AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology and Molecular Biology Reviews

Genetics and Molecular Biology | Review

RecBCD enzyme: mechanistic insights from mutants of a complex helicase-nuclease

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SUMMARY RecBCD enzyme is a multi-functional protein that initiates the major pathway of homologous genetic recombination and DNA double-strand break repair in *Escherichia coli*. It is also required for high cell viability and aids proper DNA replication. This 330-kDa, three-subunit enzyme is one of the fastest, most processive helicases known and contains a potent nuclease controlled by Chi sites, hotspots of recombination, in DNA. RecBCD undergoes major changes in activity and conformation when,

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The authors declare no conflict of interest.

See the funding table on p. 27.

Published 4 December 2023

during DNA unwinding, it encounters Chi (5′-GCTGGTGG-3′) and nicks DNA nearby. Here, we discuss the multitude of mutations in each subunit that affect one or another activity of RecBCD and its control by Chi. These mutants have given deep insights into how the multiple activities of this complex enzyme are coordinated and how it acts in living cells. Similar studies could help reveal how other complex enzymes are controlled by inter-subunit interactions and conformational changes.

KEYWORDS RecBCD enzyme, *E. coli*, DNA helicase, DNA nuclease, mutants, genetic recombination, Chi hotspots of recombination, DNA replication, crystal and cryoEM structures, enzyme conformational changes, recombination models

INTRODUCTION: IMPORTANCE OF DNA BREAK REPAIR, REPLICATION, AND GENETIC RECOMBINATION

Determining how basic biological processes occur, from enzyme catalysis to growth of organisms, has been greatly aided by the combination of genetics and biochemistry. Fusion of these approaches into molecular biology occurred roughly 75 years ago, and the molecular biology of microbes has been at the forefront since that time. A case in point is genetic recombination, which was reported in the fruit fly *Drosophila* as early as 1913 (1), but its mechanism remained unknown for decades. Understanding the molecular mechanism of recombination has come mostly through studies of microbes—bacteria and fungi. The discovery of recombination in *Escherichia coli* and its phages 75 years ago (2–4) soon led to both genetic and biochemical analyses of recombination [see reference (5) and below for further discussion of this early history]. In this review, we describe how combining these approaches has led to ever-deeper understanding of a key enzyme, RecBCD, necessary for the major pathway of *E. coli* recombination and, as now recognized, repair of DNA double-strand breaks (DSBs) and proper DNA replication.

Repair of broken DNA is essential for life (6–9). DNA DSBs can occur during ever-present DNA replication and transcription or transient exposure to chemicals and radiation. Faithful repair of broken DNA requires regulation of multiple enzymes, including DNA helicases and nucleases [reviewed in references (10–12)]. Their activities must produce functional intermediates that interact with homologous DNA to form joint molecules subsequently resolved into intact, repaired DNA. Uncontrolled action by nucleases or helicases could render the DNA unrepairable, and cell death would follow. A well-studied example of a highly controlled enzyme is the large, multi-functional RecBCD helicase-nuclease of *E. coli*, responsible for the initial events in DSB repair and recombination (13–17). Its multiple activities must be properly coordinated, as discussed below, to produce intermediates for these crucial events.

Genetic recombination, often initiated by DSBs, is important for long-term evolution [see, e.g., references (18–21)]. Recombination can generate new combinations of gene alleles on which natural selection can act to propel evolution. In bacteria, DSBs are thought to arise most frequently during replication, once every few cell cycles, as noted below. The enzymatic reactions of DSB repair and homologous recombination are, however, closely related [reviewed in references (10, 11, 22)]. Homologous recombination can occur following introduction of DNA into an *E. coli* cell in any of several ways. In each case, linear DNA from one cell (the donor) is introduced into the recipient cell; all or part of the donor DNA can be substituted for the homologous region of the recipient chromosome to generate a recombinant bearing markers from both parents. In conjugation, a donor cell [designated *h*igh-*f*requency *r*ecombination (Hfr)] injects part of its chromosome as single-stranded DNA (ssDNA) into a recipient cell, where complementary strand synthesis forms double-stranded DNA (dsDNA) for recombination. In transduction, typically done with phage P1 in *E. coli*, donor cell dsDNA is packaged into a P1 virion and injected into a recipient cell. In transformation, linear dsDNA isolated from the donor cell enters the recipient cell, previously treated to make it amenable to DNA uptake. In phage infection, phages with different genotypes can infect the same cell and produce recombinant phage. The ease of quantifying recombination in each of these

situations makes its assay simpler or more direct than other assays such as cell viability after replication problems or introduction of DSBs by chemicals or radiation. Below, we focus on recombination assays for determining the activity of mutants relative to wild type (wt) (see Supplemental Information for descriptions of genetic and enzymatic assays for RecBCD and Chi).

RecBCD does, however, have an important role in maintaining viability of *E. coli* cells. Recovery of viability after DNA damage caused by exposure to UV- or X-irradiation or certain chemicals requires repair by recombination-promoting enzymes. Indeed, *recB* and *recC* mutants were first isolated as being sensitive to UV-irradiation (8). They were later found to be recombination deficient (8, 23), indicating the close relation of DNA repair and recombination. A key study showed that in *recA* mutants, lacking the RecA DNA strand-exchange protein essential for recombination, the *E. coli* chromosome became acid soluble after UV-irradiation, but solubilization was much slower in *recB*, *recC*, and *recA recB* (or *recC*) double mutants; *recA* mutants were thus classified as "reckless" and *recB* and *recC* mutants as "cautious" (24). This behavior was soon understood when the *recB* and *recC* mutants were found to lack a potent ATP-dependent nuclease designated RecBC or exonuclease V (25), now called RecBCD enzyme (26).

Even in the absence of overt DNA damage, *recB* and *recC* null mutants grow slowly, and their cultures contain many inviable cells—those unable to form a visible colony (Table S1) (27). Pedigree analysis shows that frequently a mutant cell divides once, twice, or thrice (or more), but all of the progeny cells at the first, second, third, etc., generations cease dividing (28). This feature, called lethal sectoring, is exhibited by *recB* and *recC* null mutants, as well as *recA* and *uvrA* mutants; to our knowledge, *recD* mutants have not been tested. Other studies show DNA replication problems in *recBCD* mutants. For example, a ~50% deficiency of DNA is observed in the ~0.5-Mb region around the terminus of replication in *recBC* null mutants and a ~10% increase in a *recD* null mutant (17). When blocked, replication forks can reverse direction, forming a structure with four dsDNA arms (a Holliday junction); one arm has an end potentially open to digestion by RecBCD. Replication across a nick can lead to a one-ended DSB (fork collapse); via recombination, RecBCD may rejoin this end to the intact dsDNA to reform a functional replication fork. Replication is aided by the Rep helicase; in its absence, viability depends on RecB and RecC, but not on RecA or RecD [except in a *rep recA recD* triple mutant, which is inviable (29)]. This outcome suggests that either RecBCD- or RecBC-promoted recombination (in $recA^+$ strains) or degradation (in $recA\ recBCD^+$ strains) is essential for viability in *rep* mutants. These replication problems and RecBCD's role in resolving them have been extensively reviewed (30–33). Here, we focus on RecBCD's role in recombination, for which sensitive, easily quantifiable assays, both in cells and with purified components, allow mechanistic interpretations of RecBCD's multiple enzymatic activities. RecBCD may play the same or similar roles in maintaining viability after spontaneous or radiation-induced DNA damage or replication errors that lead to DSBs. The dozens of non-null mutants discussed here may be useful in further explorations of these roles for RecBCD.

OVERVIEW OF RecBCD ENZYME'S STRUCTURE AND ACTIVITIES

To understand the multiple controls of RecBCD enzyme, including that by Chi sites in DNA, we first discuss the structure of RecBCD enzyme and its multiple activities. Critical to this understanding was the determination of the crystal and cryoEM structures of RecBCD bound to DNA (Fig. 1) (34, 35). In accord with studies of the purified enzyme (26, 36), RecBCD in these structures is a heterotrimer, containing one copy of each subunit—RecB (134 kDa), RecC (129 kDa), and RecD (67 kDa)—bound to a dsDNA end. The free 3′-end of the blunt-ended DNA in the first crystal structure (PDB 1W36) is bound to RecB, and the 5′-end to RecC headed toward RecD; in solution, the 3′-end readily UV-crosslinks to RecB and the 5′-end to both RecC and RecD (37).

Upon addition of ATP as an energy source, RecB moves along the 3′-ended strand, and RecD moves along the 5′-ended strand (38). However, RecD moves about twice as

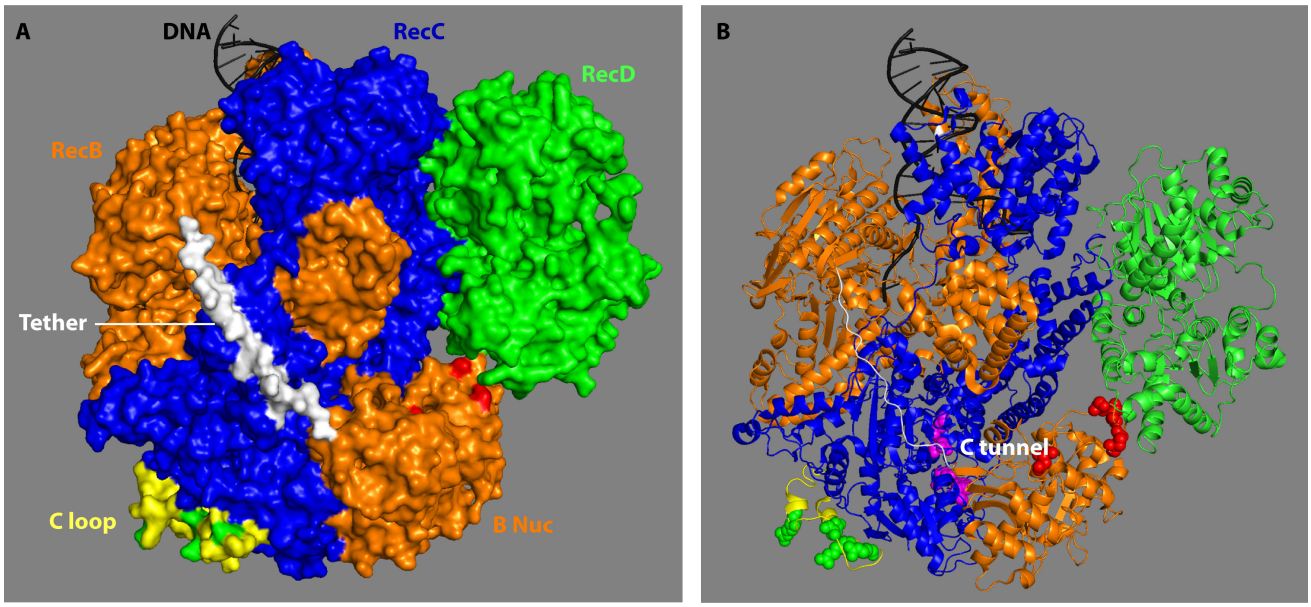

FIG 1 Crystal structure of RecBCD showing parts critical for Chi's control of RecBCD. RecBCD bound to blunt-ended dsDNA (black) was determined by X-ray crystallography (PDB 1W36) (34); surface (A) and cartoon (B) views are shown. Except for certain parts emphasized in this review, RecB is orange; RecC is blue; and RecD is green. The RecC surface loop is yellow, except for four amino acids (green spheres) in RecC postulated to dock with four amino acids (red spheres) in the RecB nuclease domain. The 19-amino-acid RecB tether connecting the helicase and nuclease domains is white in the surface view (A). The RecC tunnel amino acids required for Chi hotspot activity are magenta spheres in the cartoon view (B).

fast as RecB, so that an ssDNA loop arises, expected to be ahead of RecB, and grows with time of incubation; two ssDNA tails also arise and grow with time (39). These "loop-tail" structures (Fig. 2A) can be converted into "twin-loop" structures (Fig. 2B) by the annealing of the 3′-tail to its complement on the 5′-tail; the remaining part of the 5′-tail forms a second ssDNA loop. RecBCD unwinds DNA as rapidly as 1 kb/s (39, 40), making it one of the fastest DNA helicases known. A single RecBCD can move at least 100 kb along DNA without dissociation, making it one of the most processive helicases known (39) (A.F. Taylor, personal communication).

As RecBCD unwinds DNA, it can also cut the DNA using the RecB nuclease domain, which is positioned in the crystal and cryoEM structures near the exit of a tunnel in

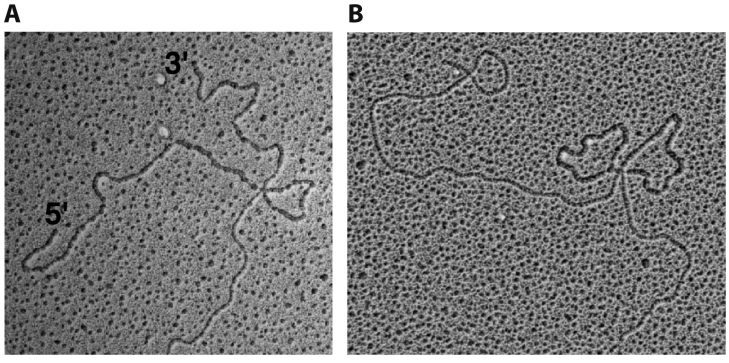

FIG 2 DNA unwinding structures formed by RecBCD. DNA briefly reacted with RecBCD was examined by transmission electron microscopy after heavy-metal staining and shadowing (39). ssDNA, bound by single-strand binding protein, appears thick, and dsDNA appears thinner. (A) Loop-tail structure showing longer 5′-ssDNA tails than 3′-ssDNA tails, the consequence of RecD helicase moving faster than RecB translocase (38, 39). (B) Twin-loop structure, the result of annealing of the 3′-ssDNA tail to its complement on the 5′-ssDNA tail but remaining unannealed over the region of the loop on the 5′-ended strand, due to topological constraints. Courtesy of Andrew Taylor.

RecC from which the 3′-ended DNA strand likely emerges during unwinding (Fig. 1) (34, 35, 41). This arrangement would predict that the nuclease cuts the 3′-ended strand. However, the nuclease domain is attached to the RecB helicase domain by a 19-amino-acid tether (Fig. 1A), which likely allows the nuclease domain to move and cut the 5′-ended strand as well. Depending on the reaction conditions (e.g., the ATP:Mg^{2+} ratio), either strand can be cut, as discussed below. The essential part of the Chi recombination hotspot is 5′-GCTGGTGG-3′, which must be on the strand with the 3′-end at which RecBCD initially bound the DNA for Chi to modify RecBCD's activities (42–46). Several amino acids lining part of a tunnel in RecC are essential for Chi activity (see below). Current information indicates that, when Chi is at that point in the RecC tunnel, the RecB nuclease nicks the 3′-ended strand about five nucleotides to the 3′-side of the Chi octamer, consistent with the distance in the cryoEM structure between the Chi octamer and the nuclease active site (the Mg^{2+}-binding site) in RecB (35, 41, 45) (Fig. 1) (see further discussion below). RecBCD also cuts DNA without Chi, which raises the critical question of how RecBCD is controlled by Chi. After Chi, RecBCD loads RecA onto the newly generated 3′-ending ssDNA (47). Below, we describe multiple classes of *recBCD* mutations that allow deep insight into the numerous RecBCD reactions at the heart of the control of homologous recombination and DNA break repair (information on mutants representative of the classes discussed here is in Table 1; multiple mutants of each class are in the supplemental tables).

A BRIEF HISTORY OF RecBCD ENZYME

The first ATP-dependent nuclease, as RecBCD is, was discovered in 1964 in extracts of *Micrococcus lysodeikticus* (66). It was odd that a nuclease, which should release energy upon hydrolyzing nucleic acid, required an energy source (ATP) for activity. The solution to this puzzle came about 15 years later, when it was recognized that the related *E. coli* enzyme (RecBCD) is also a helicase, which requires energy to unwind double-stranded nucleic acid (DNA in this case) (39, 67, 68). In 1965, the first *recB* and *recC* mutants were discovered as UV-sensitive mutants of *E. coli* (6, 8). These mutants were shown in 1968 to lack an ATP-dependent nuclease (69). Detailed biochemical characterization of *E. coli* RecBCD came with its purification in several labs [e.g., see references (25, 70–72)], which showed that the enzyme has multiple activities—solubilization of either dsDNA or ssDNA, each strictly dependent on a ribo- or deoxyribonucleoside triphosphate (NTP) (Fig. 3). Curiously, nuclease activity, even on ssDNA, required NTPs, and NTP hydrolysis required DNA, indicating close cooperation of these two activities. Whereas nucleases generally produce mononucleotides (dinucleotides by a few), RecBCD initially produces DNA hundreds or thousands of nucleotides long; eventually, it produces a mixture of oligonucleotides mostly three to six nucleotides long (the limit digest) (25, 72). These observations were accounted for by a model in which RecBCD initially produces two classes of intermediates: dsDNA with long ss tails and ssDNA hundreds of nucleotides long (67). RecBCD attacks each type of molecule, producing ssDNA from the tailed dsDNA molecules and shorter ssDNA from the ssDNA. This process is repeated until the DNA is degraded to oligonucleotides (25, 67, 73).

In the mid-1960s, researchers sought *E. coli* mutants defective in recombination or sensitive to DNA damaging agents, such as UV as noted above. These searches yielded mutations in *recA*, *recB*, and *recC* (6, 8). *recA* mutants were more deficient than *recB* or *recC* mutants in both recombination and DNA damage resistance (77). The similarity of *recB* and *recC* mutants and the closeness of their genes were consistent with each lacking one or another polypeptide that co-purified with the ATP-dependent nuclease activity in wt but lacking in these mutants (23, 71, 72). These results showed that RecB and RecC are part of the enzyme designated exonuclease V (Exo V) of *E. coli* (25). A later study, aimed at determining if the nuclease activity was needed for DNA repair and recombination, screened for nuclease-deficient mutants that were still UV-resistant and recombination-proficient (51). Mapping of the mutations and analysis of extracts uncovered a new gene (*recD*) and a polypeptide previously associated with the purified enzyme but present in

TABLE 1 Representatives from several classes of *recBCD* mutants

Class	Mutant description[a]	Genetic assays		Biochemical assays[i]			Reference
		Chi hotspot activity[b]	Hfr recombination (relative)[c]	Chi cutting[d]	Unwinding activity[e]	Nuclease activity (relative)[f]	
WT	+	5.0 (4.9)	1.0	+	+	1.0	(48)
Null	Complete deletion	1.0	0.004	–	–	< 0.02	(49)
Null	*recB* or *recC*	1.0	0.005	–	–	< 0.02	(50)
Null	*recD*	1.0	0.53	–	+	< 0.02	(51)
RecB ATPase	K29Q	1.1	0.006	–	+weak	< 0.02	(49, 52)
RecD ATPase	K177Q	3.3	0.56	+	+	0.5	(53, 54)
RecB tether	Δ881–899	1.1	0.02	–	+	0.17	(48)
RecB tether	Δ893	3.3	0.52	+weak	+	0.25	
RecC tunnel	S39E	1.0 (1.9)	0.06	NT[g]	NT	NT	(55, 56)
RecC tunnel	K88I	1.6 (4.3)	0.17	NT	NT	NT	
RecC loop	Δ252–291	1.9	0.77	+	+	0.42	(57)
RecC-RecD assembly	G905E in RecC	1.0	0.5	–	+	< 0.02	(58)
RecB nuclease active site	D1080A	1.0	0.005	–	+	< 0.02	(59–61)
	D1080A *ΔrecD*	0.9	0.64	–	+	< 0.02	
"Chi activated" in RecB ATPase site	Y803H	0.95	0.004	– (+close to end)	+	0.76	(49)
Signal transduction[h]							
Step 2 (RecC-RecD)	ΔC2 (541–544) ΔD2 (97-99)	1.2	0.65	–	+	0.06	(62)
Step 3 (RecD-RecB)	D3Ala (523–526) B3Ala (634, 635, 639, 643, 644, 646)	1.5	0.43	–	+	0.15	
Step 4 (RecB-RecC)	ΔB4 (913–922) ΔC4 (599–608)	1.2	0.16	–	+	0.06	

[a]The indicated *recBCD* alleles were on derivatives of plasmid pSA607 (*recBCD*$^+$) in *E. coli* strain V2831 (*ΔrecBCD*) except for the *recC* null, which was assayed as a chromosomal allele. The indicated amino acids were altered by substitution or deletion. Complete genotypes and allele numbers are in Tables S1–S9.
[b]Chi hotspot activity in λ vegetative crosses (Fig. S1) was determined as described (63). For Chi context dependence in RecC tunnel mutants, Chi hotspot activity (in parentheses) was determined with *χ*$^+$*L252* (between *J* and *cI* and inactivating *gam*) as clear: turbid plaque (c:t) ratio among J$^+$ R$^+$ recombinants in crosses between *susJ6 red3 χ*$^+$*L252 cI857* and *red3 χ*$^+$*L252 cI*$^+$ *susR5* (55). In *recBCD*$^+$, c:t was 4.9 with *χ*$^+$*L252* and 1.9 with *χ*$^-$*L252*; in *ΔrecBCD*, c:t was 1.3 and 1.1, respectively.
[c]Frequency of His$^+$ [Str^R] recombinants per viable Hfr parent relative to that in the concurrent *recBCD*$^+$ cross where wild-type frequency was about 5% (range 3.8%–7.3%).
[d]Appearance of a DNA fragment cut near Chi with cell extracts or purified enzyme (45, 64).
[e]Appearance of ssDNA from end-labeled dsDNA after reaction with cell extracts or purified enzyme (45, 64).
[f]Nuclease activity in extracts of cells with the *recBCD* genes on a pBR322-derived plasmid relative to that with *recBCD*$^+$ (~500 units/mg of protein, where 1 unit releases 1 nmol of acid-soluble dNMP/min under standard reaction conditions) (65).
[g]NT, not tested.
[h]In parentheses are the amino acids deleted or changed to alanine. Step 1 (Chi-RecC) mutants are RecC tunnel mutants (above).
[i]See Supplemental Information for descriptions of genetic and biochemical assays.

recB and *recC* mutant extracts (26, 51, 78). (Note that before 1986, RecBCD enzyme was called "RecBC," but that notation now means the enzyme without RecD.) Later analysis of purified enzyme from *recD* mutants showed that the remaining enzyme (RecBC) had DNA unwinding activity but no detectable nuclease activity (79). RecBC binds DNA less tightly than RecBCD and thus requires about 10–1,000 times higher DNA concentration, depending on the structure of the DNA end, for unwinding than does RecBCD (53, 79, 80) (A.F. Taylor, unpublished data). This feature and the requirement for single-strand binding protein (SSB) to prevent reannealing behind an enzyme with just one helicase, such as RecBC, likely explain why helicase activity was initially missed in *recD* mutant extracts (26).

The DNA sequences of the *recBCD* genes showed that both RecB and RecD have amino acid sequence homology with other ATP-dependent helicases and that a part of the C-terminal quarter of RecB has similarity to phage λ exonuclease (Fig. 4); these helicase and nuclease assignments were confirmed by site-directed mutagenesis (52, 59, 81–86). A search for pseudorevertants of *recB* and *recC* null mutants that regained DNA damage resistance yielded mutations in *recC*, closely linked to the original *recC* mutation, that restored recombination and nuclease proficiency but not activity of Chi

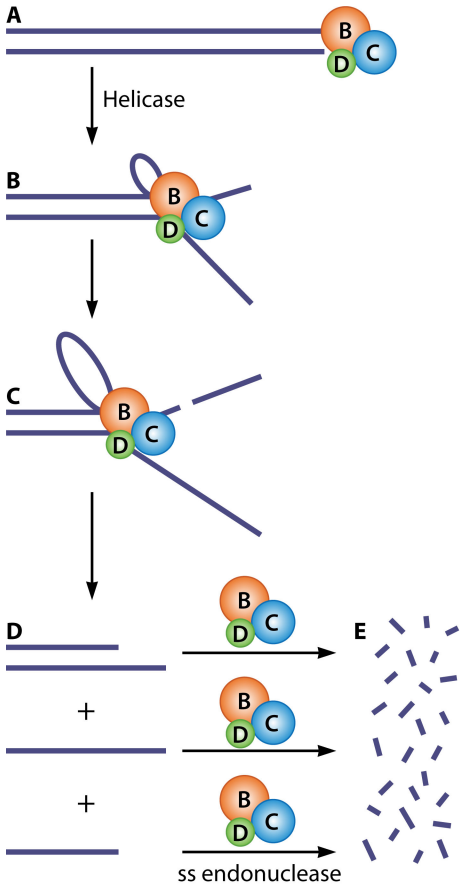

FIG 3 Formation of final DNA digestion products requires multiple passages of RecBCD enzyme(s). RecBCD binds an end of dsDNA (A) and unwinds it, producing a loop-tail structure (B) (Fig. 2A). An occasional endonucleolytic cut (C) and continued unwinding produce a mixture of ssDNA and dsDNA with a long ssDNA tail (D). These intermediates are attacked by other RecBCD molecules. Their endonucleolytic cuts, accompanying their translocation on ssDNA or unwinding of dsDNA, produce shorter DNA fragments, which eventually become the limit digestion products of oligonucleotides, mostly four to six nucleotides long (E). This mode of action raises confusion about RecBCD being an exonuclease or an endonuclease, because there are two definitions of exonuclease and endonuclease. By one definition, an exonuclease requires an end for its action, as RecBCD does (it has weak activity on ssDNA circles). By another definition, an exonuclease cuts the substrate at the terminal (or penultimate) nucleotide and produces mononucleotides or dinucleotides, which RecBCD does not do. For clarity, RecBCD should be considered a DNA end-requiring helicase (or translocase on ssDNA) with ssDNA endonuclease activity dependent on the helicase or translocase activity. The frequent illustration of RecBCD as a "Pac-Man" eating DNA from the end [e.g., see references (74–76)] does not represent the mechanism of RecBCD's complex digestion of DNA.

recombination hotspots (see next paragraph) (87). These mutants implicated RecC in Chi recognition. The *recD* mutants mentioned above lacked both Chi activity and nuclease activity (26, 51). These studies showed that the three RecBCD subunits interact in a complex way to generate helicase, NTP hydrolysis, nuclease, and Chi hotspot activities.

CHI RECOMBINATION HOTSPOTS

An earlier, independent line of research of phage λ recombination led to the discovery that RecBCD enzyme is essential for Chi hotspot activity. λ encodes its own recombination system (Red) comprising an Exo and a DNA strand-annealing protein (Beta) [reviewed in reference (88)]. Adjacent to the two overlapping λ genes *exo* and *bet* is *gam*,

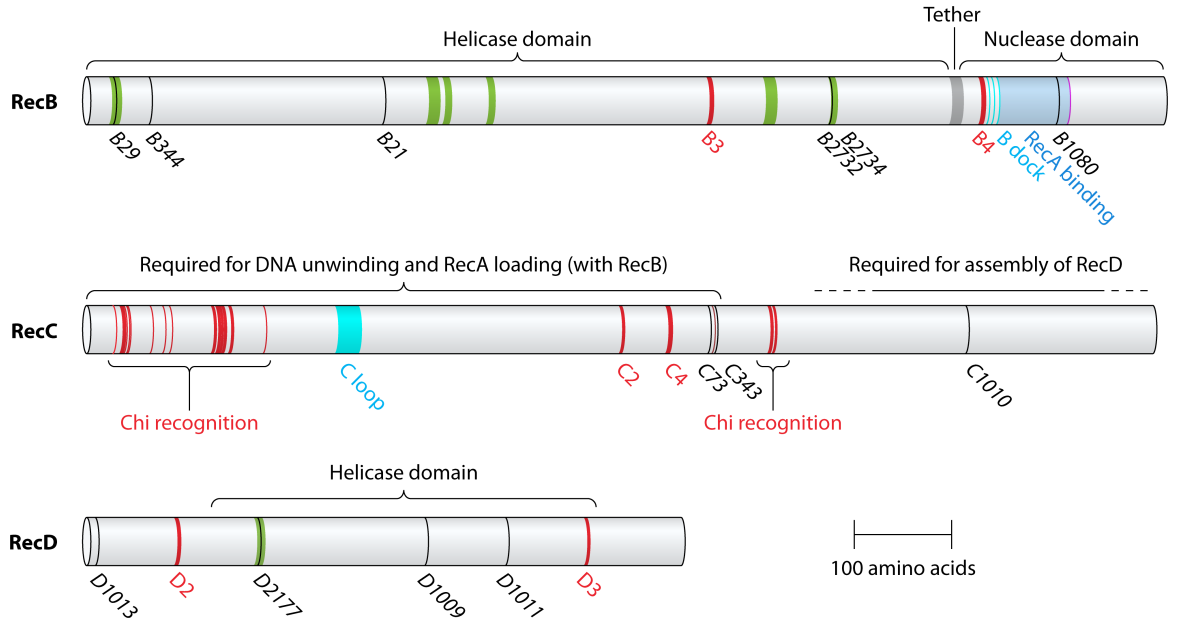

FIG 4 Positions of *recBCD* mutations and proposed signal transduction points within the multiple activity domains of RecBCD. The open horizontal bars, representing the three RecBCD polypeptides, are drawn to scale. The approximate regions for each activity domain are indicated by brackets above or below these bars. *recBCD* mutant alleles are in italic type; see Tables S2–S9 for the corresponding amino acid changes. Inter-subunit contacts for the proposed signal transduction pathway are in roman type and in red shading on the bars, as are the RecC tunnel amino acids required for Chi recognition. The 40-amino acid RecC surface loop (proposed "storage" site for inactive RecB nuc) and RecB amino acids involved in docking there are cyan. The RecA-binding domain on RecB is blue. The helicase domains in RecB (P08394) and RecD (P04993) annotated in UniprotKB are lime green.

which encodes Gamma, a small protein that binds to the DNA-entry site of RecBCD and inhibits it (89–91). Deletions removing these three λ genes result in small phage plaques, the result of RecBCD blocking the switch from circular (theta, θ) phage replication to rolling circle (sigma, σ) replication (92, 93). Large-plaque mutations (designated by the Greek letter *χ*, or Chi) arise at four different sites in λ and stimulate RecBCD-dependent recombination at and near the *χ* mutation analyzed (63, 94, 95). ("Chi" stands for *c*rossover *h*otspot *i*nstigator.) Some *E. coli* DNA fragments carried in λ *red gam* mutants also result in large plaques, the consequence of a Chi site in *E. coli* DNA (96). DNA sequencing of the λ *χ* sites and the *E. coli* Chi sites, both the active and inactive states differing by single-bp changes, showed that 5′-GCTGGTGG-3′ is necessary and sufficient for Chi hotspot activity (43, 97, 98). Chi is active with the RecBCD pathway present in wt *E. coli* but not with the λ Red pathway or the alternative *E. coli* pathways RecE and RecF active in *recBCD* mutants bearing suppressor mutations [reviewed in reference (10)]. Stimulation by Chi decreases roughly exponentially, with a half-distance of ~2 kb (99). Since the mean distance between *E. coli* Chi sites is 4.6 kb (100), these results are consistent with most *E. coli* recombination (Hfr, transduction, and transformation) likely being Chi-stimulated. The one *E. coli* Chi site tested, in *lacZ*, increases recombination between linked markers in P1 transduction by factors of up to 20 (101). Transformation of a chromosomal locus is stimulated ~50-fold by Chi on both ends of the entering linear DNA (102). A Chi site in λ prophage also increases transduction and Hfr-mediated recombination by factors of up to 3 (103). In each case, the Chi effect is RecBCD dependent. These observations support the model in Fig. 5 being generally applicable to homologous recombination in wild-type *E. coli*. This model is further supported by the properties of the many *recBCD* mutants discussed below. These mutants also allow assessment of which of the two models for the RecBCD-Chi interaction discussed below pertains to living cells.

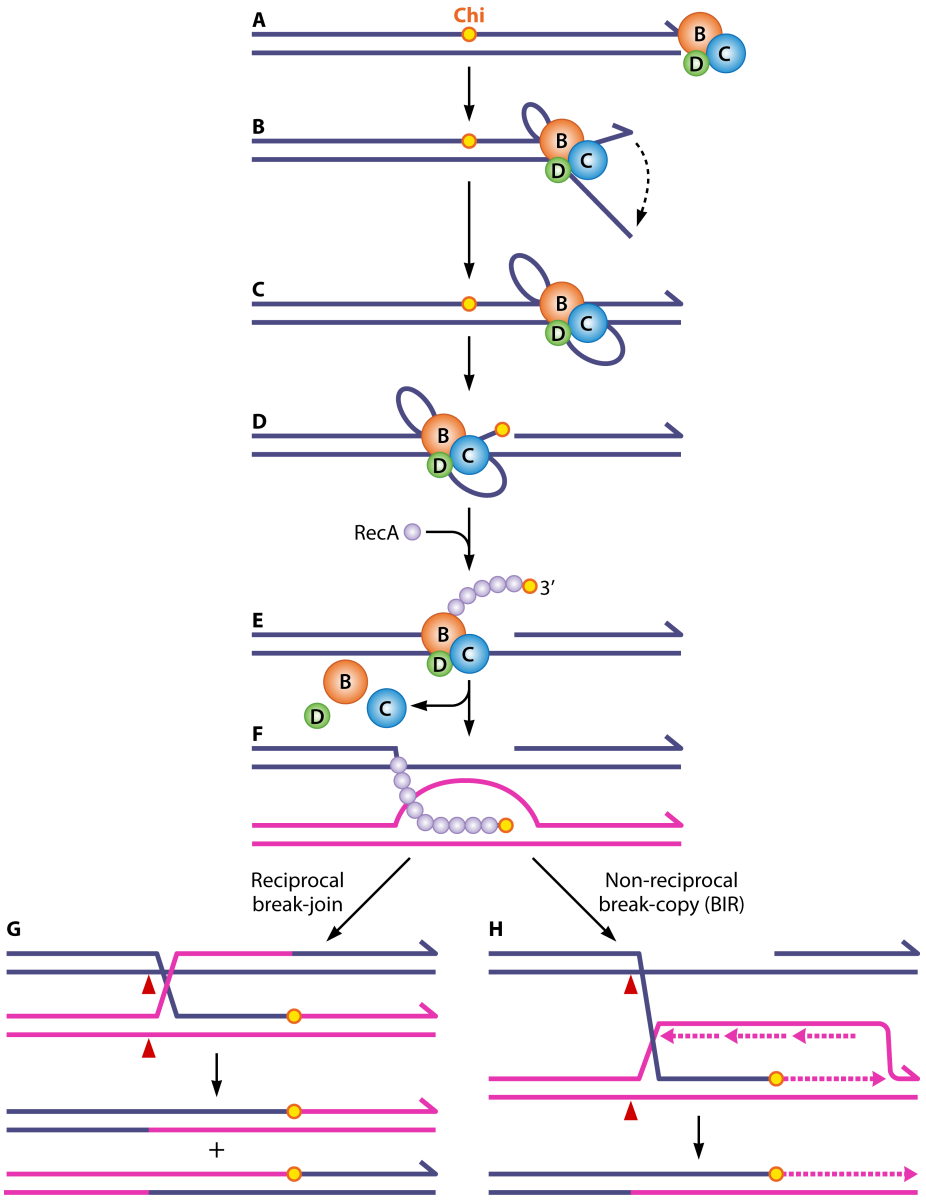

FIG 5 Model for recombination promoted by RecBCD enzyme based on nicking at Chi. (A) RecBCD binds to a dsDNA end. (B) With ATP present, RecD helicase moves on the 5′-ended strand, while the slower RecB translocase moves on the 3′-ended strand, resulting in a long 5′-ssDNA tail, a short 3′-ssDNA tail, and a ssDNA loop on the 3′-ended strand. (C) The ssDNA ends can anneal to produce two ss loops, which continue to grow as RecBCD moves along the DNA. (D) When Chi (5′-GCTGGTGG-3′, orange circle) on the strand with 3′ at the entry point is encountered, RecBCD nicks the 3′-ended strand a few nucleotides to the 3′ side of Chi. (E) RecBCD loads RecA strand-exchange protein onto the newly generated 3′-ended strand; RecBCD continues to unwind DNA, but at some point, its three subunits dissociate into inactive enzyme. (F) The ssDNA-RecA complex engages homologous dsDNA, such as a sister chromatid, and forms a D-loop via DNA strand exchange. The D-loop can be converted into a Holliday junction (G), which can be resolved into reciprocal recombinants, or prime DNA synthesis (H), which can lead to non-reciprocal recombinants. Straight arrows indicate steps in the reaction series, as in chemistry. Curved arrows indicate movement of DNA or proteins during these reactions. Redrawn from references (16, 104).

MODELS FOR RECOMBINATION PROMOTED BY RecBCD ENZYME AND CHI SITES

Figure 5 shows a model of *E. coli* recombination and DSB repair initiated by the RecBCD enzyme. RecBCD binds avidly to DSB ends, whether blunt or with short ss tails (105), and

unwinds the DNA, producing long ss tails (Fig. 2A). During unwinding and after RecBCD nicks one strand at a Chi recombination hotspot, it loads onto the newly generated 3′-end the RecA strand-exchange protein, which couples this DNA to homologous DNA, such as the intact chromosome in replication fork repair, conjugation, transduction, or transformation, or another DNA molecule in phage infection. This joint molecule is then resolved into intact DNA or recombinants. RecBCD is involved in three key steps: DNA unwinding, cutting the DNA at Chi to produce the required invasive ssDNA with a 3′-end, and loading RecA protein onto this ssDNA (47). This pathway was proposed from both

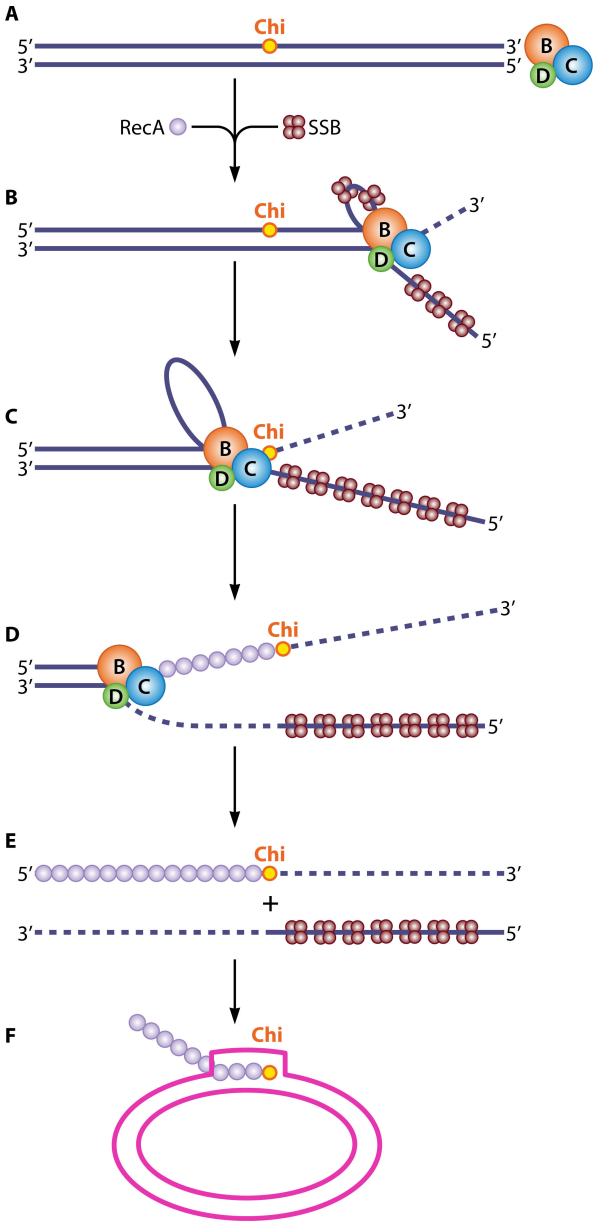

FIG 6 Model for joint DNA molecule formation promoted by RecBCD based on degradation up to Chi. (A and B) RecBCD unwinds DNA, as shown in Fig. 2A, while degrading the top (3′-ended) strand; SSB binds the single-stranded loop and tail. (C) At Chi, degradation switches from the top strand to the bottom strand, and RecBCD begins to load RecA onto the 3′-ended strand with Chi near its end. This RecA-ssDNA filament (D and E) invades intact DNA to form a D-loop (F). Redrawn from Anderson and Kowalczykowski (106).

genetic and biochemical results and was fortified by mutants blocked at, or altered in, one or another step as discussed below.

Several models in addition to that in Fig. 5 have been proposed to account for Chi's regulation of RecBCD enzyme. In a frequently cited model (14, 47) (Fig. 6), RecBCD "degrades" one DNA strand, that with a 3′-end at the RecBCD entry point, up to Chi, then nicks the other strand and continues to "degrade" that strand. "Degrades" is not specified but presumably means endonucleolytic cleavage of ssDNA to produce ssDNA fragments of unspecified length, as shown in drawings of the model. RecBCD loads multiple copies of RecA onto the strand with Chi near its newly generated 3′-end, and the ssDNA-RecA filament invades an intact dsDNA to form a joint molecule. These last two reactions are the same as those in the model in Fig. 5, but to our knowledge, this model has not specified how the DNA joint molecule proceeds to a recombinant. These models are often referred to as the "degradation-up-to-Chi" model (Fig. 6) and the "nick-at-Chi" model (Fig. 5).

Other models include one in which RecBCD enters a Holliday junction, which is made in an unspecified manner, travels along one dsDNA arm until it meets a properly oriented Chi site, reverses direction, and returns to the Holliday junction, which it resolves (42). This model was ruled out by the finding that Chi's orientation dependence is with respect to the cohesive end site (*cos*), at which a dsDNA end is generated during packaging of lambda DNA and at which RecBCD gains entry to promote recombination (44). In the "split-end" model, RecBCD degrades both strands upon entry, but after Chi, it loads RecA protein onto surviving tails of each strand to give a gene conversion (localized, non-reciprocal recombination) by substitution on either strand (107, 108). In yet another model, the RecD subunit is altered, inactivated, or ejected at Chi, to convert RecBCD from a "degrading machine" into a "recombination machine" (109–113). This model was ruled out by the findings that RecBCD retains nuclease activity, which depends on RecD, after acting at Chi (114) and that RecD is not ejected at Chi (40, 115).

A combination of genetic and biochemical data argues for the nick-at-Chi model (Fig. 5) and against the degradation-up-to-Chi model (Fig. 6) (16, 116). Both reactions by purified RecBCD on DNA are observed, but which is observed depends on the reaction conditions:

a. With ATP in excess over Mg^{2+}, RecBCD unwinds DNA, makes a nick a few nucleotides to the 3′ side of 5′-GCTGGTGG-3′, and continues unwinding but without the ability to act at a second Chi site (45, 64, 114). At some point, likely at the end of the DNA with purified components, the three RecBCD subunits disassemble, thereby inactivating the enzyme in a Chi-dependent manner and making RecBCD unable to act at Chi on another DNA molecule (action of Chi in *trans*) (117).

b. With Mg^{2+} in excess over ATP, RecBCD unwinds DNA, makes occasional nicks on the 3′-ended strand, makes the last nick at or near Chi, nicks the opposite strand, and continues unwinding but nicking only the 5′-ended strand; thus, both strands are "degraded" but on opposite sides of Chi (106, 118, 119). The enzyme remains active and can act on another DNA molecule (no action of Chi in *trans*) (117).

Which of these reactions (***a*** or ***b***) occurs in living cells is not directly demonstrable, for the effective concentrations of ATP and Mg^{2+} in cells are uncertain (12, 16), and the apparent transience of RecBCD reaction intermediates has precluded their detection in cells. Therefore, it is important to compare both the biochemical data with purified components and the genetic data in living cells. Relevant observations are the following [see references (16, 116) for further discussion]:

1. The activity of Chi depends on nucleotides 4, 5, 6, and 7 to the 3′ side of Chi in cells (55). The Chi sequence's context-dependence is observed with purified RecBCD

and DNA with ATP in excess over Mg^{2+} (reaction ***a***) but not with Mg^{2+} in excess over ATP (reaction ***b***) (120). Thus, the behavior of purified RecBCD and Chi with excess ATP appears to more nearly reflect that of RecBCD and Chi in cells than does purified RecBCD and Chi with excess Mg^{2+}. This context dependence supports the nick-at-Chi model (Fig. 5). Note that two other *E. coli* recombination-promoting enzymes, RecG and RecQ, also require ATP in excess over Mg^{2+} for optimal activity (121, 122).

2. A Chi site on one DNA molecule reduces Chi hotspot activity on another DNA molecule (in *trans*) in cells (113, 123). Chi-dependent disassembly of purified RecBCD is observed with ATP in excess over Mg^{2+} but not with Mg^{2+} in excess over ATP, as noted above (117). This effect is the action of Chi in *trans* and supports the nick-at-Chi model (Fig. 5).

3. A class of *recC* mutants, including nonsense and deletion mutations (see Table S3 below), retains Chi hotspot activity in cells but lacks detectable intracellular and extracellular nuclease activities other than nicking at Chi (50, 58). These mutants show that "degradation" of DNA is not necessary for Chi hotspot activity and are consistent with the nick-at-Chi model (Fig. 5) but are not expected by the degradation-up-to-Chi model (Fig. 6).

4. In phage lambda crosses, recombination has been reported to be either reciprocal or non-reciprocal (95, 111, 124–128). The nick-at-Chi model leaves DNA to both sides of Chi, albeit initially ssDNA to the 3′-side before this strand, if long enough, anneals with its complement to reform dsDNA (Fig. 5, steps B through C). DNA to both sides provides a ready source of genetic information to make reciprocal recombinants. The degradation-up-to-Chi model (Fig. 6) involves a DSB formed at Chi and loss of one or the other strand to each side of Chi, making the formation of reciprocal recombinants complicated. Although the reciprocality of Chi-dependent recombination remains an unsettled question, the nick-at-Chi model (Fig. 5) can more readily account for reciprocality (Fig. 5G) where it is reported. Note that the nick-at-Chi model also accounts for non-reciprocal recombination (Fig. 5H).

5. Frequently, Chi's stimulation of high-molecular-weight (HMW) DNA formation is cited as evidence that RecBCD degrades DNA without (or up to) Chi (11, 111, 129–131). HMW DNA formation requires RecA, indicating that Chi alone does not stop degradation by RecBCD (129, 130, 132). Rather, recombination, perhaps formation of "dumbbell"-shaped (endless) DNA (16), is required to counter degradation. RecBCD degrades other DNAs that cannot recombine. One example is DNA in *E. coli recA* mutants after irradiation (reckless degradation) or without irradiation (spontaneous degradation); in both cases, degradation to acid-soluble products requires RecBCD and a combination of ExoI, ExoVII, and SbcCD nucleases (24, 133, 134). Note that RecBCD-dependent degradation of *E. coli* DNA occurs, in recombination-deficient situations, even though the *E. coli* chromosome contains a thousand Chi sites. Another example of RecBCD-promoted degradation without recombination is DNA in phage T4 *gene 2* mutants, which lack a DNA end-binding protein, at low multiplicity of infection (<<1), as used for efficiency-of-plating determinations (135). These examples indicate that RecBCD does not indiscriminately digest DNA if it can recombine.

6. Acquisition of spacers into the CRISPR locus depends on RecBCD and is reduced to the 5′-side of Chi (5′-GCTGGTGG-3′) over about 5–10 kb, the region in which Chi stimulates recombination (99, 136, 137). To the 3′-side of Chi, acquisition is the same as that >10 kb to the 5′-side of Chi (for spontaneous, naive acquisition) or

elevated between an induced DSB and the first Chi site (for either I-*Sce*I-induced naive acquisition or primed acquisition). These results are consistent with the nick-at-Chi model (Fig. 5); the reduction on the recombinogenic (5′) side of Chi likely reflects RecA binding to that ssDNA and blocking CRISPR acquisition. These results are not expected by the degradation-up-to-Chi model (Fig. 6).

7. Transformation of *E. coli* to kanamycin resistance with 6.5-kb linear DNA containing 3.0 kb of DNA homologous to the *E. coli* chromosome is stimulated 77-fold by oppositely oriented Chi sites immediately flanking the homologous segment containing the *kan* insertion; individual Chi sites stimulate 9- and 17-fold (102). With two Chi sites, degradation beyond either Chi site would block activity of the other Chi, since the 5′-strand degradation after Chi with excess Mg^{2+} would degrade the GCTGGTGG-containing strand of the other Chi.

We are not aware of corresponding observations favoring the degradation-up-to-Chi model (Fig. 6) over the nick-at-Chi model (Fig. 5).

We next discuss a hundred RecBCD mutants, the alterations of their genetic and biochemical properties, and the mechanistic inferences they provide. We extensively discuss *E. coli* RecBCD, but related helicase-nucleases are found in nearly all bacteria (138), and their mutants have been studied in many species (139–148).

recB AND *recC* NULL MUTANTS LACK ALL ACTIVITIES; *recD* NULL MUTANTS LACK NUCLEASE ACTIVITY BUT RETAIN DNA UNWINDING AND CHI-INDEPENDENT RECOMBINATION PROFICIENCY

Null mutations, including nonsense, frameshift, and insertion mutations, in *recB* or *recC* reduce *E. coli* Hfr recombination by a factor of ~10^2 (87) and transduction by ~10^1 (8, 87, 149) (Table 1; Table S2). In contrast, a *recA* null mutation reduces recombination by a factor of >10^5 (6). Thus, RecBCD is very important for recombination, but there are alternative mechanisms in its absence (low level activity of the RecE and RecF pathways noted above but in the absence of highly effective suppressor mutations) (10). Extracts of *recB* and *recC* mutants lack detectable ATP-dependent nuclease activity (69, 70), indicating that RecBCD is the major *E. coli* enzyme with this property. [SbcCD has low-level ATP-dependent nuclease activity (150).] Extracts of a *recBCD* deletion mutant also lack detectable unwinding activity and Chi-cutting activity (57). In *recB* and *recC* null mutants, the residual λ recombination is insensitive to Chi (63, 94). Thus, RecB and RecC are essential for Chi-stimulated wt homologous recombination.

recD null mutations have a markedly different phenotype. In *recD* null mutants, *E. coli* Hfr recombination and P1 transduction of chromosomal DNA are reduced by factors of only ~2 or less, but recombination in phage λ *red gam* mutants is insensitive to Chi (51). Extracts of *recD* mutants lack detectable ATP-dependent nuclease activity, and the purified RecBC enzyme (i.e., lacking RecD) lacks nuclease activity with or without ATP (26, 79, 80). As noted above, RecBC does unwind DNA, but its unwinding produces only Y-shaped molecules and depends on SSB; no ssDNA loops are seen as with RecBCD (38). This is consistent with RecBC having just one helicase (RecB), whereas RecBCD has both a fast helicase (RecD) and a slow translocase (RecB) (38). (We use "translocase" for RecB in contexts about its moving along ssDNA made by the faster RecD helicase and "helicase" when it unwinds dsDNA or in reference to its helicase domain.) Even without Chi, RecBC also loads RecA at the DNA end at which unwinding began (151). The lack of nuclease activity in *recD* mutants could stem from the nuclease active site being in RecD. More detailed analysis, however, showed the nuclease domain is in RecB but fully depends on RecD for its activity (see below).

In contrast to nearly wt frequencies in conjugation and transduction recombination, intra-plasmid recombination and λ *red gam* recombination in *recD* null mutants are increased by factors ranging from ~3 to ~70, leading some investigators to call *recD* mutants "hyper-Rec" (51, 110, 111, 149, 152–155). HMW plasmid DNA arises in *recD*

mutants (152), likely from the lack of RecBCD nuclease activity to block the transition of θ to σ replication of plasmids, as noted above for λ *red gam* replication in *recBCD* mutants. The linear ends of σ DNA molecules, like other DNA ends, are likely highly recombinogenic. This feature can account for plasmid and λ recombination appearing hyper-Rec in *recD* mutants, but based on the frequency of recombinants per DNA end, recombination in *recD* mutants may be the same as in wt. Based on standard Hfr recombination and transduction, in which the donor DNA is not replicated, *recD* mutants are not hyper-Rec and may be slightly recombination deficient relative to wt (Table 1).

Analysis of nonsense, frameshift, and insertion mutations in *recB*, *recC*, and *recD* indicates two operons (*recB-recD* and *recC-ptrA*) governing expression of the genes for RecBCD and periplasmic protease III (PtrA) (Fig. 7). (PtrA has no known interaction with RecBCD, and *ptrA* mutants have no detectable recombination phenotype. The presence of *ptrA* here remains a mystery.) The classical *recB21* mutation contains the 1,338-bp insertion sequence IS*186* at codon 305 (of 1180 in *recB*) (26) (Fig. 4). With respect to recombination proficiency and Chi hotspot activity, *recB21* fails to complement *recD* mutations, which led to the initial assignment of *recD* mutations to *recB* (51). Analysis of polypeptides produced from plasmid-borne (and thus overexpressed) genes after UV-irradiation of cells ("maxicell" analysis) showed that *recB21* abolishes both *recB* and *recD* expressions and that *recC22*, a nonsense mutation at codon 524 (of 1122 in *recC*), abolishes both *recC* and *ptrA* expression but not that of *recB* or *recD* (26). Curiously, *ptrA* overlaps *recB* by 8 bp but is separated from *recC* by 165 bp; this observation and likely additional computational analysis apparently led to the assignment on RegulonDB (http://regulondb.ccg.unam.mx/index.jsp) and elsewhere of a transcriptional promoter for *recC* and a separate one for *ptrA-recB-recD*. The functional analysis noted here and additional deletion and cloning analyses (S. K. Amundsen, unpublished data) lead us to conclude there are two operons (*recC-ptrA* and *recB-recD*) with a promoter at each left end and an additional promoter near the 3′-end of *recB* that can express *recD*. These observations emphasize the need for direct analysis, both genetic and physical, to draw conclusions.

DNA sequence, mutational, and structural analyses show that RecB has both an N-terminal helicase domain and a C-terminal nuclease domain (34, 52, 59). RecD has a helicase domain (34, 54, 85, 86, 156, 157), and RecC appears to have defunct helicase and nuclease domains (34) (Fig. 1 and 4). These activity domains were confirmed by point mutations discussed below. The helicase domains place both RecB and RecD in helicase superfamily SF1 (158); the RecB nuclease domain is in the λ exonuclease family (34). Thus, RecBCD is structurally related to other bacterial helicases and nucleases, but it and its relatives, such as AddAB in many Gram-positive bacteria, are to our knowledge the only bacterial enzymes that have both activities in one complex. [Eukaryotic replication protein Dna2 is a helicase with weak structure-specific endonuclease activity; reviewed in reference (159). Human BLM helicase binds to Exo5 nuclease to maintain genome integrity; e.g., see reference (160).]

recC C-TERMINAL MUTANTS FAIL TO LOAD RecD AND MIMIC *recD* NULL MUTANTS

RecC is an essential part of RecBCD but lacks detectable enzymatic activity; isolated RecB and RecD have DNA-dependent ATPase and ATP-dependent unwinding activities, and

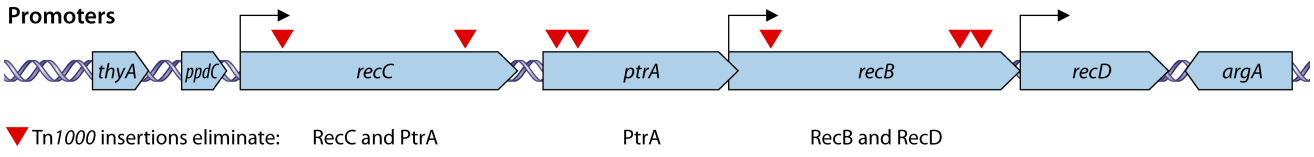

FIG 7 *E. coli* chromosomal region containing *recB*, *recC*, and *recD*. Genes are shown as open boxes, drawn to scale, with an arrow pointing in the direction of transcription. Transposon Tn*1000* insertions (red arrowheads) in *recC* are polar on *ptrA* but not on *recB* or *recD*; those in *recB* are polar on *recD*. Insertions in *ptrA or recD* are not polar on the other genes shown (26). These features indicate two operons—*recC-ptrA* and *recB-recD*.

RecB has weak, ATP-stimulated ss endonuclease activity (53, 59, 161–164). RecC appears to be a structural scaffold essential for the robust activities of RecBC and RecBCD. Consistent with this view, C-terminal deletions of *recC* appear to lack assembly of RecD into the trimeric complex (58) (Table S3). This conclusion stems from the early observation of *recC1010* (G905E) having the same phenotype as *recD* null mutants—recombination proficient but Chi inactive (51, 58) (Table 1). Deletion of 4 *recC* C-terminal codons leaves wt phenotype, but deletion of 38 or 141 codons produces the RecD$^-$ phenotype. Curiously, further deletion of 200 or 332 codons, like the *recC1041* (W841*) nonsense mutation in that interval, produces a novel phenotype—significant Chi hotspot activity but no nuclease activity as assayed by ability of phage T4 *gene 2* mutants to form plaques [*gene 2* protein appears to bind to the end of T4 DNA in the virion and thereby protect T4 DNA from RecBCD upon injection into cells (135)]. Further deletion of 444 or more codons produces the *recC* null phenotype. We infer that RecC C-terminal amino acids (roughly 900–1,118) are required for RecD to bind to the RecBC complex. This view was later confirmed by the crystal and cryoEM structures (Fig. 1) (34, 41), in which there is extensive contact between a RecC folded domain (amino acids 828–1,122) and highly folded RecD. RecC is important not just for RecBCD assembly but also in the signal transduction pathway with RecD and RecB for its proper function—control of the enzyme by Chi hotspots (see text and Fig. 10 below).

recB NUCLEASE ACTIVE-SITE MUTANTS ARE UNWINDING PROFICIENT BUT RECOMBINATION DEFICIENT

Confirmation of the nuclease domain being in RecB came from the analysis of *recB1080* (D1080A) (59), which removes the carboxyl group that binds Ca^{2+} in crystal structure PDB 1W36 published later (34). The *recB1080* mutant is as recombination deficient as a *recBCD* deletion mutation (Table 1; Table S4). It is also nuclease deficient (59); Mg^{2+} ion, which is essential for nuclease activity (25, 70), likely binds to this site. As expected, Ca^{2+} is a competitive inhibitor of the nuclease activity (25, 165). *recB1067* (D1067A) and *recB1082* (K1082Q) are also nuclease deficient (59, 60) and are altered in amino acids that bind Ca^{2+} in crystal structure PDB 1W36. Thus, there is only one nuclease site in RecBCD even though it can, depending on reaction conditions, digest either the 3′- or 5′-ended strand. As noted above and discussed more below, the nuclease being on a tether may allow the nuclease domain to assume different positions or conformations and thus have different activities, including the loading of RecA onto ssDNA during unwinding (47).

recB ATPase-SITE MUTANTS LACK 3′-STRAND TRANSLOCATION ACTIVITY, BUT *recD* ATPase-SITE MUTANTS RETAIN WEAK 5′-STRAND TRANSLOCATION ACTIVITY

The ATPase sites of RecB and RecD are similar in amino acid sequence and structure to those of other SF1 helicases and are classified as Walker A motifs (158). As noted above, isolated RecB has DNA-dependent ATPase activity (161). A lysine residue (K29 in RecB and K177 in RecD) is essential for ATPase activity of the respective subunits, as shown by their alteration to glutamine [*recB29* (K29Q) and *recD2177* (K177Q)] (52–54). *recB29* is as recombination deficient and Chi-non-activating as a *recBCD* deletion mutation, but *recD2177* retains weak recombination and Chi activities (Table 1 and Table S4; S.K. Amundsen, unpublished data). These results indicate that RecB is an essential translocase for RecBCD and that RecD, even though it is the faster helicase, might be considered an auxiliary helicase important for Chi hotspot activity. That *recD2177* does not have the same phenotype as a *recD* null mutation suggests that RecD, separate from its helicase activity, modulates RecBCD's activities, consistent with the signal transduction model discussed below.

During DNA unwinding by the RecB (K29Q) mutant, the 3′-end of the DNA substrate remains bound to the enzyme, indicating that this mutant RecB translocase is inactive (38). The 5′-end, however, is extruded as a free ssDNA tail, indicating that RecD still unwinds DNA, about half as rapidly as wt RecBCD unwinds DNA. During DNA unwinding

by the RecD (K177Q) mutant, the 3′-end is extruded but at about 20% of the rate as with wt RecBCD, indicating RecB helicase is functional in the *recD2177* mutant but is less active than with a functional RecD helicase present. [In wt RecBCD, RecB translocates along the DNA at about half the rate of RecD (39).] Unexpectedly, the 5′-end is also extruded but only ~5% as rapidly as with wt RecBCD. This result may reflect the *recD2177* (K177Q) mutation not completely inactivating the RecD ATPase site or the RecB ATPase site sending energy to RecD for unwinding or both. (The double mutant *recB29 recD2177* mutant does not detectably unwind DNA.) The latter possibility is consistent with the manifold interactions among the three RecBCD subunits, as noted above and expanded below.

recC TUNNEL MUTANTS HAVE REDUCED OR NO CHI HOTSPOT ACTIVITY, DEPENDING ON THE DNA SEQUENCE NEAR CHI

To gain genetic evidence that RecBCD directly interacts with Chi, Schultz et al. (87) sought *recBCD* mutants that retained all RecBCD activities except Chi hotspot activity. Success came from pseudorevertants of the *recC73* null mutant: four ethylmethane sulfonate (EMS)-induced mutants (*recC1001–1004*) regained partial or complete enzymatic and genetic activities but lacked detectable Chi hotspot activity (Table S5). Concurrent studies of nitrosoguanidine- and EMS-induced mutants with increased frequency of excision of Tn*10* in *lacZ* yielded two mutants (*recB344* and *recC343*) with reduced Chi hotspot activity but nearly wt for other activities (87, 166). Soon after, wt RecBCD was shown to nick DNA near Chi, but the mutants tested (*recC1001*, *recC1004*, and *recC343*) had little or no Chi nicking activity (64). These results established that RecBCD directly recognizes Chi and is activated by Chi to nick DNA (Fig. 5 and 8).

DNA sequencing showed that these *recC* mutations alter RecC in an interesting, informative way. *recC73* is a −1 frameshift in codon 647, and the pseudorevertant mutations are compensating frameshifts altering six to nine amino acids (codons 647–655) (167). *recC343* changes P666 to L (62). These amino acids are along or near a tunnel in RecC through which the 3′-ended strand travels before emerging near the RecB nuclease domain (34) (Fig. 1). CryoEM structure 6SJB (Fig. 8) was later obtained with

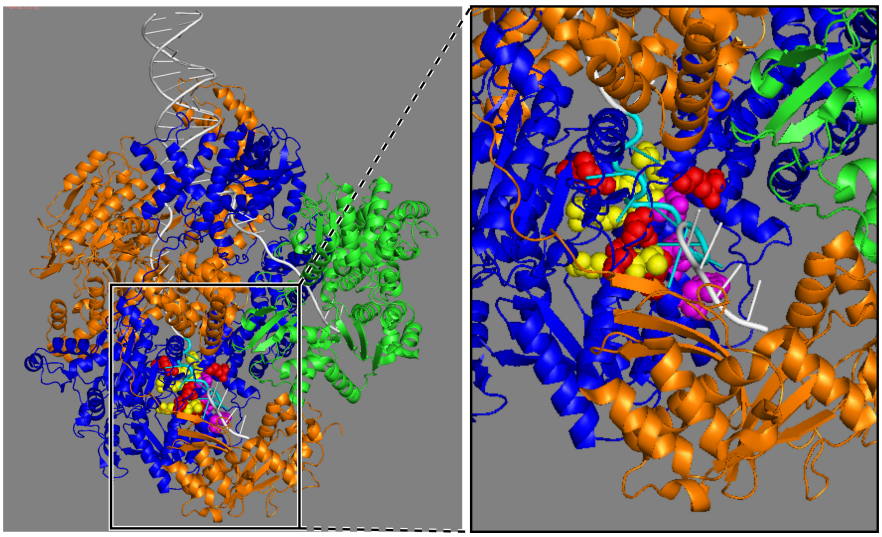

FIG 8 CryoEM structure of RecBCD with Chi DNA in the RecC tunnel. RecBCD bound to dsDNA with a 15-nucleotide 5′-tail and a 20-nucleotide 3′-tail containing Chi (light blue) four nucleotides from the 3′-end was visualized by cryoEM (PDB 6SJB) (41). Note that Chi is near the amino acids in the RecC tunnel shown by mutational analysis (Table S5) to be required for Chi hotspot activity. Amino acids in red were identified by Amundsen et al. (55); those in magenta and yellow were identified by Handa et al. (56) as type 1 and type 2, respectively. The nucleotides of Chi are in a kinked arrangement, perhaps to momentarily pause RecB translocase at Chi.

RecBCD bound to dsDNA with long 5′- and 3′-tails (41). The 3′-tail has the Chi octamer followed by four nucleotides to the 3′-end. DNA with the Chi octamer is bent into a "switchback" conformation in the RecC tunnel, suggesting that the RecB translocase may momentarily pause at Chi to initiate the "signal transduction" discussed below. DNA of the same length but without Chi is partially disordered without the switchback in the tunnel.

These results provided the foundation for more precise mutational analyses of the RecC-Chi interaction. Handa et al. (56) changed to alanine 35 individual amino acids lining the RecC tunnel and found two classes of mutants (Table S5). Type 1 ("Chi non-recognition") mutants lacked Chi hotspot activity, and λ *red gam* χ^+ phage made small plaques on them (like λ *red gam* χ^o phage on wt cells). Type 2 ("relaxed recognition") mutants had much reduced Chi hotspot activity, but λ *red gam* χ^o phage made medium to large plaques on them (like λ *red gam* χ^+ phage on wt cells), suggesting that these *recC* mutants recognize one or more DNA sequences other than Chi in λ. Amundsen et al. (55) randomly mutagenized regions of *recC* encoding amino acids lining the RecC tunnel and thoroughly analyzed 25 mutants with a spectrum of Chi activities, from undetectable to only slightly less than wt. But the hotspot activity depended on the Chi site(s) used for the assay; i.e., there was DNA sequence-context dependence to the ability of the *recC* mutants to stimulate recombination. The few type 1 (Chi non-recognition) mutants isolated by Handa et al. (56) and analyzed this way by Amundsen et al. (55) also showed context-dependent hotspot activity.

A concurrent study showed Chi context dependence biochemically: wt RecBCD nicks DNA at a frequency dependent on the nucleotides to the 3′-side of the Chi octamer (55, 120). A DNA substrate with the Chi octamer flanked on each side by 10 random bp was partially reacted with RecBCD, and the cut and uncut DNAs were deep sequenced. The results showed that cutting was essentially independent of the 5′-flank but strongly dependent on the 3′-flank: DNA molecules enriched for A or, to a lesser extent, G at nucleotides 4–7 were cut more often than DNA enriched for C or T at those positions. Since nicking occurs at nucleotides 4–6 (45), the 3′-context dependence may simply reflect the preference for RecB nuclease to cut at or near A or G. Nevertheless, these results reveal the complexity of Chi hotspots and their interaction with RecBCD.

CHI's SEQUENCE CONTEXT DEPENDENCE INDICATES THE RELEVANT INTRACELLULAR REACTION—NICKING OF THE 3′-ENDED STRAND AT CHI

The same context dependence is observed in cells (in λ hotspot crosses) and with purified RecBCD with excess ATP but not with excess Mg^{2+} (120). These observations provide strong evidence for the relevant reaction of RecBCD at Chi in living cells. The initial observation of nicking of DNA at Chi, on the 3′-ended strand, used purified RecBCD with ATP in excess over Mg^{2+} ions (e.g., 5 mM ATP and 2.5 mM Mg^{2+}) (45). Later observations of degradation of the 3′-ended strand up to Chi used Mg^{2+} in excess over ATP (e.g., 8 mM Mg^{2+} and 1 to 5 mM ATP) (118, 168). (ATP must chelate Mg^{2+} to be hydrolyzed and thus be active for unwinding, but unchelated Mg^{2+} is essential for the nuclease to be active, yielding a complex situation.) Further investigations using excess Mg^{2+} showed that the 5′-ended strand was also cut at Chi (119) and that this strand was further degraded to the 5′-side of Chi (106). Both reactions produce 3′-ended ssDNA ending near Chi and extending to Chi's "left," as written here (with RecBCD entering the right end of the dsDNA substrate; Fig. 5 and 6). As noted above, nicking at Chi leaves the 5′-ended strand intact to both sides of Chi and would allow reciprocal recombination to occur, but degradation on both sides of Chi (on opposite strands) complicates formation of reciprocal recombinants. Some studies but not others (95, 111, 125–128) [see also reference (124)] show that RecBCD-dependent Chi-stimulated recombination is reciprocal, suggesting, at least in the former cases, that nicking of DNA is the relevant reaction in living cells. Although the evidence for and against reciprocality is equivocal, the model in Fig. 5 readily accounts for either type of recombination, but the model in Fig. 6 predicts non-reciprocality. Multiple other observations also indicate nicking at Chi,

as noted above. These observations illustrate the importance of using both genetic and biochemical methods (i.e., molecular biology) to determine what happens in living cells.

THE RecB HELICASE-NUCLEASE TETHER MUST BE THE RIGHT LENGTH AND STIFFNESS FOR CHI HOTSPOT ACTIVITY

Nicking of DNA at Chi requires the RecB nuclease domain, including the active site that binds Mg^{2+}, as noted above (59, 61). In the crystal and cryoEM structures [e.g., PDB 1W36, 5LD2, and 6SJB (Fig. 1 and 8)] (34, 35, 41), the nuclease domain is at the exit of the RecC tunnel, from which the 3′-ended strand emerges during unwinding. If it were always positioned there, one might expect the enzyme to act as a 3′-exonuclease, releasing mononucleotides or dinucleotides from that end from the beginning of the reaction. Short-term reactions, however, show that RecBCD first generates dsDNA fragments up to several kb long with ssDNA tails (67) (Fig. 2 and 3). As the reaction proceeds, this DNA becomes progressively shorter, eventually becoming oligonucleotides mostly three to six nucleotides long (72). Furthermore, >20 ATP molecules are hydrolyzed per inter-nucleotide scission (72, 169), and ~2 ATP molecules are hydrolyzed per bp unwound (170, 171), indicating multiple passages of RecBCD through DNA for each DNA nick made. These observations suggest that RecBCD cuts dsDNA rarely during its unwinding and produces shorter-than-full-length ssDNA, which the enzyme subsequently degrades (in an ATP-dependent manner), eventually to oligonucleotides (Fig. 3). This scenario suggests that the nuclease is not always positioned to cut DNA; the high preference for nicking of DNA at Chi suggests the same. So, what controls when, or where, RecBCD cuts DNA?

The RecB nuclease globular domain (Nuc), which we define as RecB amino acids 900–1,180, is connected to the RecB helicase globular domain by a 19-amino acid linear tether (amino acids 881–899 in our definition) (Fig. 1 and 4). [Another definition of this region, designated as "linker," is RecB amino acids 870–940 (35), which includes about 20–30 amino acids in each of the globular RecB helicase and nuclease domains (Fig. 1).] This tantalizing feature suggests that Nuc may swing on this tether away from the RecC tunnel exit to a position in which it cannot cut DNA but then swing back to the active position under the appropriate conditions (Fig. 9; Video S1). Support for this idea came from analysis of RecBCD with and without DNA bound (172). Without DNA, the enzyme is quite sensitive to proteases, such as trypsin, at a site on RecC ~50 Å from the RecC tunnel exit (at R278 for trypsin and at other positions between R278 and W303 for other proteases) (Fig. 9, top). This site becomes much less protease sensitive when DNA is bound (in the absence of ATP, so that the enzyme remains at the dsDNA end). Small angle X-ray scattering (SAXS) analysis of RecBCD with and without DNA bound is concordant with these observations—protein mass at the position of Nuc without DNA moves to the position of the protease cutting sites upon DNA binding (Fig. 9, bottom) (172). SAXS resolution is not sufficient to identify this mass, but its change of position is consistent with Nuc moving as inferred from the protease experiments. In addition, the protease-sensitive site remains resistant when ATP is added to allow DNA unwinding, but this site returns to protease sensitivity when, during active unwinding, Chi is encountered (172). Thus, these physical experiments support the Nuc swing model (Fig. 9): upon DNA binding, Nuc moves to an inactive ("storage") position and remains there during unwinding, but when Chi is encountered, Nuc moves back to the RecC tunnel exit and cuts the 3′-ended strand near Chi.

This model was also supported genetically by showing that the RecB tether (amino acids 881–899) must be just right for full Chi activity (48). Deleting even one amino acid (RecB 893) significantly reduces Chi activity from 5.1 in wt to 3.3, or down to 1.1 with longer deletions, in standard hotspot crosses in phage λ (Table 1; Table S6). [Chi hotspot activity is the ratio of the recombinant frequency in an interval with Chi to that in the same interval without Chi; a ratio of 1 indicates no Chi activity (63) (Fig. S1).] Chi activity is also reduced when the tether contains either more prolines (likely stiffening the tether) or more glycines (likely making the tether more flexible) or when it is lengthened by

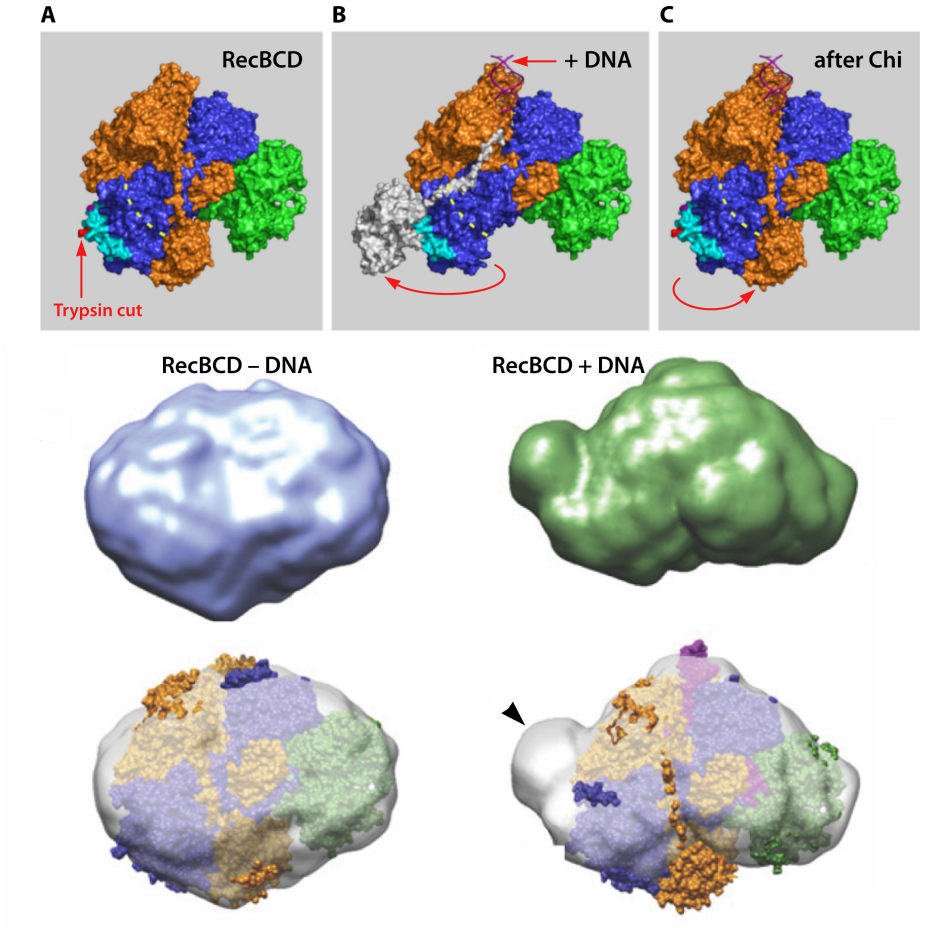

FIG 9 RecB nuclease-swing model for control of RecBCD enzyme by Chi hotspots. (A) Before DNA binding, the RecB nuclease domain (Nuc) is at the exit of the RecC tunnel (yellow dashed line). The SAXS structure (shown below, left) is similar to the superimposed crystal structure (PDB 3K70) (173). (B) Upon DNA binding, Nuc swings to the "left," where it protects amino acids in the RecC surface loop (cyan) from proteases such as trypsin (172). The SAXS structure of the RecBCD-DNA complex (shown below, right) has more mass near the RecC surface loop (black arrowhead) and less mass near the RecC tunnel exit compared to the superimposed crystal structure. (C) When Chi is encountered, Nuc swings back to the RecC tunnel exit and cuts the 3′-ended strand near Chi. Redrawn from reference (172). See Video S1 for animation of Nuc moving from the tunnel exit to the RecC surface loop.

insertion of 19 or 38 amino acids. These reductions in Chi hotspot activity are paralleled by reductions of Chi nicking activity in extracts. Thus, both biochemical and genetic data support the nuclease-swing model, but presence of Nuc at the protease-sensitive site in RecC has not yet been reported in crystal or cryoEM analyses.

CHI HOTSPOT ACTIVITY DEPENDS ON WILD-TYPE RecB, RecC, AND RecD INTERFACES—A SIGNAL TRANSDUCTION PATHWAY WITHIN RecBCD ENZYME

An amplification of the nuclease-swing model posits that recognition of Chi in the RecC tunnel triggers a pathway of signals from one subunit to another to effect swinging of Nuc and thus nicking of DNA at Chi (Fig. 5 and 10) (172). In step 1, RecC recognizes Chi and signals RecD helicase to stop (step 2). When stopped, RecD signals RecB Nuc to swing from its storage site to the RecC tunnel exit (step 3) and to nick at Chi (step 4). A further change of Nuc's position, orientation, or conformation allows it to load RecA DNA strand-exchange protein onto the newly generated 3′-ended ssDNA (step 5). [Purified RecBCD had been shown earlier to load RecA onto this "Chi tail" in a Chi-dependent

manner (47), and RecA binding to purified RecB Nuc requires the middle part of RecB Nuc—amino acids 1,034–1,100 (174).]

Examination of the crystal structure PDB 1W36 (34) revealed points of contact between each pair of RecBCD subunits that might be required to transmit the signals. Indeed, mutations, either missense or deletion, of these contact points reduce or eliminate Chi hotspot activity in cells and Chi nicking activity in extracts (62). For example, deletion of RecC amino acids 541–544 (QGEW; *recC2820*, also called ΔC2) or of RecD amino acids 97–99 (PTP; *recD2824*, also called ΔD2) or both eliminates Chi hotspot activity but reduces Hfr recombination by a factor of <2 (Table 1; Table S7). Thus, these changes eliminate Chi's control of RecBCD but leave functional the many activities required for recombination. Change of these amino acids to Ala reduces Chi activity to ~2.5 but Hfr recombination by a factor of <2, indicating their specificity for Chi action. Chi-nicking activity is undetectable in all these cases. Similar analyses identified RecD-RecB contacts for step 3 and RecB-RecC contacts for step 4 (Table S7). Although the amino acids identified for each step are in close contact in cryoEM structure PDB 5LD2 (35), the contact points for step 3 are not close in crystal structures 1W36 and 3K70 (34, 173). In addition, a 10-amino acid alpha helix proposed to block access of DNA to the nuclease active site in RecB and called B4 in step 4 (Fig. 10) is disordered in eight cryoEM structures but is ordered in the two crystal structures (34, 35, 41, 91, 173, 175). This outcome indicates conformational flexibility at the contact points, as expected from the nuclease-swing model (Fig. 9). Note that these three sets of contact points and the Chi-RecC tunnel contacts are widely distributed on the heterotrimer. All of these sets are important for transmitting Chi recognition to the nuclease domain.

The distance over which this signal transduction is hypothesized to travel is ~185 Å (62). The maximal breadth of RecBCD, from one edge to another, is ~140 Å (34, 35). The proposed signal appears to travel in a loop—from the RecC tunnel, to RecD, to the RecB helicase domain, along the RecB tether, and to the RecB Nuc domain to position it at the RecC tunnel exit. In one scenario, we imagine that the RecB helicase domain, when signaled by halted RecD, yanks on the RecB tether, which moves RecB Nuc into position

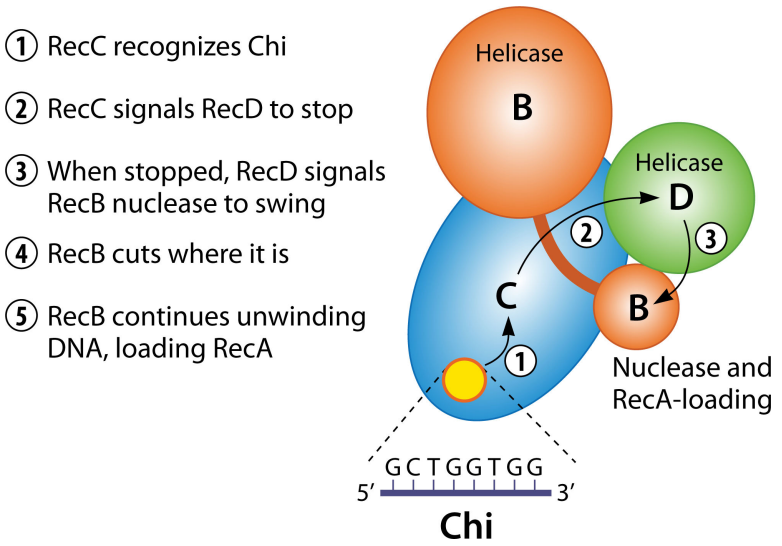

FIG 10 Signal transduction model for control of RecBCD enzyme by Chi hotspots. During DNA unwinding by RecBCD, the 3′-ended strand moves from the RecB helicase domain into a tunnel in RecC (see Fig. 1 and 8). When the Chi sequence 5′ GCTGGTGG 3′ is properly positioned in the RecC tunnel (yellow disc, step 1), RecC signals RecD to stop unwinding (step 2). When RecD is stopped, it signals RecB to swing the RecB nuclease domain (Nuc) from the "left" surface of RecC back to the RecC tunnel exit (step 3) (see Fig. 8 and 9). In this position, Nuc can cut the DNA a few nucleotides 3′ of the Chi octamer (step 4) and load RecA onto the emerging, newly generated 3′-ended strand (step 5). Redrawn from reference (49).

to nick at Chi. Nuc then rotates to put its RecA loading surface in position to load RecA onto the emerging ssDNA with Chi a few nucleotides from its 3′-end. RecA continues to be loaded onto DNA to the "left" (away from the 3′-end of the nicked DNA) for several kb as RecB helicase continues along this strand. Chi-stimulated recombination would thus be "leftward" from Chi, as observed (125).

RecB ATPase SITE MUTANTS AND A SMALL MOLECULE INHIBITOR CONVERT RecBCD INTO A CHI-ACTIVATED STATE

The signal transduction model arose from the properties of two mutants altered, unexpectedly, in the RecB ATPase site (49). That study set out to find *recB* mutants defective in RecA loading, as mutants that were recombination deficient but still nuclease proficient, the converse of the screen that led to *recD* mutants noted above (51). The *recB* DNA encoding Nuc was randomly mutagenized, and colonies were screened for Hfr recombination deficiency and resistance to phage T4 *gene 2* mutants (an intracellular assay for RecBCD nuclease activity, as described above). This screen yielded two especially interesting mutants, but the mutations were not in the *recB* region encoding Nuc; rather, they were almost at the edge of the restriction fragment luckily chosen for convenient mutagenesis. These mutations, *recB2732* (Y803H) and *recB2734* (V804E), alter amino acids in motif VI of the RecB ATPase site (Fig. 4) and physically close to RecB K29 essential for RecB's ATPase activity (Fig. 1 and 2), as discussed above. These mutants are as recombination-deficient as *recB* null mutants and lack Chi hotspot activity; they retain at least 50% of the wt nuclease activity but lack Chi-nicking activity (Table 1; Table S4). Most remarkably, they produce an ssDNA fragment cut not at a special DNA sequence but at a certain percent of the length of the DNA substrate (18% and 26%, respectively). Somehow, these mutants can measure the length of the DNA substrate, calculate a certain percent of that length, and nick the DNA at that position. The solution to this puzzle is that, in these mutants, the ratio of the RecB translocase and RecD helicase rates, determined by EM after brief unwinding of long DNA, corresponds to the fraction of the DNA length at which cutting occurs. Thus, when the faster helicase RecD reaches the end of the 5′-ended strand, it must signal RecB to cut where it is at that moment (18% of the DNA length in one mutant and 26% in the other). These observations were quickly extended into the multi-step signal transduction model (Fig. 10) (49), which was later supported by the tether and signal transduction mutants described above.

Additional support for this model came, again unexpectedly, from a study of small-molecule inhibitors of RecBCD (176). These inhibitors were found in a screen for inhibition of purified RecBCD helicase-nuclease activity, as potential novel, sorely needed antibiotics [see also reference (177)]. One inhibitor (NSAC1003; Fig. S2) appeared highly interesting because it mimics the effect of the two RecB ATPase site mutants discussed above. NSAC1003 inhibits RecB nuclease with an IC_{50} of ~10 or 100 μM with 25- or 400-μM ATP, respectively. This apparent competition with ATP is concordant with computational docking of NSAC1003 to the RecB ATPase site (Fig. S2) with a calculated K_D of ~0.3 μM (in the absence of ATP). In the presence of increasing concentrations of NSAC1003, nicking at Chi is replaced with nicking at a novel site ever closer to the 3′-end at which RecBCD initiated unwinding. At a given NSAC1003 concentration, this nick occurs at nearly the same fraction of the length of the DNA substrate regardless of its length (from 1.4 to 4.4 kb), as observed for the ATPase-site mutants noted above. These data show that NSAC1003, like the ATPase-site mutants, converts RecBCD into a Chi-activated state—nicking DNA when RecD stops—and lend further support to the signal transduction model.

A FLEXIBLE SURFACE LOOP ON RecC, FAR FROM THE CHI RECOGNITION AND NUCLEASE SITES, IS REQUIRED FOR FULL CHI HOTSPOT ACTIVITY

To test the signal transduction model further, we sought mutants altered where Nuc is postulated to sit in the storage condition (after DNA binding but before Chi recognition), i.e., near the site of differential protease sensitivity (Fig. 9; Video S1) (172). Candidate sites

were identified by computational docking of RecB Nuc onto the RecC region surrounding the protease cleavage sites (57). The best candidate docked four amino acids of Nuc against four amino acids of RecC, forming pairs of amino acids with opposite charge (e.g., RecB E936 against RecC R278, the site of differential trypsin cleavage noted above). Change of these four amino acids, in either RecB or in RecC or both, to Ala reduces Chi hotspot activity to 2.0–2.5 but leaves Hfr recombination nearly wt (65%–86% of wt frequency) (Table S8) (57). The RecC amino acids in this docking are part of a loop (amino acids 252–291) on the surface of RecC (Fig. 1 and 9). Deletion of this loop strongly reduces Chi hotspot activity (to 1.9) and leaves nearly wt Hfr recombination (77% of wt frequency) (Table 1; Table S8). This loop is ordered in one of the molecules in the asymmetric unit of PDB 1W36 (34) but disordered in the other, indicating its flexibility. The loop is displaced upon binding of Abc2, a protein of phage P22 that blocks RecBCD nuclease and Chi hotspot activities (178, 179). We posit that this RecC loop moves slightly to accommodate RecB Nuc after it has swung. Pleasingly, the tether is long enough to allow this change of Nuc position, a move of ~50 Å (57) (Video S1). These mutants also illustrate how sites on RecBCD far from the Chi recognition point are important for Chi's control of RecBCD, in line with the long-range signal transduction model described above (Fig. 10).

UNTETHERED RecB NUCLEASE HAS UNCONTROLLED NUCLEASE ACTIVITY

Above, we have discussed how the RecBCD nuclease is controlled. What happens if it is untethered from the rest of the enzyme? It appears to become uncontrolled and potentially lethal, a state we refer to as that of the "rogue nuclease." As noted earlier, purified full-length RecB (amino acids 1–1,180) has weak DNA-dependent ATPase and ATP-independent endonuclease activity on ssDNA circles (about one DNA cut per hour per enzyme molecule) but lacks detectable ds endonuclease activity (53, 59). Intact RecBCD makes about one cut per 5 min on ssDNA circles and is stimulated about 10-fold by ATP (72). An altered form of RecB Nuc, here called Nuc′ (amino acids 928–1,180) with N-terminal extensions, also has ssDNA endonuclease activity that, in the cases tested, is ATP independent. This activity is altered by N-terminal extensions in a curious manner. Assays with phage M13 ssDNA circles (6407 nucleotides long) show that Nuc′ with an N-terminal extension of six contiguous His residues embedded in a 21-amino-acid "tag" (His_6) makes about one cut per 400 hour (~2 weeks) (180). His_6-gp32-Nuc′, with an insertion of the ssDNA-binding protein of *gene 32* of phage T4, makes about one cut per 5 hour, suggesting that Nuc′ binds ssDNA poorly (59). Thrombin-induced removal of the His_6 tag, leaving four amino acids GSHM on the N-terminus of Nuc′, gives a protein that makes about one cut per min (181). Thus, the activity of Nuc′ is regulated over a 25,000-fold range by adjacent amino acids, including inhibition by N-terminal extensions. Of these proteins, only GSHM-Nuc′ cleaves dsDNA circles (about one cut per 3 min), unlike intact RecBCD or its subunits, alone or in combination (181). These derivatives of Nuc′ lack the RecB alpha helix, amino acids 913–922, designated B4 (Table S7) in the signal transduction studies discussed above (34, 62).

These observations indicate that uncontrolled ("untethered" or rogue) Nuc may make lethal lesions and imply that "domesticating" RecB Nuc by the rest of the complex enzyme is essential for Nuc to maintain cell viability. These observations with mutant forms of RecBCD illustrate additional means by which RecBCD helicase-nuclease is controlled, as emphasized throughout this review.

ADDITIONAL MUTATIONS SCATTERED THROUGHOUT RecBCD DEPRESS CHI ACTIVITY AND RECOMBINATION

During the investigations discussed above, additional *recBCD* mutants have been described (Table S9), but their molecular interpretations remain cloudy. We describe here those with the most dramatic phenotypes.

An early investigation randomly mutagenized the entire *recBCD* gene cluster (on an F′ episome or a pBR322-based plasmid) and screened for Hfr recombination-deficiency (182). Among the dozen mutants analyzed, *recB2153* (I427T) stands out for being as recombination deficient as *ΔrecBCD* in Hfr and λ crosses yet retaining nearly full unwinding, Chi-nicking, and nuclease enzymatic activities, RecA-loading activity, and Chi hotspot genetic activity (182) (S.K. Amundsen, unpublished data). The altered amino acid is ~15 Å from the RecB ATPase site in crystal structure PDB 1W36 (34). *recB2153* does not make detectable λ heteroduplex DNA during infection, unlike wt, which makes readily detectable, RecA-RecBCD-dependent heteroduplex DNA stimulated by Chi (three- to sevenfold) (183). Perhaps *recB2153* makes recombination intermediates but degrades them; the few that survive are Chi stimulated. Indeed, purified RecBCD cleaves D-loop intermediates (~20 times more rapidly than circular ssDNA on a per-nucleotide basis) but leaves the annealed strands intact (184). The *recB2153* enzyme may degrade such intermediates in an uncontrolled fashion. These results suggest that RecBCD has an important role in recombination after loading RecA, an interpretation proposed long ago (185) but later set aside (186). Further studies of *recB2153* may revive this idea and reveal additional functions of RecBCD in recombination and DSB repair.

recB2152 (T807I) and the closely related mutant *recB2109* (G493S T807I) are also as recombination deficient as *ΔrecBCD* and retain unwinding and nuclease activities but lack Chi genetic and nicking activities (182). T807 is located near the RecB ATPase site, and the mutants have reduced affinity for ATP (182, 187, 188). The lack of Chi activity may partially account for the strong recombination deficiency, since RecC tunnel mutants without Chi activity, such as *recC2777* (S39E), have reduced recombination potential (~6% of wt) (55). Another mutant with a nearby amino acid change [*recB2154* (R800C)] and another with two distant changes [*recC2145* (R186H, G304S)] also lack Chi activity and have low recombination potential (~1% of wt) (182). *recB29* (K29Q) lacks RecB ATPase activity and is strongly recombination deficient (52) (S.K. Amundsen, unpublished data). These mutants illustrate the importance of RecB helicase activity in promoting recombination, perhaps the need for RecB to move along the 3′-ended strand and to load RecA distant from the initiating DSB.

Among the many *recBCD* mutants we have analyzed, none are dominant to wt *recBCD*. A mutation called *rorA* and mapping as a *recB* or *recD* mutation is partially dominant with respect to sensitivity to UV-irradiation; it retains full ATP-dependent nuclease activity (189–191). To our knowledge, *rorA* has not been further characterized. One might expect the double ATPase site mutant *recB29* (K29Q) *recD2177* (K177Q), which binds DNA tightly but does not detectably unwind it (38), to be dominant. Even when overexpressed (~25-fold) on a plasmid, however, it is not dominant to chromosomal *recBCD*$^+$ (S.K. Amundsen, unpublished data). Evidently, wt RecBCD is powerful enough to remove this potential impediment, just as it removes other tightly bound proteins including nucleosomes, LacI repressor, RNA polymerase, and nuclease-negative *Eco*RI (192–194). Such overwhelming power may be necessary for RecBCD to move along a broken chromosome "searching" for a Chi site to repair the chromosome and thereby maintain life. In contrast, RecBCD does not displace DNA-bound Cas1-Cas2, the protein that inserts new DNA sequences into the CRISPR locus (see below) (137). Presumably, activation of the Cas-CRISPR defense system is too important to let even RecBCD interrupt it; indeed, RecBCD and Chi play important roles in CRISPR adaptation and perhaps interference (see below).

POTENTIALLY INFORMATIVE, CURRENTLY UNKNOWN *recBCD* MUTANTS TO SEEK

Two types of potentially informative mutants have been sought but not found or reported. First are *recBCD* mutants unable to load RecA but with retention of other activities. The ability of RecBCD to load RecA after cutting at Chi (47) and the ability of RecB Nuc to bind RecA (174) predict mutants altered in Nuc that would not load RecA. Such mutants would show that this feature is important for recombination in living cells.

Purified RecA can load onto DNA by itself (195–197), and the RecFOR complex aids RecA loading (198–200), leaving open the possibility that in cells, RecBCD's loading of RecA is not essential, or the only avenue, for recombination. Indeed, *recF* mutants have reduced Hfr recombination proficiency [about 50% of wt (149, 201, 202)]. Perhaps the right assays have not been used to find RecA-loading-deficient *recBCD* mutants. Alternatively, the surface on Nuc that putatively binds RecA may be so extensive that one or a few changes of amino acids may not block RecA binding or may also inactivate the nuclease and divert such mutants from further analysis.

Mutants in *recD* that are strongly recombination deficient have also not been reported, although they exist in *recB* and *recC*. The RecD ATPase site mutant *recD2177* (K177Q) and the *recD* complete deletion mutant are about 50% as proficient for Hfr recombination as wt (Tables S2 and S4). Other changes of RecD might interfere with one or another of the many RecBCD activities essential for recombination and described above. Studies of such mutants would likely reveal unknown features of RecBCD.

Mutants activated by a sequence other than Chi would be predicted to arise by appropriate alterations of the RecC tunnel (Fig. 8). Indeed, *recC1004*, a suppressor of the *recC73* frameshift discussed above, is activated by 5′-GCTGGTCTCG-3′ to increase the plaque size of λ *red gam* phage, but it does not have recombination hotspot activity (the definition of Chi) in λ *red gam* crosses (74). Note that this sequence is 11 nucleotides long vs 8 for Chi itself. With increased computing power now available (203), one might be able to alter the RecC tunnel to recognize sequences differing from Chi at one or a few nucleotides. It is interesting that *Pseudomonas syringae* RecBCD enzyme cuts DNA with high specificity at 5′-GCTGGCGC-3′ (204) closely related to Chi, although to our knowledge, the *P. syringae* sequence has not been shown to be a hotspot of recombination. Perhaps someday, recombination hotspots and their activating enzymes will be custom made.

ADDITIONAL PHYSIOLOGICAL ROLES FOR RecBCD AND CHI: CRISPR, RETRONS, AND SELF-DEFENSE

Above, we have emphasized the role of RecBCD in recombination, replication, and DSB repair, but recent studies have revealed additional physiological roles for RecBCD. We discuss two here.

A potent defense mechanism widespread in bacterial species is the cleavage of DNA at sequences incorporated into the bacterium's *c*lustered *r*egularly *i*nter-spaced *s*hort *p*alindromic *r*epeats (CRISPR) locus. Cleavage is promoted by a protein encoded by a gene neighboring the CRISPR locus when that protein is bound to an RNA copy of one of the spacers between two repeats. One such protein, Cas9 from *Streptococcus pyogenes*, is widely used in genetic engineering and DNA analysis (205). The DNA between the spacers can come, e.g., from a previous phage infection that the bacterium luckily survived. When a phage of this type infects progeny of this altered bacterium, the phage DNA is destroyed, and the bacteria survive.

Critical to this immunity system is insertion of the spacer DNA into the CRISPR locus. This DNA can come from either an infecting phage or, surprisingly, from the bacterium's own genome. In the latter case and perhaps also the former, RecBCD is involved in a Chi-regulated way. This was first observed in *E. coli* expressing the Cas1-Cas2 proteins for spacer acquisition but not the Cas proteins required for destruction (136). Deep sequencing of DNA extracted from these cells showed acquisition of *E. coli* chromosomal DNA fragments (~33 bp long in this case) into the CRISPR locus. However, the frequency of acquisition from around the chromosome is reduced by ~35% immediately to the 5′-side of 5′-GCTGGTGG-3′ (Chi) and returns to genome median ~5 to 10 kb from Chi. Note that this is the direction and distance over which Chi stimulates recombination (95, 99, 206) . In *ΔrecB*, *ΔrecC* or *ΔrecD* mutants, acquisition is reduced by ~50% and shows no reduction near Chi. These studies show that RecBCD and Chi are intimately involved in spacer acquisition.

In a strain briefly induced for I-*Sce*I endonuclease, to make a single DSB in the *E. coli* chromosome, acquisition of DNA is stimulated to both sides of the DSB but is reduced to the 5′ side of the nearest 5′-GCTGGTGG-3′ on each side of the DSB (i.e., the "top" strand on one side and the "bottom" strand on the other). These results show a role for DSBs, RecBCD, and Chi in governing the frequency of acquisition of DNA sequences into the CRISPR locus and the potential for destroying DNA with these sequences when it enters the cell again. Since *E. coli* DNA was inserted into the CRISPR locus, presumably these cells would commit suicide if, as in wt cells, the proteins for CRISPR-directed destruction were present. This may be the price cells must pay for the other cells, which incorporate foreign DNA, to survive infection and allow the population to survive. The low frequency of acquisition (136) seems to allow this possibility in the evolution of a species.

A recent study (137) found that RecBCD and RecJ ssDNA exonuclease are required to convert long DNA fragments into what appear to be the immediate precursors to insertion into the CRISPR locus by Cas1-Cas2, encoded by genes near the CRISPR locus. DNA with 23 bp and four ss tails up to 19 nucleotides long are bound by purified Cas1-Cas2; purified RecBCD and RecJ then trim this DNA to the proper form (two 33-nucleotide-long complementary strands with one four-nucleotide 3′ extension) for its insertion by Cas1-Cas2 into the CRISPR locus. Deep sequencing of DNA from cells induced for Cas1-Cas2 to acquire an *E. coli* DNA insert in CRISPR confirmed that RecB, RecC, RecD, and RecJ are required to varying extents (36%–90%) for acquisition. In one scenario, Cas1-Cas2 binds long ssDNA containing the *p*rotospacer *a*ssociated *m*otif (PAM) unique for each Cas system, such as 5′-AAG-3′ for *E. coli* Type I-E. (The PAM is adjacent to, not within, the 33 bp to be inserted into the CRISPR locus and is required for destruction when DNA enters the cell. This requirement prevents self-destruction at the CRISPR locus). Cas1-Cas2 then anneals this long DNA to its complement, which may have been made by RecBCD unwinding DNA from a DSB that initiated acquisition. Cas1-Cas2 may not bind ssDNA already bound by RecA, which would account for the decreased spacer acquisition to the 5′-side of Chi. RecBCD removes most of the DNA surrounding the site bound by Cas1-Cas2, and RecJ removes the final nucleotides to make the 33/37 nucleotide form bound by Cas1-Cas2 and inserted by it into the CRISPR locus. This scenario remains to be tested. The *recBCD* mutants discussed here should be informative in studying this defense system.

Many authors have viewed RecBCD itself as a defense system that uses Chi to distinguish self- from non-self DNA. Often, these views have claimed the destruction of invading phages by RecBCD, perhaps based on the absence of Chi in phage λ, but λ grows in wt *E. coli*. Many phages related to λ have abundant Chi sites, and many non-λ phages and bacteria, including non-enteric species, have Chi at higher density (number/Mb) than does *E. coli* [see reference (116) for a recent review]. Thus, Chi cannot be a feature for self- vs non-self-determination. These views often state that Chi occurs at higher-than-expected frequency, evidently assuming random nucleotide association in chromosomes but which is unlikely to make a living organism. The frequency of Chi in *E. coli* is readily accounted for by the high frequency of codons within Chi, such as 5′-CTG-3′ (the most frequent codon for leucine, the most abundant amino acid). It seems more likely that *E. coli*'s RecBCD evolved to recognize a frequent DNA sequence (Chi, the 21st most abundant of *E. coli*'s 65,484 octamers) to efficiently repair DSBs to live and to efficiently promote recombination to evolve.

A known bacterial system for protection against phage infection senses when RecBCD has been inhibited, e.g., by a protein such as λ Gam, and signals the cell to commit suicide so that no phage is produced and the bacterial population is saved. A clear example is the *E. coli* retron Ec48, which encodes a reverse transcriptase (RT) that uses the 2′OH on a guanosine residue in the RT mRNA to initiate DNA synthesis (207). After ssDNA 48 nucleotides long has been synthesized, RNase H trims the mRNA to leave ~119 nucleotides of RNA in the complex. This RNA-DNA hybrid somehow activates an effector protein when RecBCD has been inhibited (208). The effector protein then signals the cell to commit suicide. The effector protein for Ec48 is encoded by

the retron and appears to be a transmembrane protein, which may, upon activation, make the cell membrane permeable, allowing small molecules, such as metabolites, to escape and lead to cell death. As expected, Ec48 is lethal in a *ΔrecB* mutant, even in the absence of phage infection, presumably because the cell "surmises" that RecBCD has been inhibited. Surprisingly, Ec48 is not lethal in a *ΔrecC* or *ΔrecD* mutant, suggesting that RecB, and perhaps either its helicase domain or its nuclease domain, is sensed by Ec48 to activate cell death. Further tests with non-deletion *recBCD* mutants may help clarify the mechanism by which inhibited RecBCD, in conjunction with the RNA-DNA hybrid, leads to cell death.

With both CRISPR and retron Ec48, RecBCD is required for altruistic suicide (cell death to save the rest of the population). During DNA repair and genetic recombination, RecBCD is required for saving or genetically modifying an individual cell. The molecular mechanisms involved in these two disparate schemes (population survival vs self-survival) appear to differ fundamentally. This raises the intriguing question of which of these physiological roles of RecBCD may have evolved first. Further research on mechanisms, including the use of *recBCD* mutants, may lead to better understanding of these processes.

CONCLUSIONS FROM DECADES OF RecBCD STUDIES

RecBCD was described genetically and biochemically over 50 years ago (see Introduction). Genetic analysis since that time has been crucial in understanding not only RecBCD's physiological roles in cells but also the molecular mechanism by which this complex acts on DNA and how it is controlled. The initial observation of *recB* and *recC* mutants being sensitive to DNA damage soon led to realization that it is required for homologous genetic recombination. These observations likely played an important role in the development of recombination models emphasizing DSBs as initiators of recombination in both prokaryotes and eukaryotes (104, 209). Testing these models, by deducing the biochemical mechanisms of the required proteins, has been led by studies of RecBCD and its mutants. Early studies showed that the enzyme binds DSB ends, from which it initiates unwinding. The production of ssDNA from the end (or from a Chi site) leads to RecA's formation of joint molecules, such as Holliday junctions (Fig. 5), long postulated to be essential steps in recombination (210). Although RecBCD appears unable to resolve Holliday junctions (211), it may be involved in steps after joint molecule formation (183, 185). The basic steps of homologous recombination—DSB formation, ssDNA formation at the ends, strand invasion into homologous DNA to form a joint molecule, and resolution of these joints into recombinants—appear the same in prokaryotes and eukaryotes, although the proteins and their activities involved differ in some cases. Nevertheless, the concepts developed to understand prokaryotic recombination played significant roles in elucidating eukaryotic recombination (212). RecBCD has been central to these studies and may continue to do so as its "atomic biology" is further explored.

ACKNOWLEDGMENTS

We thank Andrew Taylor and Yihua Zhu for helpful comments on the manuscript and unpublished data, and Andrei Kuzminov, Mark Dillingham, and two anonymous reviewers for helpful suggestions. Research in our laboratory is supported by Maximizing Investigator Research Award R35 GM118120 from the National Institutes of Health of the United States of America.

We are grateful to the many people in the RecBCD and recombination communities and members of our lab who have contributed to our understanding of RecBCD and Chi, including Andrée Bruschi Epstein, Andrew Taylor, Dennis Schultz, John Swindle, Kathy Triman, Abed Chaudhury, Sam Kunes, Don Ennis, Fred Ponticelli, Keith Cheng, Chris Roberts, Aaron Neiman, Stephanie Thibodeaux, Sue Holbeck, Niki McKittrick, Patrick Dabert, Joe Farah, Sam Ganesan, Gary Braedt, Gareth Cromie, Manju Reddy, Luz Londono, Ahmet Karabulut, Jake Sharp, Alex Richardson, Khoi Ha, and Suriyen Subramaniam.

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FUNDING

Funder	Grant(s)	Author(s)
National Institutes of Health (NIH) GM	R35 GM118120	Gerald R. Smith

AUTHOR CONTRIBUTIONS

Susan K. Amundsen, Conceptualization, Data curation, Investigation, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing | Gerald R. Smith, Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental Information (MMBR00041-23-s0001.docx). Supplemental text, Fig. S1 and S2, and legend to Video S1.
Supplemental tables (MMBR00041-23-S0002.docx). Tables S1 to S9.
Video s1 (MMBR00041-23-S0003.mp4). Video of nuclease swing model.

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