

Reversible inactivation of the *Escherichia coli* RecBCD enzyme by the recombination hotspot χ *in vitro*: Evidence for functional inactivation or loss of the RecD subunit

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ABSTRACT Genetic recombination in *Escherichia coli* is stimulated by a RecBCD enzyme-mediated event at DNA sequences known as Chi (χ) sites (5'-GCTGGTGG-3'). Previously, it was shown that χ acts to regulate the nuclease activity of RecBCD; here, we demonstrate that, under appropriate conditions, interaction with χ sites can also result in an inactivation of helicase activity of RecBCD. The unwinding of double-stranded DNA-containing χ sites, under conditions of limiting Mg^{2+} ion, results in the reversible inactivation of RecBCD; addition of excess Mg^{2+} to the reaction reactivates all activities of RecBCD. Inactivation is the consequence of a χ -dependent modification of RecBCD that appears to result from an inability of the χ -modified RecBCD to reinitiate unwinding of intact DNA molecules. This characteristic behavior of RecBCD and χ is displayed by the reconstituted RecBC (i.e., without the RecD subunit), except that it is not dependent on χ interaction. This biochemical similarity between the χ -modified RecBCD and RecBC enzymes implies that recognition of χ results in a dissociation or functional inactivation of RecD subunit and lends support to the hypothesis that interaction with χ results in ejection of the RecD subunit.

The RecBCD enzyme is an essential component of the main pathway of homologous recombination in *Escherichia coli*, the RecBCD pathway of generalized recombination (reviewed in ref. 1). *In vitro*, RecBCD enzyme degrades both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) by means of its ATP-dependent exonuclease activities and its ATP-stimulated ssDNA endonuclease activity. In addition to these degradative activities, the enzyme is a DNA helicase that can processively unwind large tracts of dsDNA (>30 kb) at rates up to 1000 bp/sec (2–4). During dsDNA unwinding, the ATP-dependent degradation of DNA is asymmetric, with the 3'-terminal DNA strand at the entry site for RecBCD being degraded more vigorously than the 5'-terminal strand (5–8).

The χ recombination hotspots are composed of the DNA sequence 5'-GCTGGTGG-3' (9, 10); recombination events are 5–10 times more frequent near χ than far from it and these exchanges are *recBC*-dependent (11–13). Stimulation of recombination by χ occurs primarily downstream (5' side) of the χ site and extends, with decreasing magnitude, for >10 kb from χ (12, 14). *In vitro*, recognition of χ by RecBCD results in a single-strand break in the DNA strand containing the χ sequence, 4–6 nucleotides to the 3' side of the χ sequence; this occurs during the unwinding of dsDNA and is orientation dependent (15, 16). These properties led to models which proposed the interaction between RecBCD and χ served to initiate the RecA-dependent DNA strand invasion (17, 18).

Recently, the formation of χ -specific homologously paired joint molecules was demonstrated *in vitro* using a reconstituted recombination reaction consisting of purified RecA, RecBCD, and ssDNA-binding protein (SSB) (7). These results supported most predictions of the nick-initiation model (18), but they also uncovered an added feature of the RecBCD- χ interaction (7, 8). χ recognition resulted in attenuation of the nuclease, but not the helicase, activity of RecBCD (7, 8). The attenuation remained in effect for the duration of unwinding of the χ -containing DNA molecule but, upon exiting the DNA molecule, the nuclease activity of RecBCD was immediately and fully restored. The process was completely catalytic, with each RecBCD capable of undergoing multiple rounds of nuclease attenuation and restoration upon χ recognition and DNA exit, respectively (7, 8).

The biochemical basis for this χ -dependent attenuation of nuclease activity remained unknown; however, the interpretations of genetic experiments propose a testable hypothesis (19, 20). To explain the hyper-recombination phenotype of *recD* null mutations, it was proposed that interaction with a χ site activates the nonrecombinogenic RecBCD by the removal of the RecD subunit, producing a RecBC(D⁻) protein that is changed to become a recombinogenic resolver of Holliday junctions (19, 20). In this report, we describe experiments which test the RecD subunit-removal concept. We demonstrate that during the process of DNA unwinding at conditions of low nuclease activity (i.e., low Mg^{2+} concentration), the specific protein-DNA interaction between RecBCD and χ causes a reversible inactivation of RecBCD's helicase activity and, we presume, its nuclease activity as well. This χ -specific inhibition is fully reversible, and all RecBCD activity is restored by addition of excess Mg^{2+} . Interestingly, unwinding of DNA by RecBC reconstituted from purified RecB and RecC subunits (i.e., lacking the RecD subunit) shows the same Mg^{2+} dependency but is independent of χ interaction. These results, together with our previous data (7, 8), support the idea that χ is an unusual regulatory element or allosteric effector that alters the recombinational properties of the RecBCD through modification of the RecD subunit (19, 20) to produce an enzyme that becomes a proficient initiator of DNA strand-invasion events (7).

EXPERIMENTAL PROCEDURES

DNA and Protein Isolation. Plasmids pBR322 χ^0 (wild type) and pBR322 χ^+ FH (8) were purified by CsCl density gradient centrifugation (21) followed by chromatography on Sepha-

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

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cryl S-500 (Pharmacia). They were linearized with *Nde* I restriction endonuclease. The DNA was end labeled at the 5' termini by sequential reaction with calf intestinal phosphatase and T4 polynucleotide kinase in the presence of [γ - 32 P]ATP (ICN).

RecBCD was purified as described (3). The specific activity of the enzyme preparation was 5.4×10^4 nuclease units/mg of protein (22) or 1.1×10^4 helicase units/mg of protein (3).

The individual RecB and RecC proteins were purified as described (23–25). RecB and RecC concentrations were determined using molar extinction coefficients of 1.6×10^5 M $^{-1}$ cm $^{-1}$ and 2.0×10^5 M $^{-1}$ cm $^{-1}$ at 280 nm, respectively. The preparations of RecB and RecC proteins were approximately 90% and 95% pure, respectively, as determined by SDS/PAGE; the reconstituted RecBC preparation was free of contaminating DNA nuclease activity [0.1 ± 0.05 μ M nucleotides were made acid soluble in 30 min by 7.0 and 11.6 nM RecB and RecC, respectively (22)]. The DNA helicase activity of the reconstituted RecBC preparation was 1.5×10^2 helicase units/mg of protein at 7.0 and 11.6 nM RecB and RecC proteins, respectively (3), and DNA unwinding saturated at a stoichiometry of 1.6 RecC monomers per RecB monomer. The low apparent specific activity for this preparation of RecBC results from either a low apparent affinity of RecBC for DNA ends (i.e., high K_m) or a low percentage of active reconstituted enzyme; it is not a consequence of a low intrinsic unwinding rate (k_{cat}), because the maximum rate of unwinding at higher RecBC concentrations is at least half the rate observed for RecBCD enzyme (J.J.C. and S.C.K., unpublished observations).

SSB was purified as described (26, 27).

Reaction Conditions. The standard unwinding reaction mixture consisted of 25 mM Tris acetate (pH 7.5), 1 mM magnesium acetate, 5 mM ATP, 1 mM dithiothreitol, 1 mM phosphoenolpyruvate, pyruvate kinase at ≈ 4 units/ml, 1.44 nM dsDNA ends (6.28 and 6.23 μ M in nucleotides of linear pBR322 χ° and pBR322 χ^+ FH, respectively), 1.25 μ M SSB, and the indicated total RecBCD enzyme. The functional RecBCD concentration is 29% of the total enzyme concentrations given (data not shown; ref. 3). Reactions were initiated with the addition of ATP and pyruvate kinase or dsDNA after preincubation of all other components for 2 min at 37°C.

Agarose Gel Electrophoresis. Reactions were performed in 330 μ l using standard reaction conditions; to each time-point aliquot (40 μ l), 10 μ l of stop buffer (0.1 M EDTA/2.5% SDS/40% glycerol/0.125% bromophenol blue/0.125% xylene cyanol) was added to deproteinize the sample. Electrophoresis was performed in a 1.2% agarose gel for 18 hr at 1.6 V/cm in TAE (40 mM Tris acetate, pH 8.0/2 mM EDTA). The gels were dried and autoradiography was at -20° C using Kodak XAR-5 film and an intensifying screen. Autoradiograms of different exposures were used to quantify production of ssDNA with a BioImage system (Millipore).

Fluorescence Helicase Assay. The helicase activity of RecBCD was assayed by observing the quenching of the intrinsic protein fluorescence of SSB upon binding to ssDNA (3) under standard reaction conditions. The decrease in fluorescence was digitized at 20-sec intervals with a Shimadzu RF-5000 spectrofluorophotometer and the data were analyzed as described (3, 4).

RESULTS

Reversible Inactivation of RecBCD by χ . During the course of our examination of the effects of various reaction conditions on the ability of RecBCD to recognize χ , we observed that under certain conditions RecBCD was incapable of unwinding all of the DNA present in the reaction, but only

when that DNA contained χ sequences. These conditions are those where the ATP concentration exceeds the Mg $^{2+}$ concentration, resulting in a low free Mg $^{2+}$ concentration; under these conditions the dsDNA exonuclease is inhibited, but DNA unwinding is not dramatically affected (28). Fig. 1A illustrates the difference in unwinding of either χ^+ or χ° linear pBR322 DNA under these conditions. The left side of the autoradiogram demonstrates that χ° DNA was fully unwound ($96 \pm 1\%$) after 20 min. In contrast, the χ^+ DNA was unwound considerably more slowly, and unwinding appeared to cease at 10–20 min, with only $39 \pm 3\%$ of the dsDNA molecules unwound. Densitometric analysis yielded the data shown in Fig. 1B. Consistent with the low level of nuclease activity evident from Fig. 1A, production of acid-soluble oligonucleotides was undetectable in 30 min under the low-Mg $^{2+}$ conditions used ($<2\%$ of unwound linear pBR322 χ° dsDNA was acid soluble; D.A.D. and S.C.K., unpublished observations; ref. 28).

When Mg $^{2+}$ was added (10 mM) to the χ -inhibited RecBCD unwinding reaction mixture, complete unwinding of the remaining DNA occurred. This result suggests that, under the conditions of limiting free Mg $^{2+}$ concentration, the presence of a χ sequence in the DNA being unwound prevents RecBCD from subsequently acting on intact DNA molecules that have not yet been unwound. However, this inhibition is fully reversible, with reactivation of enzymatic activity occurring upon the subsequent increase in Mg $^{2+}$. Evidence for reactivation of the nonspecific dsDNA exonuclease activity is also seen in Fig. 1. Under conditions of limiting Mg $^{2+}$, 41 $\pm 3\%$ of the unwound pBR322 χ^+ FH dsDNA was converted to intact full-length ssDNA; however, this amount of full-length ssDNA did not increase upon the addition of excess Mg $^{2+}$, demonstrating that the degradative nuclease activity of RecBCD was also reactivated. The levels of nuclease activity seen in the reactivated enzyme agree with the amounts that we reported previously (8).

To quantitatively examine this inhibitory effect of χ on the dsDNA unwinding, we used a fluorescence helicase assay (3). Fig. 1B shows the time course of enzymatic unwinding of both χ^+ and χ° DNA under conditions identical to the agarose gel assay. The marked effect that χ has upon RecBCD enzyme activity can be seen in the limited extent of unwinding of pBR322 χ^+ FH: only 35% of the dsDNA molecules were unwound, compared with 95% of the χ° DNA molecules. In agreement with the agarose gel results, the χ -inactivated RecBCD enzyme was fully reactivated upon addition of Mg $^{2+}$ (Fig. 1B). As expected from Fig. 1, the inactivation of RecBCD by χ was continuous and was not specific to the remaining χ^+ dsDNA, because χ° dsDNA added subsequent to inactivation also was not unwound (data not shown). A "premature cessation" of RecBCD enzyme activity was reported previously (29); though the cause of the observed "silencing" remains obscure, KCl, but not bovine serum albumin, was capable of enzymatic reactivation (29). We have found that the χ -inactivated RecBCD cannot be reactivated by the addition of either KCl or bovine serum albumin (unpublished observations), suggesting that our phenomenon is different. The χ -dependent inactivation could result from the selective dissociation and sequestration (e.g., binding to DNA) of a subunit of the enzyme. To test this possibility, purified RecB, RecC, and RecD proteins were added both separately and in pairwise combinations to χ -inactivated reaction mixtures; none of the additions stimulated continued unwinding, arguing that reactivation was not limited by the absence of free subunits (data not shown). The modest difference detected in the rate of unwinding χ^+ and χ° dsDNA seen in Fig. 1B was observed at both subsaturating and saturating RecBCD concentrations (data not shown). This apparent decrease in rate is consistent with the previous

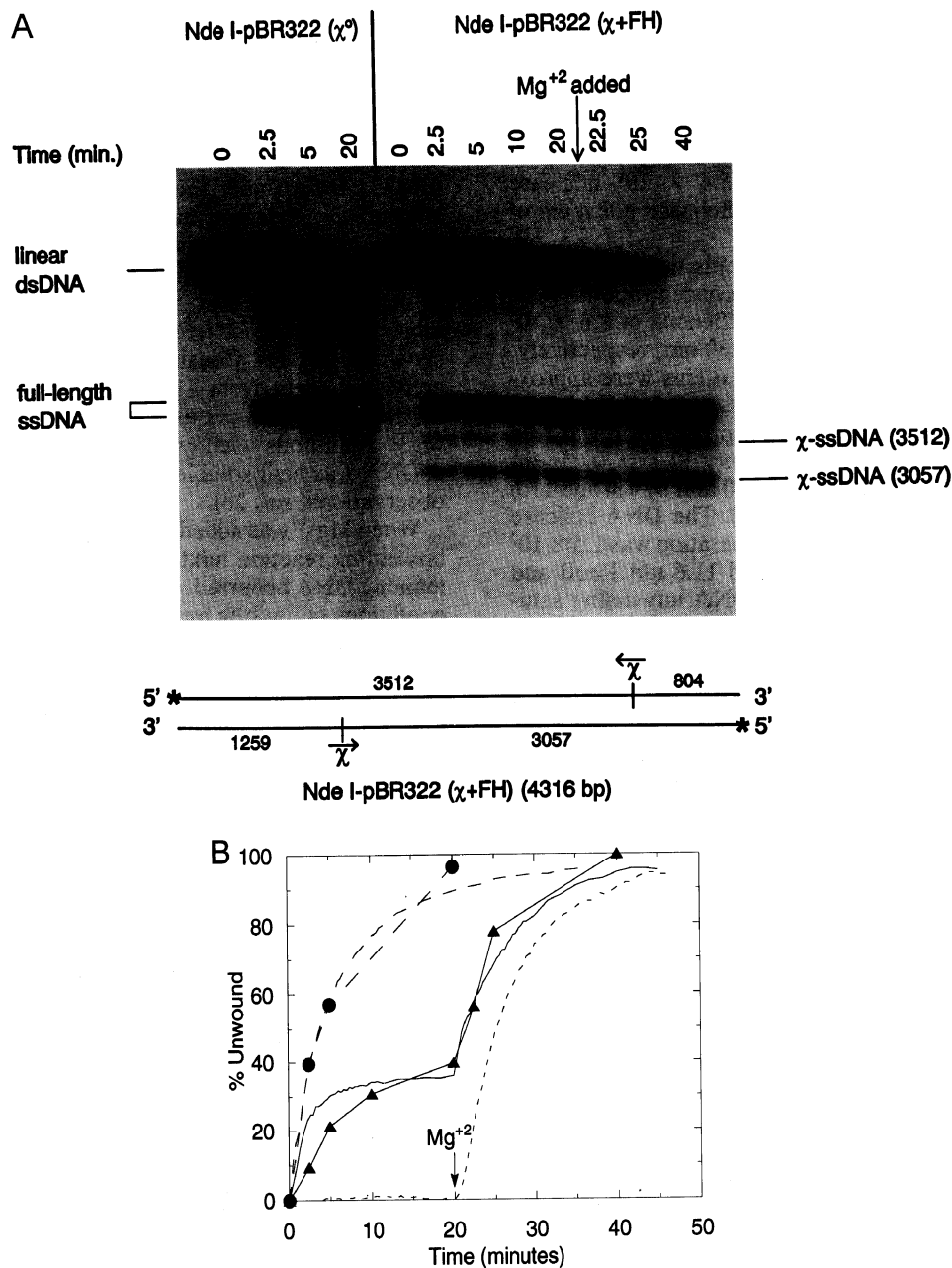


FIG. 1. χ -dependent inhibition of dsDNA unwinding by RecBCD enzyme. (A) *Nde* I-cut pBR322 χ^+ and *Nde* I-cut pBR322 χ^+ +FH were radioactively labeled with ³²P at the 5' ends and unwound under standard reaction conditions using 0.14 nM RecBCD (10 DNA ends per enzyme molecule). Aliquots were analyzed by agarose gel electrophoresis. The products corresponding to full-length ssDNA and χ -dependent ssDNA (χ -ssDNA) of lengths of 3512 and 3057 nucleotides are indicated. The doublet bands corresponding to full-length ssDNA are presumed to result from dissimilar conformations of the two DNA strands. Magnesium acetate (Mg²⁺) was added (10 mM) to the *Nde* I-cut pBR322 χ^+ +FH unwinding reaction mixture immediately after the 20-min time point was taken. The substrate *Nde* I-cut pBR322 χ^+ +FH is illustrated to show the relative location and directionality of χ sites, along with the radioactive end-label (asterisks). (B) Unwinding of *Nde* I-cut pBR322 χ^+ +FH (—) and *Nde* I-cut pBR322 χ^+ (---) by RecBCD and unwinding of *Nde* I-cut pBR322 χ^+ (- - -) by reconstituted RecBC protein, measured by the fluorometric helicase assay. Standard reaction conditions with 0.14 nM RecBCD (0.18 helicase unit) or 7.0 nM RecB and 11.6 nM RecC (0.12 helicase unit) were used. For comparison, unwinding as measured by the agarose gel assay (A) is plotted as the amount of dsDNA substrate remaining for *Nde* I-cut pBR322 χ^+ +FH (\blacktriangle) and *Nde* I-cut pBR322 χ^+ (\bullet). Arrow indicates the point where magnesium acetate was added (10 mM) in the RecBCD enzyme/*Nde* I-cut pBR322 χ^+ +FH and RecBC protein/*Nde* I-cut pBR322 χ^+ unwinding reactions.

suggestion that the interaction between RecBCD and χ results in a pause in DNA unwinding (8).

The χ -dependent inhibition of unwinding DNA shown in Fig. 1 occurred at subsaturating amounts of RecBCD with respect to dsDNA ends (i.e., 10 dsDNA ends per RecBCD enzyme). To determine whether enzymatic inhibition was, in fact, due to an interaction with χ , the relationship between RecBCD concentration and extent of unwinding of χ -containing DNA was examined (Fig. 2). At subsaturating concentrations of RecBCD relative to dsDNA ends, the amount of χ^+ DNA unwound increased linearly with enzyme con-

centration. In contrast, the unwinding of linear pBR322 χ^+ dsDNA was essentially complete over all enzyme concentrations examined, except at the lowest concentration, due to slight nonspecific loss of enzyme activity at such low concentrations. If every encounter between RecBCD and χ resulted in inactivation, then complete unwinding would require one or more functional RecBCD molecules per DNA end (or site). The data in Fig. 2 suggest that the frequency of χ interaction is <100%. We can estimate the percentage of productive encounters from these data if we assume that inactivation of RecBCD to a functional concentration below

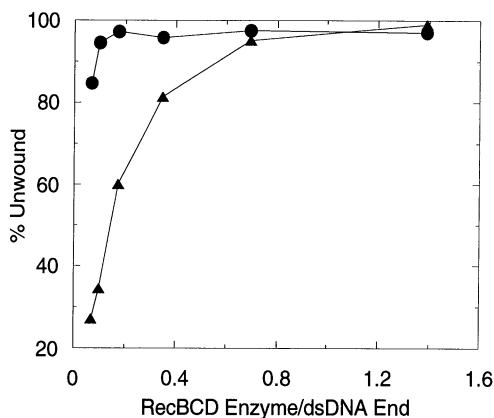


FIG. 2. Dependence of the extent of DNA unwinding on RecBCD concentration. Standard reaction conditions with various amounts of total RecBCD enzyme were used to determine the extent of unwinding of *Nde* I-cut pBR322 χ^+ FH (▲) and pBR322 χ^- dsDNA. The functional RecBCD concentration is 29% of the total enzyme concentration.

≈ 0.003 nM would result in an experimentally undetectable amount of DNA unwinding. The data in Fig. 2 can be roughly approximated if the χ -recognition efficiency is 20%. This conclusion is in complete accord with previous estimates of productive χ encounters based on the frequency of nicking at χ , which are 25–40%, suggesting that the event responsible for these two effects (i.e., χ recognition) is the same (8, 30).

RecBC Protein Is Enzymatically Equivalent to the χ -Inhibited RecBCD. Genetic studies were interpreted to suggest that the interaction between RecBCD and χ results in dissociation of the RecD subunit from the holoenzyme to yield a dsDNA exonuclease-deficient DNA helicase (19, 20, 31). To both test this hypothesis and possibly illuminate the molecular form of the χ -inactivated RecBCD enzyme, similar DNA-unwinding experiments were performed with RecBC (i.e., lacking the RecD subunit) reconstituted from individual RecB and RecC subunits. At low Mg^{2+} concentration, no detectable unwinding of dsDNA occurred, but with addition of excess Mg^{2+} (10 mM), the RecBC was activated for DNA unwinding (Fig. 1B). Addition of RecD subunit at the low Mg^{2+} concentration did not lead to unwinding, consistent with the behavior of the χ -inactivated RecBCD (unpublished observations). This parallel behavior suggests that the RecBC is functionally equivalent to the RecBCD which has interacted with χ . Identical results were obtained with χ^+ DNA (data not shown), indicating that RecBC either does not recognize or is not affected by χ . This observation is consistent with the inability of RecBC to recognize χ , as manifested by its failure both to display χ stimulation of recombination *in vivo* (20, 32, 33) and to generate a χ -specific ssDNA fragment *in vitro* (25) (J.J.C. and S.C.K., unpublished observations). Our *in vitro* results imply that χ recognition is accompanied by dissociation or functional inactivation of the RecD subunit, supporting the conclusions derived from *in vivo* experiments (19, 20).

DISCUSSION

The interaction between RecBCD and χ results in a stimulation of the exchange of genetic information. Previously, we demonstrated that χ causes an attenuation of the nuclease activity of RecBCD that is presumably responsible for the elevated recombination frequency *in vivo* (7, 8). However, the molecular basis of the event responsible for the attenuation of nuclease activity is unknown. In this report, we demonstrate the χ -specific inactivation of enzymatic activities that occurs under conditions of limiting Mg^{2+} concentration; inactivation is fully reversible upon addition of ex-

cess Mg^{2+} . This distinctive pattern of inactivation and reactivation is mirrored by the RecBC enzyme, suggesting that the interaction between RecBCD and χ is associated with loss of RecD subunit function or of RecD subunit itself.

We speculate that the χ -dependent inhibition of RecBCD is due to an inability of the inactivated enzyme to reinitiate unwinding on intact DNA molecules, rather than a prolonged pause or slow dissociation event at the χ site. If RecBCD were halted at the χ site, then the data in Fig. 1A would demonstrate the formation of a population ($\approx 10\%$) of DNA molecules that are only partially unwound to χ . Furthermore, treatment of unwound DNA with P1 nuclease results in degradation of the ssDNA present but does not result in the appearance of a shorter, discrete dsDNA species that might be expected to form if a significant population of DNA molecules had heterogeneous ssDNA tails that ended at the χ sequence (data not shown). Finally, this interpretation is also consistent with the inability of RecBC to initiate unwinding under these conditions. This last statement, coupled with the failure of free added RecD to reactivate holoenzyme, implies that, under inhibiting conditions, another limiting step is the reactivation (reassembly) of RecD; if it were not, then the action of holoenzyme would be catalytic as is seen at elevated Mg^{2+} concentrations (7, 8). Thus, it appears that upon interaction with χ , RecBC(D⁻) is modified in such a way that it can continue unwinding the DNA molecule to which it is bound but can neither reactivate (reassemble) nor initiate unwinding on a new DNA molecule. Since the physiological concentration of free Mg^{2+} is unknown, it is not clear whether cellular conditions are reactivating, where RecBCD enzyme action would be catalytic or inhibiting, or where its action would be stoichiometric.

To explain the genetic properties of *recBCD*[±] mutations, which exhibit a hyperrecombination phenotype in the absence of χ and a loss of nuclease activity (31–35), Thaler *et al.* (19, 20) were the first to suggest that χ is responsible for altering the RecBCD enzyme, through the dissociation of the RecD subunit, to yield a χ -activated RecBC enzyme. The results presented here show that, at limiting Mg^{2+} concentrations, RecBCD is functionally inactivated in both helicase and nuclease activities upon encountering a χ sequence and that this inhibition is completely reversible with the addition of excess Mg^{2+} . The identical dependency on Mg^{2+} that is seen for DNA unwinding catalyzed by the reconstituted RecBC suggests that the interaction between RecBCD and χ produces an enzyme that is functionally equivalent to the nuclease-deficient RecBC (J.J.C. and S.C.K., unpublished observations; ref. 25). Thus, the most straightforward conclusion is that χ recognition by RecBCD causes a dissociation or functional inactivation of the RecD subunit. Although these findings do not directly prove subunit loss, this proposal suggests a molecular mechanism that is consistent with the *in vivo* behavior of *recBCD*[±] mutations that produce a severely truncated RecD polypeptide (19, 20) and with *in vitro* results involving χ -dependent regulation of the enzymatic activities of RecBCD (7, 8). Despite attempts, we have yet no unambiguous biochemical evidence for RecD subunit dissociation (P. Alizadeh and S.C.K., unpublished observations).

A model incorporating the present results and previous findings is shown in Fig. 3. RecBCD holoenzyme initiates DNA unwinding at a dsDNA end; under the conditions examined here, the dsDNA exonuclease activity is reduced (8, 28). Upon encountering χ , the specific protein–DNA interaction between RecBCD and χ causes the enzyme to pause at the χ site. This pause not only reflects the specific recognition event but also represents the time required for the modification of the RecBCD (shaded RecBC enzyme) to yield an enzyme functionally equivalent to RecBC. The pause also increases the likelihood that the last nucleolytic event occurs

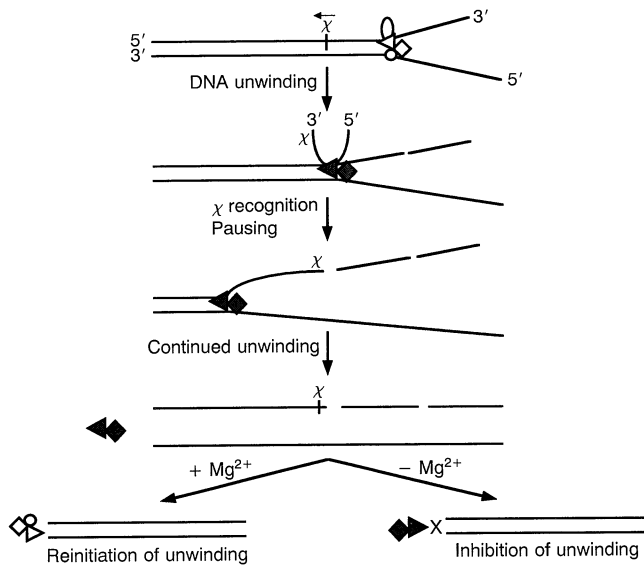


FIG. 3. Model for the effect of χ on the enzymatic activity of RecBCD. The arrow above the χ site indicates the direction that RecBCD must approach for recognition of χ . RecBCD is represented by diamond/triangle/circle. Prior to interaction with the χ site, RecBCD unwinds the DNA with limited degradation at low concentrations of free Mg^{2+} ; upon encountering a χ site, RecBCD pauses and becomes functionally equivalent to RecBC (shaded symbol). Continued unwinding produces a ssDNA fragment downstream from the χ site. With the addition of excess Mg^{2+} , the enzyme continues unwinding on subsequent DNA molecules, whereas reinitiation on another DNA molecule is inhibited under limiting amounts of Mg^{2+} . SSB is bound to the ssDNA but is not shown.

in the vicinity [4–6 nucleotides (16)] of χ . Through continued unwinding, the χ -inhibited enzyme produces a ssDNA fragment whose length is dependent upon the location of the χ site in the linear dsDNA. In the absence of excess Mg^{2+} , the χ -modified RecBC-like enzyme is blocked in both initiation of dsDNA unwinding and reassembly with (or reactivation of) the RecD subunit, resulting in inhibition of both DNA unwinding and degradation. Though the failure of RecBC to initiate DNA unwinding is sufficient to explain its lack of helicase activity at these conditions, the χ -modified RecBCD must, in addition, fail to reassemble with (or reactivate) the RecD subunit, since the unmodified RecBCD is capable of complete DNA unwinding. In the presence of excess Mg^{2+} (3, 7, 8, 28) reactivation of helicase and nuclease activity is essentially instantaneous upon DNA exit, and RecBCD action is truly catalytic. Our conclusions are in apparent conflict with a recent conclusion that, after interaction with χ , the helicase and χ -cutting activities are inhibited but the nuclease activity is not (30). Our results offer a ready explanation for this discrepancy. Those experiments were conducted under conditions that manifest reversible inhibition by χ ; when the helicase and χ -cutting activities were measured after χ inactivation, assays were performed at low concentrations of free Mg^{2+} that maintain inactivation and no activity was detected; however, when the dsDNA exonuclease activity was measured, a high concentration of free Mg^{2+} that promotes reactivation was used and exonuclease activity was detected.

Further supporting the suggestion that χ acts as a molecular switch to regulate the activities of RecBCD, our present findings provide biochemical evidence implicating loss or functional inactivation of the RecD subunit as a part of that switch. Both *in vivo* and *in vitro* observations suggest that the χ sequence protects DNA downstream from itself by atten-

uating the nuclease activity of RecBCD (7, 8, 36, 37). This model provides a specific biochemical mechanism that resolves the paradox of RecBCD being both a destructive nuclease and a constructive recombination enzyme.

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