RecBCD enzyme is essential for the major pathway of homologous recombination of linear DNA in Escherichia coli. It is a potent nuclease and helicase and, during its unwinding of double-stranded DNA, makes single-strand scissions in the vicinity of Chi recombination hot spots. We report here that both the strand that is cut and the position of the cuts relative to Chi depend on the ATP to Mg\(^{2+}\) ratio. With ATP in excess, Chi-dependent nicks occurred, as we have previously reported, four to six nucleotides to the 3'-side of the Chi octamer (5'-GCTGGTGG-3') and were detected only on the strand bearing that sequence. Three differences were seen with Mg\(^{2+}\) in excess. 1) Chi-dependent 3'-ends were produced on the GCTGGTGG-containing strand closer to and within the Chi octamer. 2) Chi-dependent cuts occurred on the complementary DNA strand. 3) RecBCD enzyme destroyed the 3'-terminated strand of DNA from its entry point up to the vicinity of the Chi site, as others have previously reported. We show here that, with Mg\(^{2+}\) in excess, the enzyme continued to travel along DNA, after encountering a Chi site, releasing both strands of the DNA distal to Chi as single strands. We discuss potential biological consequences of these two modes of RecBCD enzyme-Chi interaction.

RecBCD enzyme (EC 3.1.11.5), encoded by the recB, recC, and recD genes of Escherichia coli, is the best characterized enzyme specific to the major (RecBCD) pathway of homologous recombination in E. coli conjugation and transduction (reviewed in Ref. 1). Purified RecBCD has many activities. It has potent ATP-dependent ss and ds DNA exonuclease activities and a weak ATP-stimulated endonuclease activity on ss, but not ds, circular DNA. Under conditions that reduce these nucleolytic activities (e.g. 5\(\text{mM}\) ATP and 1\(\text{mM}\) Mg\(^{2+}\)) the enzyme totally unwinds ds DNA, up to 40 kilobase pairs or longer in the presence of SSB (2–4). Electron microscopy revealed that the enzyme travels unidirectionally and highly processively from a ds DNA end through the DNA, with either transient or permanent unwinding of the DNA behind the enzyme (5, 6). Under such conditions the enzyme has a potent site-specific cleavage activity at Chi sequences (5'-GCTGGTGG-3') (7), a recombinational hotspot specific to the RecBCD pathway (reviewed in Ref. 8). This cleavage occurs only as the enzyme is unwinding the DNA from right to left, as Chi is written here (9). The enzyme makes Chi-dependent ss nicks a few bases to the 3'-side of Chi on the GCTGGTGG-containing ("Upper") strand\(^2\) but makes no detectable Chi-dependent nicks on the complementary strand (7, 9) (Fig. 1, left). The activities of the purified enzyme, under these conditions, correlate well with the properties of Chi deduced from genetic studies and support a previously proposed model of Chi-stimulated recombination by the RecBCD pathway (8, 10) (see "Discussion").

RecBCD enzyme has been coupled with RecA and SSB proteins to produce Chi-dependent joint molecules from linear Chi-containing ds DNA and supercoiled Chi-free ds DNA (11–13). This reaction requires [Mg\(^{2+}\)] to be in excess over [ATP] (e.g. 8\(\text{mM}\) Mg\(^{2+}\) and 1\(\text{mM}\) ATP) to allow RecA protein to promote homologous strand exchange between the linear DNA unwound by RecBCD enzyme and the supercoiled ds DNA. Under this condition RecBCD enzyme has a different reaction at Chi: it degrades the Upper strand from its 3'-end up to Chi, which attenuates the exonuclease active under this condition (Fig. 1, right). Unwinding continues, to produce ss DNA with a 3'-end near Chi, which is used by RecA and SSB proteins to make a joint molecule.

Under both reaction conditions ([ATP] > [Mg\(^{2+}\)] or [Mg\(^{2+}\)] > [ATP]) RecBCD enzyme thus produces ss DNA with a 3'-end near Chi, the postulated substrate for RecA protein. With [ATP] > [Mg\(^{2+}\)] RecBCD enzyme unwinds DNA up to Chi, cuts the Upper strand at Chi, and continues unwinding to produce ss DNA with Chi near its 3'-end (Fig. 1, left). With [Mg\(^{2+}\)] > [ATP] the enzyme degrades the Upper strand up to Chi, ceases degradation, and continues unwinding to produce a similar ss DNA fragment (Fig. 1, right). To explore which reaction more nearly reflects that in E. coli cells, we have further characterized the reaction products under the two conditions. Markedly different products were observed, and we discuss their relationship to the types of genetic recombinants produced by the RecBCD pathway.

**EXPERIMENTAL PROCEDURES**

**Proteins**

Fraction V of RecBCD enzyme (14) was used throughout. Molar enzyme concentrations were calculated from the ds DNA exonuclease activity of the fraction (3) and the specific activity of fraction VI, which is nearly homogeneous (14) (3.3 \times 10^6 units/mg of protein). SSB was a gift from S. Kowalczykowski, University of California at Davis (12). Restriction enzymes and DNA-modifying enzymes were from New England Biolabs, Life Technologies, Inc., or Boehringer Mannheim.

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\(^1\) The abbreviations used are as follows: ss, single-stranded; ds, double-stranded; MOPS, 3-[N-morpholino]propanesulfonic acid; nt, nucleotides; SSB, single-stranded DNA binding protein from E. coli.

\(^2\) The strand nicked at Chi under these conditions (that containing 5'-GCTGGTGG-3') is designated the "Upper" strand. The other strand is dubbed the "Lower" strand.
RecBCD Enzyme Nicks Both Strands of DNA

FIG. 1. Action of RecBCD enzyme on ds DNA containing a Chi site. RecBCD enzyme is shown acting on Chi-containing ds DNA under Low Mg\(^{2+}\) conditions (ATP) > [Mg\(^{2+}\)] (7) or High Mg\(^{2+}\) conditions ([Mg\(^{2+}\)] > ATP) (13). SSB, necessarily present under high Mg\(^{2+}\) conditions, is omitted for clarity. The previously unreported Chi derivative was linearized with EcoRI.

DNA Substrates

Plasmids—Plasmid pBR322 and its Chi derivatives were purified as described elsewhere (15). Where necessary, RNA was removed by centrifugation (16) or by agarose gel electrophoresis. DNA concentrations were calculated from the specific activity of labeled DNA, which was then cut with NdeI, and the desired capped DNA fragment was purified as previously described (15). Gel electrophoresis of samples, following the EcoRI digestion, showed that 70–90% of the incorporated label was in the expected fragments.

Size Standards—A BstEII digest of phage λ DNA (New England Biolabs) was labeled as described elsewhere (14). Maxam-Gilbert degradation reactions were as previously described (19).

Reaction Conditions

"Low Mg\(^{2+}\)" reactions contained 20 mM MOPS-KOH (pH 7.0), 5 mM ATP, 3 mM magnesium acetate, 1 mM dithiothreitol (15), and 2 μM SSB. "High Mg\(^{2+}\)" reactions contained 25 mM Tris acetate (pH 7.5), 1 mM ATP, 8 mM magnesium acetate, 2 μM SSB, 1 mM dithiothreitol; the ATP-regenerating system used in the original publication (13) was omitted. All reactions were supplemented with 100 μCi/mg polynucleotide phosphorylase K-60 (Matheson, Coleman & Bell) to stabilize RecBCD enzyme in dilute solutions (20).

Sample Preparation and Electrophoresis

Native Gels—Reactions were stopped as described elsewhere (13) and loaded directly onto horizontal 12% agarose gels in Tris acetate-EDTA buffer (16). Where indicated, samples were denatured by boiling immediately before electrophoresis. Gels were run at 1 V/cm for 16 h and dried onto DEAE paper before autoradiography at −70°C with intensifying screens.

Denaturing (Alkaline) Gels—Reactions were stopped by addition of an equal volume of 10 mM EDTA, 0.6 mM sodium acetate, 10 μg/ml RNA. After addition of 5 volumes of ethanol, DNA was recovered by centrifugation for 15 min and resuspended in 10 μl of alkaline loading buffer (21), before electrophoresis on 1% agarose gels in 50 mM NaOH, 1 mM EDTA, run at 1 V/cm for 16 h at room temperature. Gels were dried and exposed as above.

"Sequencing" Gels—Reactions were stopped by addition of 9 volumes of 10 mM EDTA, 0.3 mM sodium acetate, 0.2% SDS, 80 μg/ml sonicated carrier DNA, 4 μg/ml RNA. Following phenol and chloroform extractions to remove SSB, DNA samples were recovered by ethanol precipitation and dissolved by boiling in formamide loading buffer (19). Samples were analyzed by electrophoresis on 12% urea-Tris borate-EDTA polyacrylamide gels (19), followed by autoradiography of the undried gel at −70°C with intensifying screens.

Identification of Cleavage Sites

Examination of the RecBCD enzyme cleavage products on urea-polyacrylamide gels allowed identification of bands corresponding to every nucleotide position expected for the region of the gel between the restriction enzyme markers in adjacent lanes. These identifications were confirmed by reference to the Maxam-Gilbert chemical degradation products, after allowance had been made for the migration differences caused by the two terminal phosphates on the chemical degradation products.

RESULTS

Mg\(^{2+}\) to ATP Ratio Determines Nuclease Activity—The interactions of Chi sites on ds DNA with purified RecBCD enzyme have been investigated under two different reaction conditions, one designed to minimize nonspecific nuclease activity by the enzyme (7), the other to allow RecBCD enzyme and RecA protein to function simultaneously (11). While there are several differences between the reaction conditions, the ratio of ATP to Mg\(^{2+}\) ions is the factor that determines the level of the nonspecific nuclease activity of RecBCD enzyme (22), and hence the two conditions will be referred to as "Low Mg\(^{2+}\)" and "High Mg\(^{2+}\)" respectively. They are defined under "Experimental Procedures." We assume the major factor is the presence of free Mg\(^{2+}\) ions in High Mg\(^{2+}\) conditions, most of the Mg\(^{2+}\) ion being chelated to ATP in Low Mg\(^{2+}\) conditions.

During the course of this work we examined the differences between these two reaction conditions, using Chi cleavage assays similar to those described below. We found that, in the High Mg\(^{2+}\) reactions, MOPS buffers with pH values between 7.0 and 7.5 would substitute for the Tris acetate and that omission of the ATP-regenerating system did not affect the
results. Likewise we observed that omission of SSB from the Low Mg²⁺ reactions did not alter the products observed (23).  

Using a 3′-end-labeled substrate, we examined the survival of ds DNA, and its Chi-dependent cleavage products, under conditions related to High Mg²⁺ conditions.  

Neither DNA unwound by RecBCD enzyme nor Chi-dependent ss fragments were observed if Mg²⁺ exceeded ATP either at high ATP concentrations (5 mM ATP, 8 mM Mg²⁺) or at low ATP concentrations (1 mM ATP, 8 mM Mg²⁺), even in the presence of SSB, as observed by Dixon and Kowalczykowski (13). When ATP was in excess, ss DNA reaction products survived both at high ATP concentration (5 mM ATP, 3 mM Mg²⁺) and at low ATP concentration (1 mM ATP, 0.5 mM Mg²⁺). The ratio of ATP to Mg²⁺ (presumably free Mg²⁺ ion) is thus the salient feature distinguishing the two reaction conditions, in accord with previous observations (22, 24). In the experiments reported below, either 5′- or internal ³²P labels were used, to allow detection of Chi-specific reaction products under either reaction condition.

Cuts on Both Strands at Chi under High Mg²⁺ Conditions—We show here that under High Mg²⁺ reaction conditions Chi-dependent cutting of both strands of the DNA occurs. As seen in Fig. 2, two distinct reaction products resulted from the action of RecBCD enzyme on 5′-end-labeled ds DNA bearing a Chi site. These reaction products (which are indeed ss; Fig. 2B) migrated at the positions expected for ss fragments resulting from cutting of the two strands of the DNA at, or near, Chi.

The Chi sequence is uniquely determined by the sequence 5′-GCTGGTGTTG-3′ (18), and no significant effects of flanking sequences have previously been found for the nicks on the Upper strand (7, 9, 23). Fig. 2 shows that the two Chi sequences tested, which are about 500 base pairs apart and share no obvious flanking sequence similarities (18), stimulated equivalent levels of cutting on the Lower strand of the DNA under High Mg²⁺ conditions.

The fragments just described were Chi-dependent, since they were produced at much lower levels from DNA devoid of Chi sites (lanes 10–13). In the absence of Chi the DNA was degraded, even in the presence of SSB, to generate both intermediates (mostly less than 800 nt long) and the final products of degradation by the enzyme (short oligonucleotides, seen at the bottom of the gel). Fragments apparently equivalent in size to those resulting from Lower strand nicking at χ⁻F were seen in the χ⁻ lanes but were severalfold enhanced by χ⁺F.

The products shown in Fig. 2A resulted from a single round of reaction of RecBCD enzyme with ds DNA. Heparin prevents binding of RecBCD enzyme to the ends of ds DNA but does not disrupt preformed complexes (25). In the experiments in Fig. 2, RecBCD enzyme was bound to DNA ends in the absence of ATP, and the reactions were started by addition of a mixture of ATP and heparin. In the control lanes (1, 8, and 20), addition of heparin to the DNA prior to the addition of RecBCD enzyme prevented any detectable reaction on ds DNA. It is unlikely that the reaction products resulted from the subsequent action of RecBCD enzyme on ss products released in the first round of reaction, as SSB strongly inhibits the activities of the enzyme on ss DNA (2).

The yields of the Chi-dependent fragments depended upon the RecBCD enzyme concentration. The yield of fragments resulting from nicking at Chi on the Upper strand was maximal when there was approximately one RecBCD enzyme per DNA molecule. Presumably, at higher enzyme concentrations the reaction products were destroyed during the collision between enzyme molecules traversing the DNA from opposite directions, as previously observed (13).  

³A. F. Taylor, unpublished results.
ucts were ss or ds DNA. Native (ds) and boiled (ss) size markers corresponding to the sizes expected for cutting at $\chi^+ E$ or $\chi^+ F$ were well separated by the gel (Fig. 2B, lanes 21 and 22). Migration of the RecBCD reaction products (lanes 23 and 24) was unaffected by boiling, showing that both the Upper strand and the Lower strand cut products were released as ss DNA.

Given the ss nature of the products, the conformation of the Chi-dependent species with the ss size markers confirms that the Chi-dependent cuts are in the immediate vicinity of the Chi sequences. Their exact locations are described below.

Strand Nicking during Unidirectional Travel by RecBCD Enzyme—Under Low Mg$^{2+}$ conditions, RecBCD enzyme nicked the Upper strand of DNA only if the enzyme has approached the Chi site from the biologically appropriate direction (26), that is by entering the DNA at the 3'-terminus of the strand containing the GCTGGTGG sequence (9). We considered the possibility that Lower strand Chi cleavage might be the result of RecBCD enzyme encountering Chi from the opposite direction, either by itself or as a result of a collision with a RecBCD enzyme molecule that was "paused" at the Chi site.

We tested this possibility by using synthetic hairpin-shaped oligonucleotides ligated onto one end of the DNA substrates. Hairpins prevent the entry of RecBCD enzyme under Low Mg$^{2+}$ reaction conditions (14). We first tested whether such hairpin-shaped molecules were resistant to RecBCD enzyme under High Mg$^{2+}$ conditions in the absence of SSB. Hairpin oligonucleotides, labeled at their 5'-termini, were self-ligated, and the double-length products purified (Fig. 3A). Removal of the tips, by restriction enzyme digestion, provided positive controls for RecBCD enzyme sensitivity. The "double-length" DNA was reacted with increasing concentrations of RecBCD enzyme and reaction products assayed by trichloroacetic acid precipitation (Fig. 3B). The small fraction of $^{32}$P released by low concentrations of RecBCD enzyme presumably came from double-length open-ended ds DNA molecules formed by annealing of two hairpin molecules (as shown in Fig. 3A, top). However, the remainder of the DNA was resistant to 100 times the concentration of RecBCD enzyme needed to solubilize the control DNA species (unligated hairpin oligonucleotides or double-length molecules whose tips had been removed by restriction enzyme digestion). Thus, hairpin oligonucleotide caps are indeed resistant to cleavage by RecBCD enzyme under High Mg$^{2+}$ conditions, even in the absence of SSB.

Substrates for Chi cutting were made by ligating hairpin oligonucleotides onto fragments of pBR322 with or without Chi sites (Fig. 4) to produce DNA molecules in which RecBCD enzyme could approach Chi only in the active orientation. DNA molecules were prepared uniquely labeled in the Upper or the Lower "strand" of the DNA, in the position labeled A or T in Fig. 4, to allow separate examination of the two strands of the DNA. In the $\chi^+ E$ substrates the $^{32}$P label was between the ds DNA end and Chi, allowing detection of fragments extending from Chi toward the ends of the DNA strands, while in the $\chi^+ F$ substrates the $^{32}$P label was distal to Chi, allowing detection of fragments extending from Chi toward the hairpin.

Reaction of these substrates under Low Mg$^{2+}$ conditions produced products similar to those previously observed using substrates labeled on both strands (15). Reaction of either $\chi^+$ DNA substrate produced half-length (2300 nt) molecules, resulting from RecBCD enzyme molecules passing Chi without cutting it, then cutting the hairpin from within; fragments of this length were the only specific reaction products seen with $\chi^+$ substrates (Fig. 4A, lanes 4-6 and 11-13). Such fragments were seen with substrates labeled either in the Upper or the Lower strand, confirming that there is little or no preferential degradation of either strand under Low Mg$^{2+}$ conditions. Under High Mg$^{2+}$ conditions, however, the half-length fragments are seen with Lower (5'-terminated) strand labeled substrates (lanes 2 and 3) but not with Upper (3'-terminated) strand labeled substrates (lanes 9 and 10), confirming the strand-specific degradation previously reported under these conditions (13).

Nicking of the Upper strand at $\chi^+ F$ under Low Mg$^{2+}$ conditions produced a 1300-nt fragment with the T-labeled substrate (Fig. 4B, lanes 11-13) and a 3300-nt fragment with the A-labeled DNA (Panel B, lanes 4-6). The absence of a specific 1300-nt fragment with A-labeled DNA under Low Mg$^{2+}$ conditions (Panel B, lanes 4-6) is further evidence of the lack of Lower strand cutting under these conditions.

Under High Mg$^{2+}$ conditions, a 1300-nt fragment was produced with A-labeled DNA (Fig. 4B, lanes 2 and 3), demonstrating that RecBCD enzyme molecule(s) that encounter Chi from only one direction can indeed produce Lower strand cuts at Chi. The product was Chi-dependent (compare lanes 2 and 3 of Panel B with those of Panel A) and was produced at low enzyme concentrations (lane 2 with 2 enzyme molecules per DNA end).

Experiments with 3'-labeled DNA molecules bearing Chi sites have previously been interpreted as evidence that RecBCD enzyme degrades the 3'-terminated strand until it encounters a Chi sequence (13). Such data were consistent with

![Diagram of RecBCD Enzyme Nicks Both Strands of DNA](image-url)

**Fig. 3. Resistance of hairpin ends to RecBCD enzyme degradation.** A, Construction of the substrates. The self-complementary oligonucleotide shown was 5'-end labeled with $^{32}$P, and some of it was self-ligated (14). Monomer and dimer length molecules were purified by gel electrophoresis. Some of the purified dimer DNA was treated with Clal or HaellI to produce linear ds DNA with internal labels. The intermolecular dimer shown results from isomerization of the hairpin monomer material and is not separated from the ligated dimer on gel purification. B, Reaction of hairpin DNAs with RecBCD enzyme. Reactions (10 $\mu$) contained, in High Mg$^{2+}$ reaction mix lacking SSB, the indicated concentrations of RecBCD enzyme and monomer (0.11 nM) or dimer (0.09 nm) hairpin DNAs or restricted dimer hairpin DNAs (0.14 nm). After 10 min of incubation at 37°C, the reactions were analyzed by trichloroacetic acid precipitation (3). Precipitate and supernatant fractions were counted by Cerenkov radiation. The graph plots the percentage of the counts precipitable before RecBCD enzyme reaction subsequently released by RecBCD enzyme.

**Fig. 4.** To allow separate examination of the two strands of the DNA. In the position labeled A or T in Fig. 4, to allow separate examination of the two strands of the DNA. Upper and Lower strand cutting under these conditions. The product was Chi-dependent (compare lanes 2 and 3 of Panel B with those of Panel A) and was produced at low enzyme concentrations (lane 2 with 2 enzyme molecules per DNA end). Under High Mg$^{2+}$ conditions, however, the half-length fragments are seen with Lower (5'-terminated) strand labeled substrates (lanes 2 and 3) but not with Upper (3'-terminated) strand labeled substrates (lanes 9 and 10), confirming the strand-specific degradation previously reported under these conditions (13).

Nicking of the Upper strand at $\chi^+ F$ under Low Mg$^{2+}$ conditions produced a 1300-nt fragment with the T-labeled substrate (Fig. 4B, lanes 11-13) and a 3300-nt fragment with the A-labeled DNA (Panel B, lanes 4-6). The absence of a specific 1300-nt fragment with A-labeled DNA under Low Mg$^{2+}$ conditions (Panel B, lanes 4-6) is further evidence of the lack of Lower strand cutting under these conditions.

Under High Mg$^{2+}$ conditions, a 1300-nt fragment was produced with A-labeled DNA (Fig. 4B, lanes 2 and 3), demonstrating that RecBCD enzyme molecule(s) that encounter Chi from only one direction can indeed produce Lower strand cuts at Chi. The product was Chi-dependent (compare lanes 2 and 3 of Panel B with those of Panel A) and was produced at low enzyme concentrations (lane 2 with 2 enzyme molecules per DNA end).

Experiments with 3'-labeled DNA molecules bearing Chi sites have previously been interpreted as evidence that RecBCD enzyme degrades the 3'-terminated strand until it encounters a Chi sequence (13). Such data were consistent with
RecBCD Enzyme Nicks Both Strands of DNA

Figure 4. Cleavage at Chi during unidirectional travel by RecBCD enzyme. DNA substrates were 2300-base pair fragments of pBR322, bearing the indicated Chi sites (A, χ1, B, χ1E; C, χ1F), extending clockwise from the EcoRI site (nt 4360) to the NdeI site (nt 2297), with a synthetic oligonucleotide cap (Fig. 3A) ligated onto the EcoRI site. Substrates bore 32P labels on one or the other strand of the DNA, immediately 5′ to one or both of the A or T nucleotides in the StyI site at nt 1371, as indicated in the diagram. DNA (50 μm) was reacted for 1 min with RecBCD enzyme under High or Low Mg2+ conditions as indicated and analyzed on a 1% alkaline agarose gel. Size markers (lanes 7 and 14) were a 3′-end-labeled BstEII digest of phage λ DNA. RecBCD:DNA denotes the ratio of RecBCD enzyme molecules to DNA molecules. The diagram below the figure shows the locations of the Chi sites and 32P labels, and their distances in nucleotides from the hairpin. The small diagrams next to the autoradiograms denote the parts of the molecules present in the adjacent fragments. Half-length molecules, cut at the hairpin, are not indicated in Panels B and C.

The alternative view that RecBCD enzyme removed only a few 3′-terminal-labeled nucleotides, rendering invisible (in the reported experiments) a postulated DNA fragment extending from the Chi site to near the 3′-terminus. Results in Fig. 4B, with a χ1E substrate labeled internally in the 3′-terminated strand, under High Mg2+ conditions, failed to reveal either any fragment of the postulated size (1300 nt) or any Chi-dependent smear indicative of limited degradation. The experiment thus shows that degradation of the 3′-terminated strand extends at least 900 nt from the terminus, and presumably up to Chi.

If the RecBCD enzyme molecule that nicked the Lower strand at χ1E under High Mg2+ conditions also failed to degrade the 3′-terminated strand, then a 3300-nt T-labeled fragment would be observed (analogous to the 3300-nt A labeled fragment seen in lanes 4–6 of Panel B under Low Mg2+ conditions). The failure to observe such a fragment (Panel B, lanes 9 and 10) implies that RecBCD enzyme molecules that cut the Lower strand do indeed also degrade at least part of the Upper strand of the DNA.

A capped DNA substrate, with the label distal to the Chi site, permits investigation of the nuclease activities of RecBCD enzyme after it has encountered Chi (χ1F in Fig. 4C). The oligonucleotide cap is essential to prevent RecBCD enzyme molecules approaching from the opposite direction and destroying the 3′-end of the Lower strand. Cleavage of the Upper strand at χ1F would produce, in the absence of other cuts, a 3800-nt fragment. Such fragments were the most prominent product observed, under Low Mg2+ conditions, with the 32P label in either strand (Panel C, lanes 4–6 and 11–13). Fragments of this size were much less prominent under High Mg2+ conditions (Panel C, lanes 2, 3, 9, and 10) and may be no more frequent than a contaminant band present in the substrate (lanes 1 and 8). The most prominent reaction product with χ1F DNA, under High Mg2+ conditions, was 1500 nt long, resulting from RecBCD enzyme cutting both at χ1F and at the tip of the DNA. The occurrence of this 1500-nt fragment, and the absence of a 2300-nt fragment with Upper strand labeled DNA, confirms that the nuclease activity of RecBCD enzyme on 3′-terminated strands is attenuated at Chi (13). The yield of this fragment was, however, much lower than that of equivalent 3800-nt fragment produced under Low Mg2+ conditions, showing that the attenuation under High Mg2+ conditions is only partial (Panel C, lanes 9 and 10 versus lanes 11–13). Observation of the 1500-nt fragment with Lower strand labeled DNA shows that RecBCD enzyme neither gains a nuclease activity nor loses its hairpin cutting activity upon nicking the Lower strand at Chi.

In summary, the experiments in Fig. 4 demonstrate that nicking of the Lower strand at Chi, under High Mg2+ conditions, is not a result of collisions between RecBCD enzyme molecules coming from opposite directions, or from enzyme molecules encountering Chi from the “opposite” direction. These results, when taken with the results from Fig. 2, imply that a single RecBCD enzyme, encountering a Chi in the “active” orientation, is sufficient to catalyze nicking of the Lower strand of the DNA at Chi. The scission at Chi on the Lower strand is indeed a nick (or two or more closely spaced nicks), as shown by the recovery of fragments to both sides of it, while that on the Upper strand results from degradation of the 3′-terminated strand up to Chi.

Position of Upper Strand Cuts at Chi—Analysis of RecBCD enzyme reaction products on "sequencing" gels allows determination of the position of the cut sites at Chi, using restriction enzyme digestion and chemical degradation products of the same substrate DNAs as size markers. As seen in Fig. 5 (lanes 23 and 24), RecBCD enzyme cleavage of the Upper strand at χ1F under Low Mg2+ (5 mM ATP, 3 mM Mg2+) conditions was at the same two nucleotides as previously reported (9) for somewhat different reaction conditions (5 mM ATP, 1 mM Mg2+, 100 mM NaCl). The SSB, present here but not in the previously published results (9), did not influence the positions of the cuts. Reaction under High Mg2+ conditions, however, caused a marked change in the distribution of Chi-specific Upper strand cuts (Fig. 5, lanes 1 and 2). The cuts were less pronounced, distributed over more nucleotides, and closer to (or within) the Chi octanucleotide.

The High Mg2+ and Low Mg2+ reactions were carried out with different buffers and at different pH values, and with different ATP concentrations, but the salient difference was the ATP to Mg2+ ratio. As the ATP concentration in a High Mg2+ reaction was increased (lanes 1 through 12 versus lane 23), the Chi-dependent cut sites moved toward the positions seen in the Low Mg2+ reactions. Similarly, when the ATP concentration in a Low Mg2+ reaction was decreased, the reaction products changed to resemble those of a High Mg2+ reaction (lanes 16–24 versus lane 2).
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A qualitative summary of the cut sites under High and Low Mg$^{2+}$ conditions is shown on the right of Fig. 5 and is later compared to Lower strand cuts.

Position of Lower Strand Cuts at Chi—Investigation of Lower strand cuts (Fig. 6) revealed a somewhat different picture. With 10 mM ATP, 8 mM Mg$^{2+}$ (lanes 2 and 3), Chi-dependent cuts were barely detectable, but under High Mg$^{2+}$ conditions (1 mM ATP, 8 mM Mg$^{2+}$) Chi-dependent cuts at least 9 positions were prominent. Outside that region, the patterns of cuts were indistinguishable in $\chi^o$ and $\chi$F substrates. Most of the High Mg$^{2+}$ reactions shown used heparin to prevent multiple rounds of RecBCD enzyme reaction. The enzyme titration (lanes 11–25) revealed that Chi-enhanced cuts were seen at low enzyme doses and reached a maximum at about one RecBCD enzyme per DNA, as shown by PhosphorImager analysis. Lower strand cuts were thus the result of a single round of reaction with one RecBCD enzyme molecule.

The results of PhosphorImager analysis of the $\chi^o$ and $\chi$F reaction products at the highest RecBCD enzyme concentration are shown in Fig. 6C. Maximal Chi-enhanced cleavage (~3-fold) was after the C (in the center of the Chi sequence) at nt 1497, with enhancement disappearing within 9 nt to either side. These results are shown schematically in Fig. 6 and are compared, in Fig. 7, to the positions of Upper strand cuts. Similar quantitation showed a 15-fold enhancement by Chi of the major band on the Upper strand under Low Mg$^{2+}$ conditions and a 4.5-fold enhancement of the major band produced on the Upper strand under High Mg$^{2+}$ reaction conditions.

DISCUSSION

Summary of Observations—Under Low Mg$^{2+}$ conditions ([ATP] > [Mg$^{2+}$]) a single RecBCD enzyme molecule unwinds DNA up to Chi, nicks the Upper (5'-GCTGGTGG-3' containing) strand a few nucleotides to the 3'-side of this sequence, and continues unwinding; the Lower strand is not detectably nicked (Figs. 4-7) (7, 9). This reaction produces three ss DNA fragments (Fig. 1, left). Under High Mg$^{2+}$ conditions ([Mg$^{2+}$] > [ATP]) RecBCD enzyme degrades the Upper strand from its 3'-terminus to Chi, nicks the Lower strand within or near either side of the Chi sequence 3'-CGACCACC-5', and continues unwinding (Figs. 2 and 4-7) (13, 24). This reaction produces three ss DNA fragments and oligonucleotides (Fig. 1, right). Upon cutting at Chi the enzyme loses its Chi-nicking activity (Low Mg$^{2+}$ conditions) (15) or its 3' to 5' degrading activity (High Mg$^{2+}$ conditions) (13). The yields of Chi-cut products are considerably greater under Low Mg$^{2+}$ conditions than under High Mg$^{2+}$ conditions (Figs. 4-6); SSB is required for detection of Chi-cut products under High Mg$^{2+}$ conditions but not under Low Mg$^{2+}$ conditions (Figs. 4-6) (7, 9, 23) (data not shown).

ATP and Mg$^{2+}$ Levels in E. coli—Knowledge of the levels of unbound ATP and Mg$^{2+}$ within E. coli would enable us to decide which of the reactions reported here is more pertinent to the recombination reactions catalyzed by RecBCD enzyme within the cell. The total ATP concentration in E. coli is reported to be 1.6 mM (27, 28) or 2.7 mM (29) and varies little with growth rate (27) but can vary from 0.6 mM to 2.2 mM depending on growth conditions (28). While the total Mg$^{2+}$ ion concentration in E. coli is about 100 mM (30), only between 1 and 2 mM is estimated to be "free in solution in the cellular sap" (31). This estimate of free Mg$^{2+}$ was somewhat indirect, and it is unclear whether it would have included Mg$^{2+}$ ions bound to ATP. A more direct measurement using fluorescent indicators estimates the free intracellular concentration of Mg$^{2+}$ in mouse 3T3 fibroblasts to be less than 1 mM (32) but has not, to our knowledge, been reported for E. coli. 31P NMR (33) can distinguish free ATP from Mg-bound ATP; it was used with anaerobic E. coli but was unable to separate the ATP signals from those of other nucleoside triphosphates (34). The authors observed that all detectable NTP was bound to Mg$^{2+}$ and suggested that free Mg$^{2+}$ was 1 mM. Values obtained in eu-
karyotes, in which the ATP signal can be separated from that of other nucleoside triphosphates, are also typically less than 1 mM free Mg\(^{2+}\) (33). The free ATP and Mg\(^{2+}\) concentrations in E. coli thus appear to be approximately equal, and we cannot decide on this basis which condition studied here more nearly reflects that in E. coli.

Protection of Linear DNA by Chi in E. coli—Purified RecBCD enzyme is differentially inactivated when it encounters a Chi site (13, 15). Such inactivation persists for greater than 20 min under Low Mg\(^{2+}\) conditions (15, 35) but is rapidly reversed if the reactions are switched to High Mg\(^{2+}\) conditions (35). The fate of intracellular linear DNA molecules bearing Chi sites, coupled with these observations, may reveal the conditions inside the cell.

RecBCD enzyme rapidly destroys DNA molecules with ds DNA ends generated within E. coli by the intracellular action of a type I restriction enzyme (36), by rolling circle plasmid replication (37, 38) or by the intracellular induction of phage λ's terminase (39). Such degradation is reduced by Chi on the linear DNA molecules (37–39).
tein (37–39) and SSB (39) suggests that RecBCD-mediated homologous recombination, rather than simply the inactivation of the exonuclease activity of RecBCD enzyme by Chi or the titration of the enzyme by the large number of dsDNA ends in the cell, is responsible for the apparent loss of exonuclease activity.

Two groups have investigated the trans effect of a plasmid bearing a Chi site on the survival of a compatible Chi-free plasmid in the same cell. Zaman and Boles (38) did not find extensive protection of a Chi° plasmid by a compatible Chi+ plasmid in the same cell. Kuzminov et al. (39), however, found that intracellular linearization of a plasmid bearing Chi largely prevented the degradation of an unrelated linearized Chi+ plasmid in the same cell. If, in contrast to the conclusion in the preceding paragraph, this protection reflects inactivation of RecBCD enzyme, such inactivation must be long lived. Since, however, the Chi-mediated inactivation of purified RecBCD enzyme is rapidly reversed by High Mg++ conditions (35), the conditions within the cell may more nearly resemble the Low Mg++ conditions described here.

Genetic Consequences of Upper versus Lower Strand Cuts at Chi—In Fig. 8 we explore the consequences, for a Chi initiation model of recombination (10), of the two modes of RecBCD enzyme action at Chi. We have not yet determined whether a single RecBCD enzyme can effect scissions on both strands, and so we consider several possible actions of RecBCD enzyme at Chi.

First, in Fig. 8A we diagram the essence of the previously proposed model, under conditions in which the Upper, but not the Lower, strand is nicked. RecBCD enzyme enters the right end of the black parental dsDNA, and travels along the DNA, unwinding and rewinding it (5). This rewinding occurs more frequently with long (>10 kilobase pairs) DNA used for electron microscopy (5), and likely with the DNA substrates typical in E. coli recombination, than with the short (<5 kilobase pairs) DNA used here and in other studies with Chi and purified RecBCD enzyme (7, 9, 12–15, 23, 24, 35). When the enzyme encounters Chi, it nicks the Upper strand of the DNA, and continues to unwind the DNA, resulting in extrusion of a 3'-ended single strand. SSB and RecA protein then promote in

Third, if the Lower strand, but not the Upper, strand is nicked at Chi, then recombination could occur as shown in Fig. 8A, except that the invading single strand extending to the left of Chi will have a 5'-end, rather than a 3'-end. Experiments with purified RecA protein and substrates similar to those in Fig. 8A suggest that the 3'-terminus is preferred in such reactions (41). Cleavage of the Lower strand produces a 3'-ended fragment extending to the right of Chi (Fig. 8C), which would lead to Chi stimulation of recombination to the right of Chi; this has not been observed (e.g. see Refs. 42–44). These observations question whether Lower strand cutting and, hence, High Mg++ conditions do indeed occur in E. coli.

Fourth, Chi-mediated cuts on both strands (Fig. 8C) preclude the recovery of reciprocal recombinants, for markers bracketing Chi, from just the two DNA duplexes in the diagram. If the Upper strand is degraded up to Chi (or merely nicked at Chi) and the Lower strand is nicked at Chi, then a Holliday junction can still be formed (Fig. 8C). However, as the joint molecule is missing part of one of the parental DNAs (black DNA to the right of Chi), reciprocal recombinants cannot be formed but could be recovered if the incomplete arm of the Holliday junction recombines with a third dsDNA (45). The partially unwound DNA structure in the upper panel of Fig. 8C, produced by continued unwinding by RecBCD enzyme after cutting both strands at Chi, is equivalent to the "split end" structure, hypothesized to be a recombination intermediate (46).

In summary, the models in Fig. 8, A and B, are essentially equivalent to that previously proposed (10), which can account for conjugal and transductional recombination in E. coli (47) and the stimulation at and to the left of Chi (e.g. see Refs. 42–44). The model in Fig. 8C does not allow reciprocal recombinants to emerge from a single interaction between only two parental DNA molecules but reciprocal recombinants could arise when three or more DNA molecules are involved, as in
phage λ crosses (45). This model could also account for the integration of ds DNA fragments into the E. coli chromosome during conjugation or transduction. The model in Fig. 8C, however, predicts stimulation to the right of Chi, which has not been detected (e.g. see Refs. 42–44).

Following the fate of DNA molecules during recombination in E. coli, for example by Southern blot hybridization of DNA extracted from cells, may reveal which mode of RecBCD enzyme reaction on Chi-containing DNA, as reported here, will aid the design and interpretation of such experiments.

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REFERENCES
1. Taylor, A. F. (1988) in Genetic Recombination (Kucherlapati, R., and Smith, G. R., eds), pp. 231–263, American Society for Microbiology, Washington, DC
2. MacKay, V., and Linn, S. (1976) J. Biol. Chem. 251, 3716–3719
3. Eichler, D. C., and Lehman, I. R. (1977) J. Biol. Chem. 252, 499–503
4. Rosamond, J., Telander, K. M., and Linn, S. (1979) J. Biol. Chem. 254, 8646–8652
5. Taylor, A., and Smith, G. R. (1980) Cell 22, 447–457
6. Muskavitch, K. M. T., and Linn, S. (1982) J. Biol. Chem. 257, 2641–2648
7. Ponticelli, A. S., Schultz, D. W., Taylor, A. F., and Smith, G. R. (1985) Cell 41, 145–151
8. Smith, G. R., and Stahl, F. W. (1985) BioEssays 2, 244–249
9. Taylor, A. F., Schultz, D. W., Ponticelli, A. S., and Smith, G. R. (1985) Cell 41, 153–163
10. Smith, G. R., Schultz, D. W., Taylor, A. F., and Triman, K. (1981) Stadler Genet. Symp. 13, 25–37
11. Roman, L. J., Dixon, D. A., and Kowalczykowski, S. C. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3367–3371
12. Dixon, D. A., and Kowalczykowski, S. C. (1991) Cell 66, 361–371
13. Dixon, D. A., and Kowalczykowski, S. C. (1993) Cell 73, 87–96
14. Taylor, A. F., and Smith, G. R. (1990) J. Mol. Biol. 211, 117–134
15. Taylor, A. F., and Smith, G. R. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5226–5230
16. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. Watson, N. (1988) Gene (Amst.) 70, 399–403
18. Smith, G. R., Kunes, S. M., Schultz, D. W., Taylor, A., and Triman, K. L. (1981) Cell 24, 429–436
19. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1989) Current Protocols in Molecular Biology, Vol. 1 and 2. Wiley-Interscience, New York
20. Taylor, A. F., and Smith, G. R. (1985) J. Mol. Biol. 185, 431–443
21. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
22. Eggleston, A. K., and Kowalczykowski, S. C. (1993) J. Mol. Biol. 231, 605–620
23. Cheng, K. C., and Smith, G. R. (1987) J. Mol. Biol. 194, 747–750
24. Dixon, D. A., and Kowalczykowski, S. C. (1995) J. Biol. Chem. 270, 16360–16370
25. Korany, F., and Jülin, D. A. (1992) J. Biol. Chem. 267, 3088–3095
26. Kdawashi, I., Murielado, H., Crasemann, J. M., Stahl, M. M., and Stahl, F. W. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 5981–5985
27. Kahan, A., and Orell, R. (1990) Microbiol. 62, 83–92
28. Lowry, O. H., Carter, J., Ward, J. B., and Glaser, L. (1971) J. Biol. Chem. 246, 6511–6521
29. Mathews, C. K. (1972) J. Biol. Chem. 247, 7430–7438
30. Moncany, M. L., and Kellenberger, E. (1981) Experientia 37, 846–847
31. Aalto, K., Jütte, H., Kuhn, A., and Kellenberger, E. (1986) J. Bacteriol. 162, 413–419
32. Morel, B., Salmon, J.-M., Vigo, J., and Viallet, P. (1994) Anal. Biochem. 218, 170–176
33. Gupta, R. K., Gupta, P., Yushok, W. D., and Rose, Z. B. (1983) Biochem. Biophys. Res. Commun. 117, 210–216
34. Ugur, K., Rottenberg, H., Glyn, P., and Shulman, R. A. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 2244–2248
35. Dixon, D. A., Churchill, J. J., and Kowalczykowski, S. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2980–2984
36. Simon, V. F., and Lederberg, S. (1972) J. Bacteriol. 112, 161–169
37. Dabert, P., Ehrlich, S. D., and Gruss, A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 12073–12077
38. Zaman, M. T., and Boles, T. C. (1994) J. Bacteriol. 176, 5093–5100
39. Kuzminov, A., Schattbach, E., and Stahl, F. W. (1994) EMBO J. 13, 2764–2776
40. Taylor, A. F. (1992) Cell 69, 1063–1065
41. Konforti, B. B., and Davis, R. W. (1990) EMBO J. 9, 1327–1334
42. Stahl, F. W., Stahl, M. M., Malone, R. E., and Crasemann, J. M. (1980) Genetics 84, 235–248
43. Cheng, K. C., and Smith, G. R. (1989) Genetics 123, 5–17
44. Holt, S. D., and Smith, G. R. (1992) Genetics 132, 879–891
45. Stahl, F. W., Thomason, L. C., Siddiqi, I., and Sambrook, J. (1990) Genetics 126, 519–533
46. Rosenberg, S. M., and Hastings, P. J. (1991) Biochimie (Paris) 73, 385–397
47. Smith, G. R. (1991) Cell 64, 19–27