such a temporal sequence of events may result in an apparent increase in the rate of invasion by the χ-specific ssDNA fragments, resulting in a greater yield of stable (plectonemic) joint molecules. However, this hypothesis does not explain why the total pool of heterogeneous ssDNA fragments, which must possess a 3′-end, does not contribute to an equally significant population of heterogeneously sized joint molecules. The final explanation, which is that the χ sequence itself may interact preferentially with RecA protein and/or with its target DNA sequence, seems the most unlikely. However, although both the increased survival of χ-specific fragments (relative to other discrete ssDNA fragments) and their appearance as the first and most prevalent unique 3′-terminal ssDNA species under DNA pairing conditions may be sufficient to explain their predominance as substrates for joint molecule formation, recent results support the possibility that χ-like DNA sequences behave anomalously in RecA protein-promoted pairing reactions[5] and will be described elsewhere.

The second major outcome of this work reported here was uncovered in the course of examining the effect of various reaction conditions on the RecABCD-promoted χ-dependent pairing reaction; two distinct conclusions emerged from the effects of Mg^{2+} concentration on the reaction. The first is that the minimum Mg^{2+} concentration required for χ-dependent joint molecule formation is dictated by the Mg^{2+} dependence of recA protein-promoted pairing since, at concentrations of Mg^{2+} that are too low to support RecABCD-dependent joint molecule formation (<4 mM Mg^{2+}), there nevertheless is sufficient χ-specific ssDNA fragment production by RecBCD enzyme. The second conclusion is that the amount of χ-specific ssDNA fragment production increases with decreasing Mg^{2+} concentration, to a point (Figs. 3 and 5). As elaborated elsewhere (Kowalczykowski, 1994; Kowalczykowski *et al.*, 1994), this behavior is readily explained by the reduction of nuclease activity that accompanies a decrease in the free Mg^{2+} concentration (Eggleston and Kowalczykowski, 1993a) and by the following simple but comprehensive view of the relationship between the nuclease and helicase activities of RecBCD enzyme (Fig. 8).

The nuclease activity of RecBCD enzyme is envisioned to act on ssDNA endonucleolytically during the course of DNA unwinding (translocation), and the nuclease and helicase activities are considered to be independent of one another. The helicase activity is relatively insensitive to Mg^{2+} concentration (Roman and Kowalczykowski, 1989a), but the frequency of endonucleolytic cleavage is quite sensitive (Eggleston and Kowalczykowski, 1993b). The probability of recovering an ssDNA species of a given size is a function of both translocation rate and cleavage frequency, with the average size produced increasing as the translocation rate increases and the cleavage frequency decreases. If the enzyme pauses at a sequence, as it does at χ (Dixon and Kowalczykowski, 1993), then the probability of cleaving (nonspecifically) at that sequence is increased in proportion to the length of the pause. Furthermore, despite the attenuation of nuclease activity that is elicited by χ, some nuclease activity remains "downstream" of χ. This occasional

[5] B. Tracy and S. C. Kowalczykowski, unpublished observations.

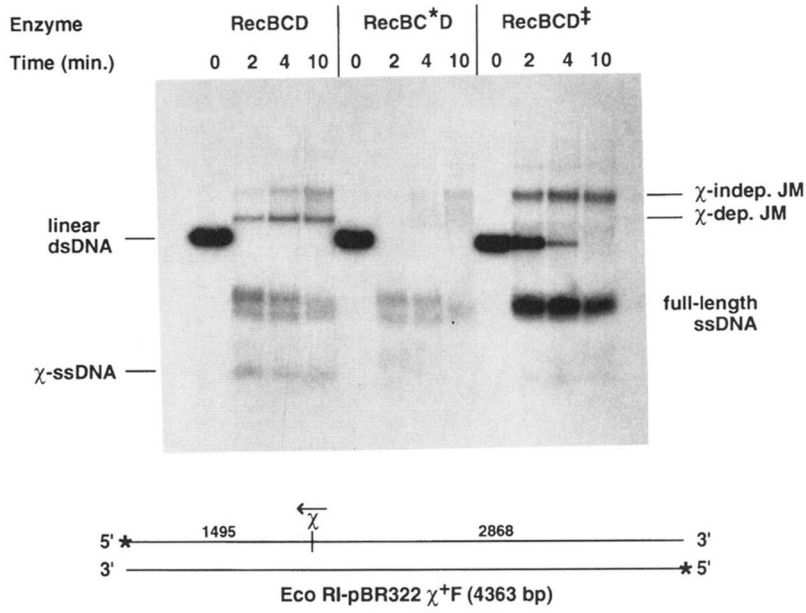

FIG. 7. **Ability of the mutant RecBC*D and RecBCD‡ enzymes to form χ-dependent and independent joint molecules.** Standard RecABCD-reaction conditions were used, except all DNA and protein components were increased by a factor of 4. The donor linear dsDNA *Eco*RI-pBR322 χ^+F (χ^+ dsDNA) was radioactively end-labeled at the 5′-end. Reactions were initiated with the addition of 1.25 nM wild type RecBCD enzyme, 0.70 nM RecBC*D enzyme, or 7.08 nM RecBCD‡ enzyme where indicated (0.025 functional enzyme/dsDNA end based on the observed stoichiometry shown in Table I). All values reported are a percentage of the total input linear dsDNA contained in a reaction that is unwound. Specific χ-dependent (*χ-dep.JM*) and χ-independent (*χ-indep. JM*) joint molecules are indicated as well as the χ-dependent ssDNA fragment (*χ-ssDNA*) and full-length ssDNA.

nicking after χ by the attenuated enzyme results in a lower net yield of the χ-specific ssDNA fragments. Reduction of the free Mg^{2+} concentration reduces the overall nucleolytic activity of RecBCD enzyme, resulting in fewer cleavage events both upstream and downstream of χ. However, further reduction of nuclease activity by reducing the concentration of Mg^{2+} to below 125 μM diminishes the overall yield of χ-specific fragment; this is due to a decrease in the probability of cleavage by RecBCD enzyme when it is paused at χ. Thus, fewer DNA molecules are cleaved in the vicinity of χ and, despite the fact that fewer molecules would be degraded downstream of χ, the net yield of χ-specific fragments is reduced; instead, because of the overall lower level of nuclease activity, more full-length ssDNA is produced.

This general idea that the detection of χ-specific ssDNA fragments *in vitro* is a trade-off between the frequency of cleaving (nonspecifically) in the vicinity of χ while paused and the frequency of cleaving (nonspecifically) while translocating through the DNA explains another feature of Fig. 1. At the lower Mg^{2+} concentrations, the χ-specific fragment derived from the DNA strand that is 3′ at the entry site (the "upstream" fragment) is detected (Ponticelli *et al.*, 1985). This follows because when both the frequency of nonspecific degradation is low and the distance to χ is short, the probability of cleavage in the "upstream" region is low. This behavior, coupled with a reasonable probability of cleavage at χ, permits detection of the upstream χ-specific ssDNA fragment under these conditions.

Finally, the data in Fig. 1 illustrate the formation of a novel χ-specific fragment at the lower Mg^{2+} concentration. This fragment has a mobility consistent with cleavage of the lower, non-χ-containing (*i.e.* χ complement) DNA strand in the vicinity of the χ sequence. Recent studies verify the identity of this species as χ-specific, and both its formation and properties will be described elsewhere.[6]

The third major outcome of the findings presented here is the demonstration that the mutant RecBC*D and RecBCD‡ enzymes are unresponsive to the presence of χ within linear dsDNA, in agreement with their phenotypic behavior (Chaudhury and Smith, 1984; Schultz *et al.*, 1983). Although the mutant enzymes are sufficiently processive to unwind completely the dsDNA substrates used (Figs. 6 and 7), they are less efficient than the wild type enzyme at joint molecule formation in the presence of χ (Figs. 4 and 5; Table I), and few or no χ-dependent joint molecules are formed (Fig. 7). However, the reason for the absence of χ-specific species is different for each mutant enzyme.

The inability of the RecBC*D enzyme to stimulate joint molecule formation in the presence of χ is most simply explained by a failure of the enzyme to recognize χ, to attenuate its nuclease activity, or both (Fig. 7). Since the mutation maps to the *recC* gene (Schultz *et al.*, 1983), these findings suggest that at least one of the domains responsible for χ recognition or attenuation of nuclease activity resides in the RecC subunit. To explain the ability of this class of mutants to promote modest levels of recombination in the absence of χ recognition, it was proposed that they are capable of recognizing and stimulating recombination at undefined sequences other than 5′-GCTGGTGG-3′ (Schultz *et al.*, 1983). Evidence for this possible explanation, however, was not seen here, perhaps because the linear donor DNA used (pBR322) lacked this postulated sequence. Thus, the phenotypic behavior of the RecBC*D enzyme resembles, to a less severe degree, that of the $RecB^{2109}CD$ enzyme, which also fails to recognize χ and/or to attenuate its nuclease activity (Eggleston and Kowalczykowski, 1993a, 1993b).

In contrast, although the RecBCD‡ enzyme failed to produce χ-specific species, its ability to form joint molecules was nearly identical with that of the wild type enzyme. But, as seen in Fig. 7, this ability is due to the substantially greater amount of full-length ssDNA produced and, consequently, of joint-molecules containing full-length ssDNA. Thus, the RecBCD‡ enzyme lacks most of the nuclease activity associated with the wild type enzyme and, hence, behaves like a constitutively χ-activated, nuclease-attenuated enzyme. Due to the nature of enhanced recombination observed in *recD* cells (Chaudhury and Smith, 1984; Lovett *et al.*, 1988; Thaler *et al.*, 1989), the inability of RecBCD‡ enzyme to promote χ-specific joint molecule formation was anticipated. Genetic studies led to the view that *recD* mutations resulted in a recombinogenic form of the enzyme, ostensibly the RecBC enzyme, that was equivalent to the χ-activated form of the RecBCD enzyme (Thaler *et al.*, 1989). The reconstituted RecBC enzyme, devoid of the RecD subunit, is a DNA helicase that neither degrades DNA nor interacts with χ sequences (Boehmer and Emmerson, 1991; Masterson *et al.*, 1992;

[6] D. Anderson and S. C. Kowalczykowski, unpublished observations.

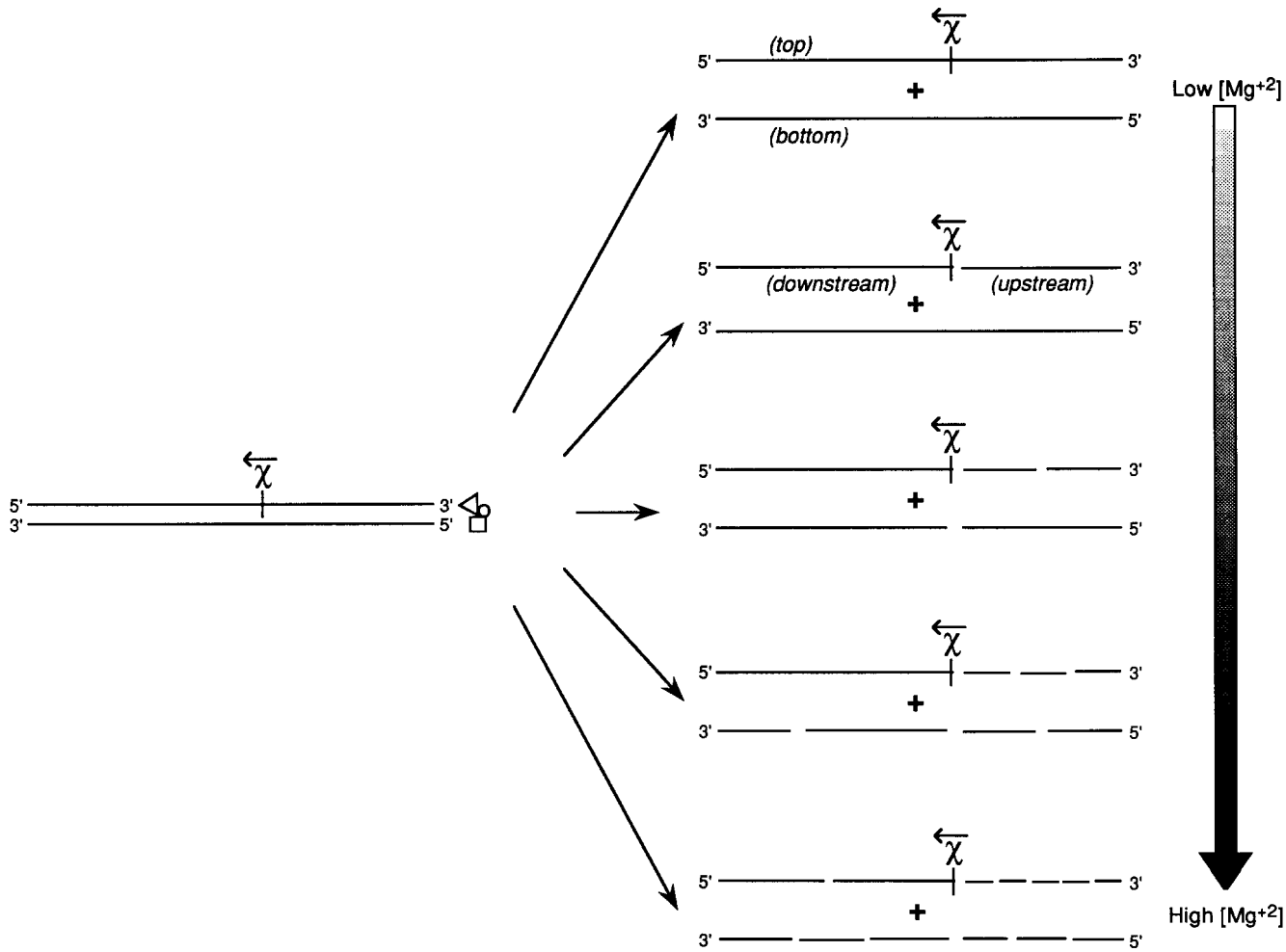

FIG. 8. **Pattern of ssDNA fragment production by RecBCD enzyme and χ at various free Mg^{2+} concentrations.** The pattern of ssDNA fragment production is strongly dependent on the free Mg^{2+} concentration. At the lowest free Mg^{2+} concentrations, nuclease activity is nearly completely suppressed but helicase activity is not, resulting in the production of predominantly full-length ssDNA regardless of the presence of a χ sequence. The pause at χ increases the probability of cleavage at χ, resulting in formation of both upstream and downstream χ-specific fragments at higher Mg^{2+} concentrations. A further increase in Mg^{2+} concentration increases nuclease activity, resulting in cleavage of the bottom DNA strand containing the χ complement, and in degradation of the top upstream fragment; the downstream χ-specific fragment is preserved due to attenuation of nuclease activity elicited by interaction with χ. Continued increase in the free Mg^{2+} concentration results in loss of all χ-specific fragments due to the increased probability of random endonucleolytic cleavage during DNA unwinding.

Korangy and Julin, 1993; Dixon *et al.*, 1994). Subsequent biochemical studies established the equivalence of the RecBC and the χ-activated RecBCD enzymes (Dixon *et al.*, 1994). Since a majority of the *recBCD*‡ mutations are amber mutations producing a severely truncated or undetectable RecD polypeptide, it is reasonable that the RecBCD‡ and the RecBC enzymes are nearly functionally equivalent.

The findings presented here suggest that χ may have an additional role in the stimulation of homologous pairing that goes beyond the crucial regulation of the nuclease activity of RecBCD enzyme. Although regulation of nuclease activity is required, as demonstrated with the RecBC*D and $RecB^{2109}CD$ enzymes, the constitutive attenuation of nuclease activity inherent in RecBCD‡ enzyme does not allow the same level of pairing *in vitro* that is observed with wild type RecBCD enzyme and χ. Though the RecBCD‡ enzyme produces even greater amounts of full-length ssDNA, the extent of joint molecule formation (when normalized for the amount of ssDNA produced) is less than that observed for χ-dependent joint molecule formation by the wild type enzyme (see Fig. 7). Thus, the possibility remains that either the manner by which χ-specific ssDNA is presented by RecBCD to RecA protein or the interaction of RecA protein with χ-specific ssDNA fragments is somehow unique.

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