Pathway of intramolecular signal transduction for Chi hotspot control of RecBCD helicase-nuclease

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Short title: RecBCD signal transduction mutants lacking Chi hotspot activation

Abstract

Life-saving repair of broken DNA by homologous recombination requires coordinated enzymatic reactions to prepare it for interaction with intact DNA. The multiple activities of enterobacterial RecBCD helicase-nuclease are coordinated by Chi recombination hotspots (5' GCTGGTGG 3') recognized during DNA unwinding. Our previous studies led to a signal transduction model in which the Chi-bound RecC subunit signals RecD, a fast helicase, to stop; RecD then signals RecB, a slower helicase, to swing its nuclease domain into position to cut DNA at Chi and load RecA protein onto the newly generated 3'-ended single-stranded DNA. Here, we report a set of mutants blocked at each step in this pathway. The mutants have helicase and degradative nuclease activities, but most have lost or reduced coordination by Chi and have reduced recombination and DNA repair proficiency. The mutant alterations are widely scattered throughout the three subunits of RecBCD; each alters the intimate contact between two subunits in the crystal or cryoEM structures, supporting the signal transduction model. Similar large conformational changes in other vital enzymes, such as DNA and RNA polymerases, may make RecBCD a paradigm for elucidating their control by the nucleic acid sites they recognize.

Author summary

Repair of broken DNA is essential for life. Faithful repair occurs by homologous genetic recombination, which also generates new gene allele combinations important for evolution. In bacteria the RecBCD helicase-nuclease enzyme is essential for most DNA break repair and is regulated by special DNA sites (Chi recombination hotspots). As it unwinds DNA, RecBCD's

activities are dramatically altered at Chi to prepare the broken DNA for interaction with intact DNA to finish repair. Here, we describe RecBCD mutants that respond weakly, or not at all, to Chi; the alterations are scattered widely throughout the enzyme, supporting our previous model of large conformational changes of RecBCD at Chi. Similar methods may help understand how other large enzyme complexes are regulated.

Introduction

Regulation of complex enzymes and reaction pathways is critical for all living organisms, because having too much or too little enzymatic activity, or having spurious activity, can be highly deleterious or lethal. This principle holds true for the repair of broken DNA, which requires in many cases large, complex enzymes. If not properly regulated, DNA repair can fail and produce incomplete or rearranged chromosomes leading to sickness or death. Here, we identify interaction domains involved in an intramolecular signal transduction pathway for regulation of the enterobacterial RecBCD helicase-nuclease by Chi hotspots of genetic recombination and DNA break repair.

When DNA is broken, its faithful repair requires interaction with intact homologous DNA, such as the sister chromosome or, in diploids, the homolog. This interaction can produce genetic recombinants if the broken and intact DNAs are genetically different, as often occurs in diploids or when linear DNA is introduced into a cell. In many bacteria, including *Escherichia coli* studied here, the major pathway of homologous recombination and DNA break repair requires RecBCD enzyme, a three subunit, 330 kDa enzyme with DNA unwinding (helicase) and DNA cutting (nuclease) activities (reviewed in [32]). RecBCD enzymes of enteric bacteria, ranging from *E. coli* to *Proteus mirabilis* and *Vibrio harveyi,* are controlled by a special DNA sequence called Chi (5' GCTGGTGG 3') [7, 12, 14]. At Chi the enzyme acts at high frequency, resulting in a high frequency of recombination. Chi is thus a genetic hotspot. After acting at Chi, RecBCD does not act at another Chi; this regulation assures a single recombination event for each DNA end and regeneration of the circular bacterial chromosome [15, 16]. The molecular mechanism of regulation by Chi has been elucidated progressively, both genetically and biochemically, since its discovery over four decades ago [1, 2, 32]. Our results reported here begin to explain at the atomic level Chi's regulation of RecBCD and may serve as an example by which other complex enzymes are regulated by large conformational changes during their reaction cycle.

The RecBCD reaction cycle begins with RecBCD binding tightly (K_D ~0.1 nM) to the end of broken DNA (Fig. 1A) [19]. The RecD helicase binds to and moves along the 5'-ended strand

rapidly (~1 kb/sec); the RecB helicase binds to and moves along the 3'-ended strand more slowly (about ½ the rate of RecD) [18, 25]. Consequently, a single-stranded (ss) DNA loop on the 3'-ended strand accumulates, presumably ahead of RecB, and grows with time [6, 13]. The crystal and cryoEM structures of RecBCD bound to DNA show that, when the 3'-ended strand emerges from the RecB helicase, it enters a tunnel in RecC (Fig. 1B) [26, 42]. Mutational analysis indicates that the Chi sequence on the 3'-ended strand is recognized within the RecC tunnel [9, 31, 34].

Chi recognition results in multiple changes in the conformation and activities of RecBCD [16, 24, 33]. A "patch" on the surface of RecC becomes sensitive to proteases when Chi is encountered during DNA unwinding (Fig. 1C – E). This patch is also protease-sensitive before DNA is bound (Fig. 1C) but becomes resistant upon DNA binding and remains resistant during initial unwinding up to Chi (Fig. 1D). Comparison of small angle X-ray scattering envelopes of RecBCD free or bound to DNA with the RecBCD crystal structure indicates that, upon binding of RecBCD to DNA, the RecB nuclease domain swings from a point near the exit of the RecC tunnel to the patch on RecC, thereby accounting for the differential protease-sensitivity of this patch (Fig. 1C, D, E) [26, 33]. Upon encountering Chi, the nuclease domain is proposed to swing back to the RecC tunnel exit (Fig. 1E), cut the emerging 3'-ended strand a few nucleotides 3' of the Chi octamer, and begin loading RecA DNA strand-exchange protein onto the newly generated 3'-ended strand [11, 21, 33]. The RecA-ssDNA filament can invade homologous DNA, and subsequent reactions can produce intact DNA and reciprocal or non-reciprocal recombinants (Fig. 1A) [8, 32]. The research reported here is focused on the events from Chi recognition to RecA loading.

Based on the remarkable behavior of novel *recB* mutants altered in the RecB helicase domain, we proposed a signal transduction model for the changes of RecBCD after Chi recognition (Fig. 1F) [28]. In this model, RecC recognizes Chi (step 1) and signals RecD helicase to stop (step 2); RecD then signals RecB nuclease domain to swing (step 3) and to cut DNA and load RecA protein (step 4). While this signal transduction pathway is consistent with the properties of RecBCD and Chi described above, direct evidence for the pathway has been lacking. The RecBCD mutants described here and previously support each of the four steps of the Chi-initiated signal transduction pathway. The principles discussed here may apply to other complex enzymes that undergo large conformational changes during their action in various types of metabolism, from fatty acid synthesis to transcription.

Results

Experimental rationale

To test the signal transduction model, we sought *recBCD* mutants altered at each of the four steps described above. Step 1 (Chi recognition in the RecC tunnel) has been tested previously and strongly supported: >30 mutations altering the RecC tunnel reduce or abolish Chi hotspot genetic activity and, in the cases tested, cutting of DNA at Chi, the most direct assays for Chi recognition [5, 9, 10, 31, 34]. (There is no direct assay for RecBCD recognizing Chi, such as binding of Chi DNA to RecBCD, since Chi is recognized only during active unwinding by RecBCD; consequently, only the effect of Chi can be assayed, such as cutting of DNA at Chi and Chi genetic hotspot activity.)

To isolate mutants altered in other steps, we examined crystal and cryoEM structures of RecBCD [26, 37] for points of contact between RecC and RecD (step 2), between RecD and RecB (step 3), and in or near the RecB nuclease domain, which binds RecA protein and can load RecA onto Chi-cut DNA (step 4) (Fig. 2; Table 1) [27]. Contact points for steps 3 and 4 were discussed by Wilkinson et al. [37] (see Discussion). We then mutated, by deletion or amino acid substitutions, these points of contact and assayed Chi hotspot activity in phage lambda crosses and recombination-proficiency in *E. coli* Hfr matings. Mutants with reduced or abolished Chi hotspot activity were assayed for DNA unwinding and cutting of DNA at Chi as well as Chi-independent nuclease activity. As described below, we isolated multiple mutants altered at each step. Many of these mutants have reduced or undetectable Chi genetic activity and have lost or retained enzymatic activities that support the signal transduction model (Fig. 1F).

RecC-RecD interaction for model step 2 – RecC signals RecD to stop unwinding

In the published crystal and cryoEM structures (Figs. 2A and 2B) [26, 37, 42], four contiguous amino acids (here named C2) in RecC make intimate contact with three contiguous amino acids (named D2) in RecD (Fig. 2C; Table 1). Deletion of C2 (C2Δ) strongly reduced Chi hotspot activity to 1.5 (from 5.1 in wild type), and deletion of D2 (D2Δ) abolished Chi hotspot activity (Chi activity = 1.0) (Table 2). [Chi hotspot activity is the ratio of the recombinant frequency in an interval with Chi to the frequency in the same interval without Chi. A value of 1 thus indicates no Chi hotspot activity; in the standard Chi hotspot crosses used here, Chi stimulates recombination in the intervals measured by a factor of 5.1 in wild-type cells [5] (Table 2).] As expected, the double mutant C2Δ D2Δ also had no significant Chi activity. Substitution of alanine for the amino acids of C2 or D2 (C2ala or D2ala) also significantly reduced Chi activity, to 3.3 or 2.7, respectively (Table S1). These mutants had slightly reduced recombination

proficiency in Hfr matings (~40 – 80% of wt); a *recBCD* null mutant's proficiency is 0.2% of wt (Tables 2 and S1; Fig. 3B). Thus, as predicted, Chi activity requires the C2-D2 contact between RecC and RecD.

RecD-RecB interaction for model step 3 – RecD signals RecB to swing its nuclease domain

In the cryoEM structures of RecBCD bound to ds DNA [37, 42], four contiguous amino acids with overall positive charge (QPSR in the center of D3) in RecD contact an α-helix of 13 amino acids (B3), six with negative charge (D or E), in RecB (Fig. 2D; Tables 1 and 3). As for the C2-D2 interaction, complete deletion of B3 or the central part of D3 eliminated Chi hotspot activity (Table 3A and B). Substitution of alanine for the central four amino acids in D3 or for the six negative amino acids in B3 or both changes strongly reduced Chi activity (to 1.7, 1.7, and 1.5, respectively; Table 3A – C). A second double mutation (B3ala D3Δ) eliminated Chi activity (1.1). Hfr recombination proficiency was reduced to 20 – 50% of the wild-type value, except in the B3Δ single mutant, which had recombination proficiency similar to that of a *recBCD* null mutant (0.1% of wild type; Table 3B). These results show that the D3-B3 contact is essential for Chi activity.

As noted above, deletion of the central four amino acids in D3 (QPSR) eliminated Chi activity. To determine which of these four amino acids is critical, we deleted separately the left two (QP) and the right two (SR) amino acids. Surprisingly, both mutants had nearly full Chi activity (4.4 and 5.0, respectively; Table S2A). We then deleted other combinations of amino acids in D3 (SVQPSRLP). Some of these deletions eliminated Chi activity (e.g., deletion of SV, of QP plus LP, or of SRLP), and others strongly reduced Chi activity (e.g., deletion of V, to 1.5). These results show that D3 and its surround are critical for Chi activity and suggest a more complex effect than simple deletion of a few amino acids as the basis for loss of Chi activity. Indeed, except in one case, substitution of alanine for one to four of the amino acids in SVQPSRLP also significantly reduced Chi activity (to 1.4 – 3.5).

We similarly explored the requirements for amino acids in B3 (DEHAWDVVVEEFD). Deletion of only 3, 5, 8, or 10 amino acids, collectively spanning all points of the 13 amino acids deleted in ΔB3, left substantial Chi activity (2.9 – 4.4; Table S2B), suggesting that no individual part of B3 is essential. Four or six alanine substitutions distributed throughout B3 substantially reduced Chi activity (to ~2.0). Other substitutions in B3 also significantly reduced Chi activity (e.g., ……E..D to ……Q..S had Chi activity of 3.4, and .R...N...QK.S had Chi activity of 3.0. Substitutions of only two D or E residues with K or R left nearly full Chi activity (4.8 and 4.1), but

similar K and R substitutions of four or five residues nearly eliminated Chi activity (1.8, 1.6, and 1.3). Thus, both the presence and the amino-acid composition of B3 are critical for Chi activity.

Double B3 D3 mutants were similar to the stronger single mutant, except in one case. An additive effect of .R...N...QK.S in B3 and ..E.DE.. in D3 was observed in the double mutant: Chi activities were 3.0, 3.2, and 1.8, respectively (Table S2). Thus, the role of the B3 and D3 amino acids is more complex than those of C2 D2, but nevertheless this couple plays an essential role in Chi hotspot activity.

RecB-RecC interaction for model step 4 – RecB nuclease domain cuts DNA at Chi and loads RecA protein

In the published crystal structures of RecBCD [26, 29], a 10-amino-acid α-helix (B4) in the RecB nuclease domain is close to a 25-amino-acid α-helix in RecC; we define C4 as the seven amino acids at the N-terminus of the α-helix plus the adjacent three amino acids, which form a turn (Fig. 2E; Table 4). The B4 α-helix has been postulated to control RecBCD nuclease activity [26, 37], but deletion of B4 only marginally reduced Chi activity (to 4.3) and had no significant effect on Chi-independent nuclease activity (Tables 4 and 5). Deletion of C4, however, eliminated Chi activity, as did the double deletion (B4Δ C4Δ) (Table 4). Deletion of seven amino acids in C4 also strongly reduced Chi activity (to 2.3) (Table 4). Substitutions with alanine of the ten amino acids in B4 or three of the ten amino acids in C4 slightly reduced Chi activity (to 3.7 and 4.4, respectively; Table S3A and B). These results show that at least part of C4 is essential for Chi activity but that B4 plays at most a minor role on its own.

We noted that the B4 and C4 α-helices ordered in the crystal structure are close to a seven-amino-acid loop of RecD (D4, amino acids 469 – 475; Fig. S1) in the cryoEM structures (PDB 5LD2, 6SJB, 6SJE, 6SJF, 6SJG, 6T2U, and 6T2V; Fig. 2B) [37, 42]. This loop in RecD and 50 surrounding amino acids are disordered in both molecules in the asymmetric unit of both crystal structures (PDB 1W36 and 3K70; Fig. 2A) [26, 29], and B4 is disordered in the cryoEM structures. This outcome indicates that B4 and the loop in RecD can adopt alternative structures. Deletion of this loop or changing each amino acid to alanine had little if any effect on Chi activity (3.9 or 4.4) or recombination potential (74 or 83% of wild type) (Table S3C). Control of step 4 thus appears to be largely independent of this loop.

Recombination proficiency is positively correlated with Chi hotspot activity

The critical role of Chi in regulating RecBCD's activities predicts that lowering the Chi hotspot activity would also lower recombination proficiency. We tested this notion by plotting the two

values for each of the mutants in the four separate steps of Chi-initiated signal transduction (Fig. 3). The correlation was significantly positive (R^2 = 0.60, 0.58, 0.55, and 0.76 for steps 1, 2, 3, and 4, respectively; p = <0.0001, 0.047, <0.0001, and <0.0001, respectively). We noted that the intercept of Hfr recombination proficiency at no Chi hotspot activity (0.27; mean of the four values in Fig. 3) was comparable to the ratio of lambda recombination without and with Chi (0.20). In other words, removing a Chi site reduces lambda recombination to about the same extent as removing the signal transduction contact points for Hfr recombination. Residual recombination reflects RecBCD's innate ability to promote recombination without Chi or the signal it elicits.

Signal transduction mutants have DNA unwinding and general nuclease activity but have lost Chi cutting

To test more directly the effect of the mutations on RecBCD activity, we assayed DNA unwinding, cutting of DNA at Chi, and "general" (Chi-independent) nuclease activities. Representative mutants altered at each step were tested. In some cases, the mutants retained approximately wild-type levels of DNA unwinding activity (Figs. 4 and S2), but in other mutants there was significant reduction. As expected, Chi cutting was observed in mutants with full or partial Chi hotspot activity (>4.3) but not in mutants lacking Chi activity (<1.6). Chi cutting was not observed in step 2 mutants with partial Chi genetic activity (2.2 – 3.4) but was observed in two step 3 mutants (B3ala and D3ala) with low Chi hotspot activity (1.7 – 1.8) (Table 5). The difference may reflect intracellular *vs.* extracellular conditions for the assays. The double step 3 mutant (B3ala D3ala) had low Chi hotspot activity (1.5) in cells and lacked Chi cutting outside cells, as expected.

Chi-independent nuclease activity was nearly wild-type in some mutants but was low in others (Table 5). In most of the latter cases, nuclease activity was about 2-fold higher with 1 mM ATP than with 25 μM ATP; wild-type RecBCD activity was about 2-fold <u>lower</u> with 1 mM ATP, as reported previously [3]. Possible explanations for this phenotype are in the Discussion. All of the mutants studied here do, however, block the growth of T4 *gene 2* mutants, showing that they retain nuclease activity in living cells [4].

Discussion

The results reported here strengthen the previously proposed signal transduction model for the control of RecBCD enzyme by Chi hotspots of recombination [28]. Points of contact between the enzyme's subunits are essential for Chi activity, and mutants altered at each step of the

model comport with the predictions of the more detailed nuclease-swing model (Fig. 1C – E) [33]. The many mutants described here provide important material to test these models further by more direct assays.

The mutants studied here have a wide range of effects on Chi activity, from no significant effect to complete loss of Chi activity. At each of the three postulated steps, we found mutants that lacked Chi activity. For step 2 (Chi-bound RecC signals RecD to stop) and step 3 (when stopped, RecD signals RecB to swing its nuclease domain), we found such mutations in each of the interacting subunits (RecC-RecD and RecD-RecB, respectively). For step 4 (the nuclease domain is positioned to cut DNA at Chi and to load RecA), mutations in RecC abolished Chi activity, but none of the RecB or RecD mutants tested dramatically lowered Chi activity. Because the nuclease active site is in RecB [23] and part of RecC essential for Chi activity (C4 in Table 4) is close to the RecB nuclease domain, it seems likely that RecC and RecB contact each other to effect this step. Thus, our choosing points of contact to mutate, based on the crystal and cryoEM structures, successfully identified areas of RecBCD far from the Chi-recognition and nuclease sites critical for Chi's control of RecBCD.

It is noteworthy that two of the points of contact are positioned differently in the crystal and cryoEM structures (Figs. 2A and 2B). The RecD-RecB contact points for step 3 intimately interact in the cryoEM structures (Fig. 2B and 2D) but are >15 Å apart in the crystal structures (Fig. 2A). Conversely, the RecC-RecB contact points for step 4 intimately interact in the crystal structures (Fig. 2A and 2E) but are >20 Å apart in the cryoEM structures. This is evidence that these points can move relative to each other, in accord with the models tested here. In addition, one of the proposed contacts for step 4 (B4) is ordered in the crystal structure but not in the cryoEM structures. This outcome also indicates flexibility in contact points, as expected for parts of the protein that interact differently during steps of the reaction cycle.

Based on the crystal and cryoEM structures of RecBCD bound to a set of forked DNA substrates with increasingly long 5' single-stranded DNA extensions, Wigley's group has discussed the roles of contact points 3 and 4. When the 5' extension was elongated from four to ten nucleotides, parts of RecD became more ordered [26, 29]. An additional two nucleotides on the 5' extension resulted in larger changes [37]. The SH3-like domain of RecD became well-ordered, and contact point D3, in the SH3 domain, touched contact point B3. There was also a major change in the B4 α-helix. The C4 α-helix moved a few Å and apparently allowed the B4 α-helix to become disordered and to move away from the RecB nuclease active site. They postulated that this movement of ~15 Å of what they termed the RecB "linker," which contains

B4, was necessary to convert RecBCD from an inactive nuclease (when bound to a ds DNA end) into an active nuclease (during unwinding).

Comparison of cryoEM structures of RecBCD bound to a 5' extension without Chi or with Chi appropriately placed revealed additional small changes in the position and orientation of the RecB nuclease domain [42]. These changes were postulated to inactivate the nuclease after making its final cleavage, on the 3'-ended strand upon encountering Chi, a property of purified RecBCD under reaction conditions with high [Mg^{2+}] but not, the evidence indicates, in living cells [17, 32, 36]. With high [Mg^{2+}], purified RecBCD begins to cut the 5'-ended strand at Chi [20, 22], but the basis for this switch in strand cutting was not addressed by Cheng et al. [42]. The predictable consequences of these structural changes were not tested, for example by the type of mutational analysis used here. Our data here demonstrate the importance of the points of contact in the structures reported by Wigley's group.

A quarter of the mutations altering the contact points (16 of 63 analyzed here) retained nearly full Chi hotspot activity (4.1 – 5.0), recombination proficiency, and the enzymatic activities tested (Tables 5 and S1 – S3; Figs. 4 and S2). Thus, some alterations as great as deletion of ten amino acids [B3Δ(10) in RecB at step 3; Tables 5 and S2B] had little effect. This outcome shows that not all structural alterations, even large ones, block Chi's control of RecBCD and its other activities. It also supports the view that the contact points in mutants that do have an effect have a direct role in Chi activity as proposed in the nuclease-swing model (Fig. 1C – E). Additional points in RecBCD are also likely critical for Chi activity, but more complex analyses, such as molecular dynamics, may be required to reliably predict points to mutate.

Here and elsewhere, we have identified five points in RecBCD enzyme at which mutations reduce or abolish Chi activity. These include mutations in the RecC tunnel (Chi recognition, step 1 [9, 31, 34]) and in each of the subunits for steps 2, 3, and 4 (Tables 2 – 4 and S1 – S3; Figs. 1 and 2). Other mutations in the RecB tether connecting the RecB helicase and nuclease domains show that the tether has to be long enough and sufficiently flexible to swing and act at Chi (Fig. S3) [38]. Each of these pairs of contact points spans a distance as great as 80 Å; the maximal distance between points in RecBCD is ~140 Å. The sum of distances from Chi recognition to point 2, then to point 3, then to point 4, and finally to the nuclease active site is ~185 Å. The signal from Chi to the nuclease domain thus traverses much of the expanse of the enzyme even though in the reported structures the Chi recognition site is only ~25 Å from the nuclease active site. This result indicates a high degree of coordination among the three subunits to control the numerous activities of RecBCD.

The mutants described here provide excellent material for more direct tests of the nuclease-swing model. Possible methods include Förster resonance energy transfer (FRET). RecBCD enzyme with fluorescent moieties at two points, for example on the nuclease domain and at the RecC tunnel exit, could show that the nuclease domain does swing as inferred from previous studies (Fig. 1C – E) [33]. Mutants altered in step 1 (recognition of Chi in the RecC tunnel), step 2, or step 3 are predicted not to change the FRET signal when RecBCD encounters Chi during unwinding, unlike wild type. Step 4 mutants may still swing the nuclease domain but not position it properly for Chi cutting, and therefore the FRET signal in step 4 mutants would change at Chi as in wild type. These structural changes might also be visible by cryoEM of RecBCD stopped during active unwinding before and after Chi. Similar predictions are also made for single-molecule unwinding experiments, such as those using DNA curtains [30]. Step 1 and step 2 mutants are predicted to have no pause in unwinding at Chi, whereas step 3 and step 4 mutants are predicted to pause. These methods require advanced equipment not currently available for our studies.

Some signal transduction mutants unexpectedly had lower “general” (ATP-dependent but Chi-independent) nuclease activity than the wild type (Table 5). These mutants’ nuclease activities were higher with 1 mM ATP than with 0.025 mM ATP, the concentration in the standard nuclease assay [3], whereas the wild-type nuclease activity is <u>lower</u> with 1 mM ATP, which is more nearly physiological. In the nuclease swing model (Fig. 1C – E), the nuclease domain can be positioned at the exit of the RecC tunnel, where it can cut DNA, or on the protease-protected “patch” of RecC, where it cannot cut DNA [33]. We propose that in these mutants the nuclease domain remains, during unwinding, more often than in wild type at the protease-sensitive patch on RecC and thus far from the RecC tunnel exit and unable to cut DNA frequently (Fig. 1C – E). In this view, the activity of the nuclease reflects the fraction of time the nuclease domain resides at the tunnel exit. This fraction may be altered by the mutations studied here and by the ATP concentration.

The approach we have used may be useful to study other complex proteins that undergo large conformational changes during their reaction cycle. *E. coli* RNA polymerase, for example, contains a 10 amino acid "trigger loop," which covers the NTP binding site when the proper NTP is bound, apparently to hold it in place for addition to the growing RNA molecule. The tip of the trigger loop moves ~20 Å when the wrong, or no, NTP is present, apparently to allow only the correct NTP to occupy the active site for RNA synthesis [41]. Mutational analysis of the corresponding part of *Saccharomyces cerevisiae* RNA polymerase II indicates that amino acids in the trigger loop interact with each other and with parts of the polymerase in other subunits,

though close in 3D space and thus near the active site for RNA synthesis [35]. We are not aware of mutational analyses of sites far from the active site of large enzymes akin to those reported here. We expect, however, that co-ordination of multiple activities of a large enzyme, such as RNA polymerase moving along the DNA template and adding correct NTPs, would require communication between distant parts of the enzyme, as discussed here for RecBCD. Kinesin and ATP synthase also undergo large conformational changes during their reaction cycles [39, 40] and may be amenable to the type of analysis used here. RecBCD is large, has a complex reaction cycle, and is not essential for growth, making it readily amenable to mutational analysis. RecBCD may therefore provide an excellent paradigm for studies in this area.

Materials and methods

Site-specific mutagenesis

Mutations were generated on plasmid pSA607, which contains the three genes of RecBCD controlled by their native promoters [33], using the QuikChange Site-Directed (Agilent Technologies) or the Q5 Site-Directed (New England Biolabs) mutagenesis kit. Oligonucleotides with the desired mutations were designed using web-based programs (Agilent Primer Design Program or NEBaseChanger) and purchased from Integrated DNA Technologies. Each mutant plasmid was confirmed by sequencing at least 300 bp on each side of the intended mutation, the only mutation detected. DNA primers for generating these mutations are available upon request. Plasmid pSA607 contains the *recC2773* mutation, which places six histidine residues on the RecC C-terminus to aid protein purification; this mutant has a wild-type phenotype [33]. Allele numbers and codon changes are in Tables S1 – S3.

Genetic assays for Chi hotspot activity and homologous recombination

Mutant plasmids were introduced by calcium-mediated transformation into strain V2831 (*ΔrecBCD2731<kan> argA21 recF143 hisG4 met rpsL31 galK2*); *ΔrecBCD2731* deletes the entire *recBCD* gene cluster [28]. These transformants were tested for Chi hotspot activity, lambda recombination-proficiency, and *E. coli* recombination-proficiency using Hfr strain S727 (PO44 *thi-1 relA1*), as described [9].

Enzymatic assays for general nuclease activity, DNA unwinding, and Chi cutting

RecBCD Chi-independent ("general") nuclease activity was assayed using uniformly [^{3}H]-labelled DNA from phage T7 [3]. Duplicate assays, each with three amounts of extract in the linear range, are given in many cases in Table 5. Background values (*i.e.,* without ATP)

determined in each set of assays were subtracted; values ranged from 21 to 58 units/mg of protein (37 ± 10 units/mg averaged over all assay sets). Protein concentrations were determined with a BioRad protein assay kit, using bovine serum albumin as standard. DNA unwinding and cutting at Chi were assayed using pBR322 with or without Chi (χ^+*F225*), linearized with *Hin*dIII, and 5'-end labeled using [γ-^{32}P] ATP and phage T4 polynucleotide kinase [38]. Reaction products were separated by agarose gel electrophoresis and transferred to Whatman 3MM paper instead of DEAE paper; oligonucleotide products, seen in previously published results (e.g., [38]), were thus not detected.

Supporting information

Tables S1 – S3 contain recombination data for additional mutants in steps 2 – 4, respectively. Fig. S1 shows contact points B4, C4, and D4. Fig. S2 shows additional unwinding and Chi cutting activities of additional mutants. Fig. S3 shows the correlation of Chi hotspot activity and Hfr recombination proficiency for RecB tether mutants.

Acknowledgments

For helpful discussions of protein structure, we are grateful to Rick Dahlquist, Brett Kaiser, Craig Kaplan, and Rick McLaughlin. We thank Randy Hyppa for helpful comments on the manuscript. This research was supported by grants R35 GM118120 to G.R.S. and P30 CA015704 to the Fred Hutchinson Cancer Research Center from the National Institutes of Health of the United States of America.

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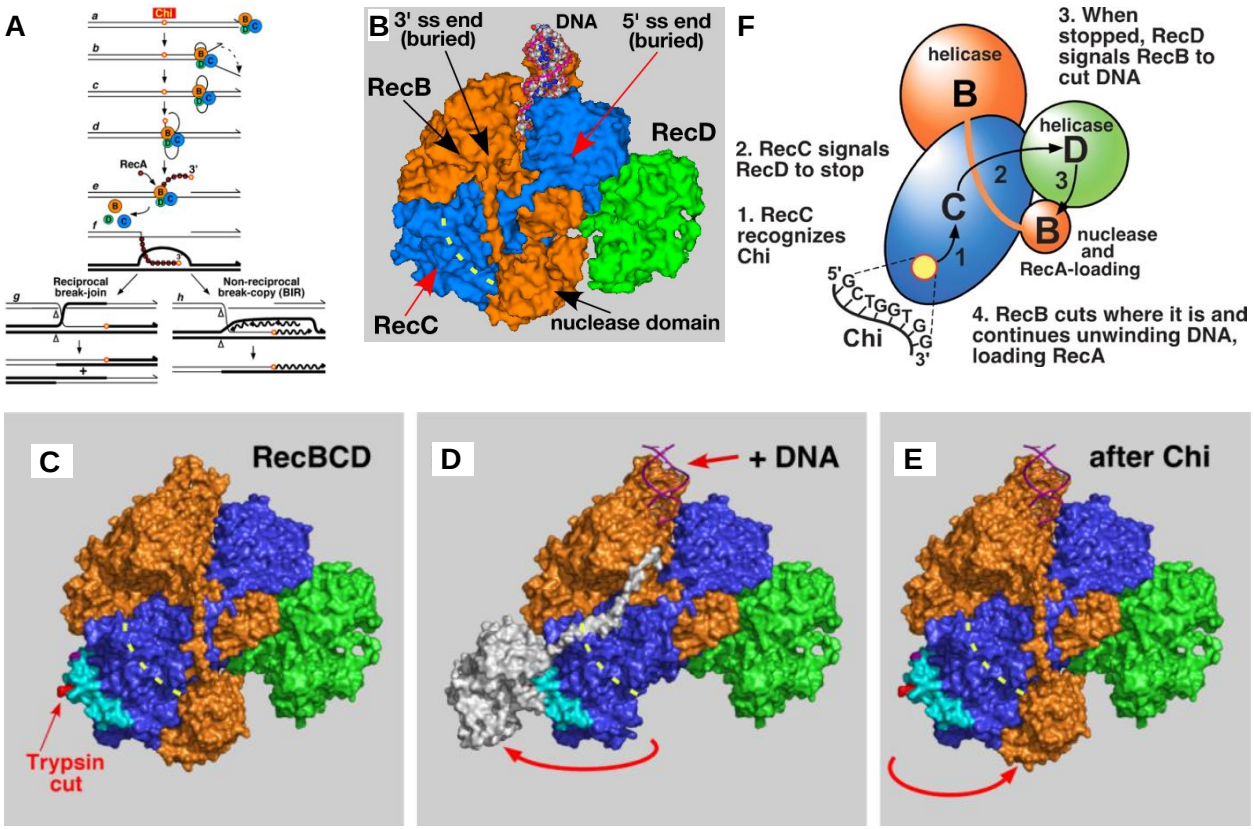

Fig. 1. Model for homologous recombination and DNA break repair by RecBCD enzyme and its control by Chi hotspots. (**A**) Pathway of RecBCD-promoted recombination and DNA break repair [8, 32]. RecBCD binds a ds DNA end (*a*) and unwinds the DNA, producing loop-tail structures (*b*) that are converted into twin-loop structures (*c*) by annealing of the tails. At Chi, it nicks the 3'-ended strand (*d*) and loads RecA (*e*). The ssDNA-RecA filament invades intact homologous DNA to form a D-loop (*f*), which can be converted into a Holliday junction and resolved into reciprocal recombinants (*g*). Alternatively, the 3'-end in the D-loop can prime DNA synthesis and generate a non-reciprocal recombinant (*h*). See ref. [32] for discussion of other alternatives. (**B**) Atomic structure of RecBCD bound to DNA (PDB 1W36) [26]. RecB is orange, RecC blue, and RecD green. Yellow dashed line indicates the RecC tunnel in which Chi is recognized. (**C, D, E**) Nuclease swing model for Chi's control of RecBCD [33]. Cyan indicates the RecC "patch" that is differentially sensitive to proteases. Grey indicates the RecB nuclease domain and the tether connecting it to the RecB helicase domain; it was positioned by hand in the middle panel (**D**). (**C**) Before DNA is bound. (**D**) After DNA is bound but before Chi is encountered. (**E**) After Chi is encountered during unwinding. (**F**) Signal transduction model for RecBCD regulation by Chi [28]. Modified from [33].

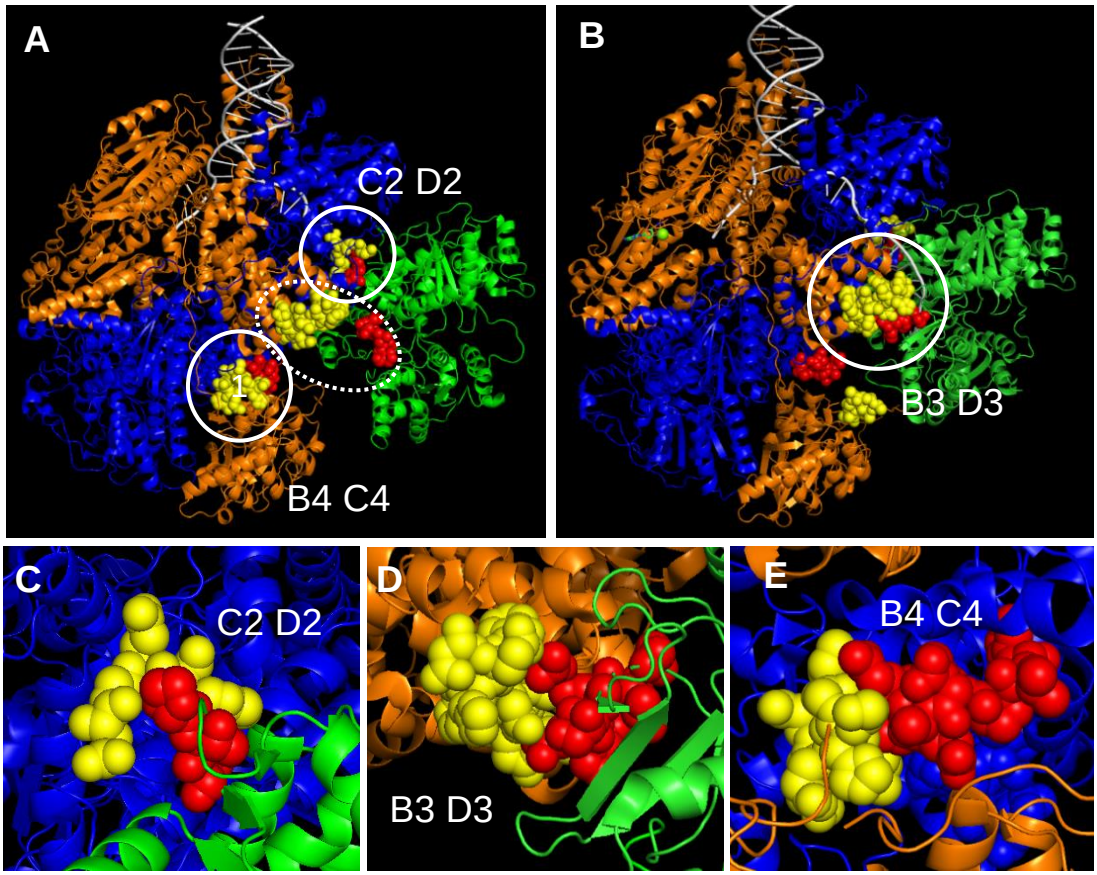

Fig. 2. Contact points between RecBCD subunits tested for a role in Chi signal transduction (steps 2, 3, and 4). Atomic structures of RecBCD showing contacts between RecB (orange), RecC (blue), and RecD (green); DNA is grey. Points of contact studied here are represented as spheres; the rest of the molecule is represented as cartoons (PyMol version 2.2.0). Note that the RecB-RecD contacts at step 3 are separated by >20 Å in the crystal structure (dotted circle in **A**) PDB 1W36 [26] but are close to each other in the cryoEM structure (solid circle in **B**) PDB 5LD2 [37, 42]. Note that, conversely, a domain of ~70 amino acids in RecD is ordered in the cryoEM structures but disordered in the crystal structures. (**C**) Step 2. C2 (amino acids QGEW at positions 541 – 544 of RecC) is yellow, and D2 (PTP at positions 97 – 99 of RecD) is red. Shown is part of the crystal structure PDB 1W36. (**D**) Step 3. B3 (DEHAWDVVVEEFD at positions 634 – 646 of RecB) is yellow, and D3 (amino acids SVQPSRLP at positions 521 – 528 of RecD) is red. Shown is part of the cryoEM structure PDB 5LD2. (**E**) Step 4. B4 (amino acids GHGIAQDLMP at positions 913 – 922 of RecB) is yellow, and C4 (amino acids FLPDAETEAA at positions 599 – 608 of RecC) is red. Shown is part of the crystal structure PDB 1W36.

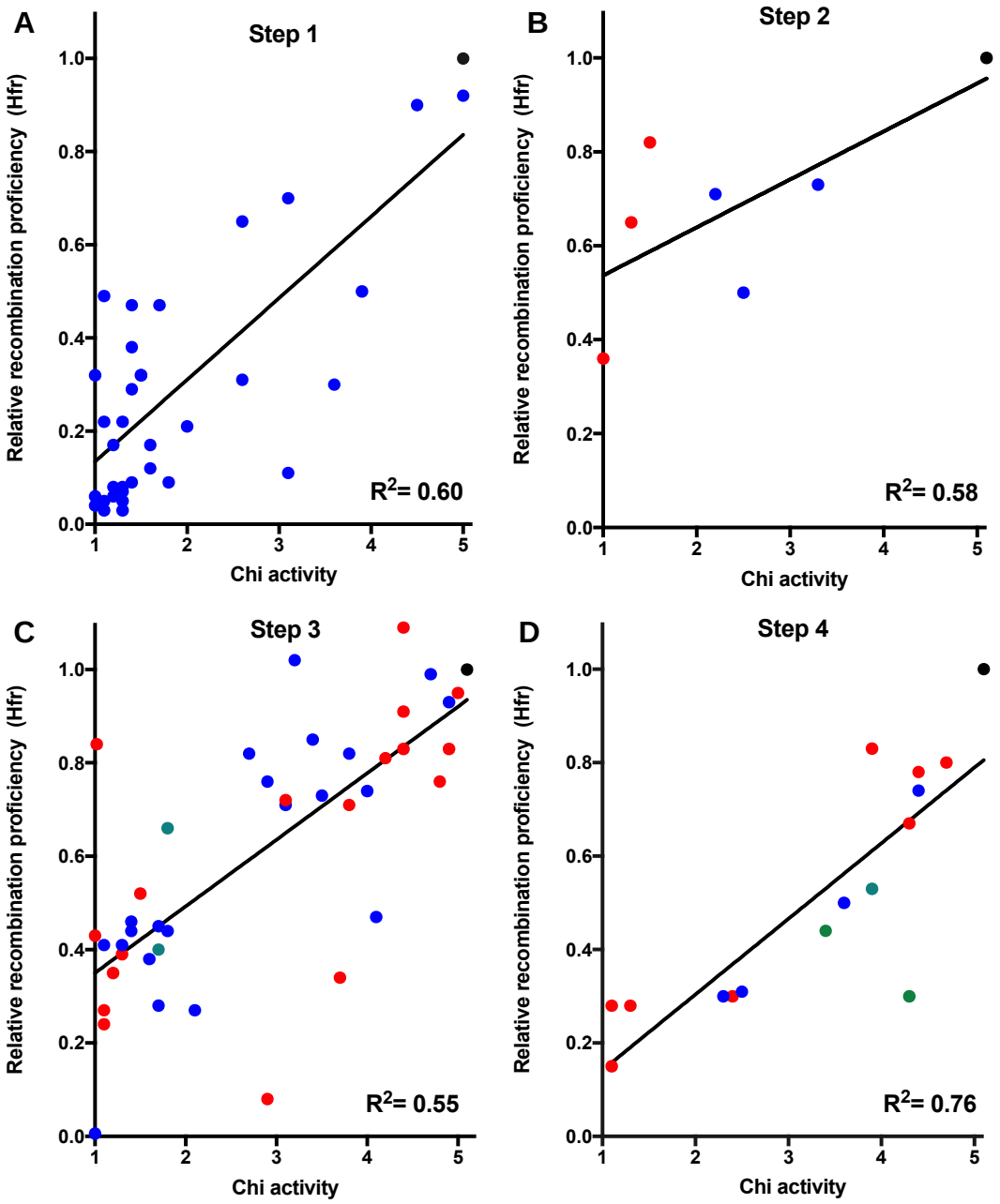

Fig. 3. Recombination proficiency is positively correlated with Chi hotspot activity. Red data points are for deletion mutants, blue for substitution mutants, and green for mutants with a substitution and deletion; black point is wild type. Linear regression lines and the coefficient of determination (R^2) are shown. Data are from Tables 2, 3, 4, S1, S2, and S3. (**A**) Step 1 mutants – RecC recognizes Chi [9, 31, 34]. (**B**) Step 2 mutants – Chi-bound RecC signals RecD to stop unwinding. (**C**) Step 3 mutants – RecD signals RecB nuclease domain to swing. (**D**) Step 4 mutants – RecB nuclease domain cuts DNA at Chi and loads RecA.

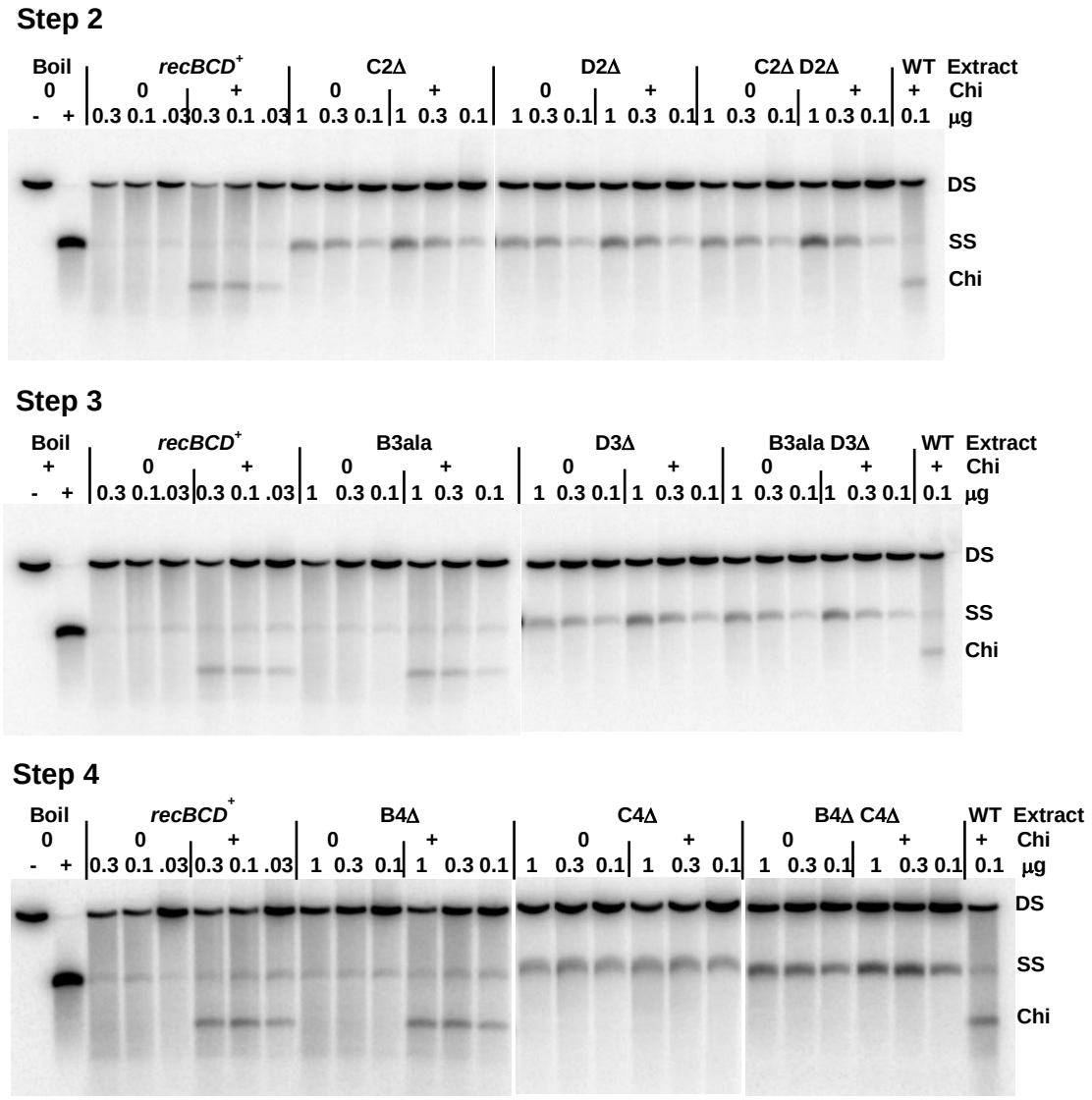

Fig. 4. Signal transduction mutants retain DNA unwinding activity but have reduced or undetectable cutting of DNA at Chi hotspots. Extracts of *recBCD*$^+$ (wild type or WT) and the indicated mutants were assayed for unwinding and cutting of linear pBR322 DNA (4.3 kb long) with or without a Chi site (χ^+*F225*) 1470 bp from the 5' [^{32}P]-labelled DNA end. ds substrate (DS), unwound ss DNA (SS), and Chi-cut DNA (Chi) are indicated.

Table 1. Steps of Chi-initiated signal transduction in RecBCD enzyme

Step in signal transduction[a]	Subunit interaction contact points[b]	Amino acid positions[b]
1. RecC recognizes Chi	DNA and RecC tunnel	38,39,41,64,70,88,133, 136,186,652,708
2. RecC signals RecD to stop	RecC2	541-544
	RecD2	97-99
3. RecD signals RecB nuclease domain to swing	RecD3	521-528
	RecB3	634-646
4. RecB nuclease domain is positioned to cut DNA at Chi and load RecA	RecB4	913-922
	RecC4	599-608

[a] See Fig. 1F and [28].

[b] See Fig. 2, Tables 2 – 4, and [9, 31, 34].

Table 2. Step 2 – RecC signals RecD to stop

Allele description[a]	C2[b] 541-544 QGEW	D2[b] 97-99 PTP	Chi hotspot activity[c]	*E. coli* Hfr cross (relative recombinant frequency)[d]
WT	. . . .	. . .	5.1 ± 0.09	≡1
C2Δ	ΔΔΔΔ	. . .	1.5, 1.4	1.0, 0.64
D2Δ	. . . .	ΔΔΔ	0.9, 1.0	0.43, 0.29
C2Δ D2Δ	ΔΔΔΔ	ΔΔΔ	1.4, 1.1	0.81, 0.49

[a] The indicated mutation was on a derivative of plasmid pSA607 (*recBCD*$^+$) in strain V2831 (Δ*recBCD2731*). Complete genotypes and the amino acids altered are in Tables S1 – S3.

[b] The contact points are composed of the indicated amino acids at the indicated positions. The alleles have mutations deleting the indicated amino acid (Δ); substitutions (when made) contain the indicated amino acid; (.) indicates that the amino acid is wild type.

[c] Chi hotspot activity in lambda vegetative crosses was determined as described in Materials and Methods (Stahl and Stahl, 1977). Chi hotspot activity = $\sqrt{(t/c)_1/(t/c)_2}$ where t/c is the ratio of turbid (*cI*$^+$) to clear (*cI857*) recombinant plaques among J^+R^+ recombinants in cross 1 (with χ^+*D123*) and cross 2 (with χ^+*76*).

[d] Frequency of His$^+$ [Str^R] recombinants per viable Hfr parent relative to that in the concurrent *recBCD*$^+$ cross. Wild-type frequency was 6.99% ± 0.46% (n = 25). The relative recombinant frequency for a null mutant (*recB21*) is 0.005.

Table 3. Step 3 – RecD signals RecB nuclease domain to swing

A.

Allele description[a]	D3[b] 521-528 SVQPSRLP	Chi hotspot activity[c]	*E. coli* Hfr cross (relative recombinant frequency)[d]
WT	.....	5.1 ± 0.09	≡1
D3ala	..AAAA..	1.9, 1.4	0.41, 0.49
D3Δ	..ΔΔΔΔ..	1.0, 1.2	0.24, 0.24

B.

Allele description[a]	B3[b] 634-646 DEHAWDVVVEEFD	Chi hotspot activity[c]	*E. coli* Hfr cross (relative recombinant frequency)[d]
WT	.....	5.1 ± 0.09	≡1
B3ala	AA...A...AA.A	1.8, 1.6	0.21, 0.34
B3lys,arg	KR...K...RR..	1.2, 1.4	0.39, 0.42
B3Δ	ΔΔΔΔΔΔΔΔΔΔΔΔΔ	0.9, 1.0	0.006, 0.009

C.

Allele description[a]	B3[b] 634-646 DEHAWDVVVEEFD	D3[b] 521-528 SVQPSRLP	Chi hotspot activity[c]	*E. coli* Hfr cross (relative recombinant frequency)[d]
WT	.....	.....	5.1 ± 0.09	≡1
B3ala D3ala	AA...A...AA.A	..AAAA..	1.5, 1.4	0.48, 0.39
B3ala D3Δ	AA...A...AA.A	..ΔΔΔΔ..	1.1 ± 0.06	0.53, 0.34

Footnotes are as in Table 2.

Table 4. Step 4 – RecB nuclease domain is positioned to cut DNA at Chi and load RecA

Allele description[a]	B4[b] 913-922 GHGIAQDLMP	C4[b] 599-608 FLPDAETEAA	Chi hotspot activity[c]	*E. coli* Hfr cross (relative recombinant frequency)[d]
WT	. . . . .	. . . . .	5.1 ± 0.09	≡1
B4Δ	ΔΔΔΔΔΔΔΔΔΔ	. . . . .	4.2, 4.3	0.81, 0.52
C4Δ	. . . . .	ΔΔΔΔΔΔΔΔΔΔ	1.1 ± 0.12	0.24 ± 0.04
C4Δ(7)	. . . . .	. . . ΔΔΔΔΔΔΔ	2.3 ± 0.12	0.32 ± 0.03
B4Δ C4Δ	ΔΔΔΔΔΔΔΔΔΔ	ΔΔΔΔΔΔΔΔΔΔ	1.1, 1.3	0.09, 0.22

Footnotes are as in Table 2.

Table 5. Summary of signal transduction mutant genetic and enzymatic activities

Step	Allele description[a]	ATP-dep. nuclease activity (units/mg protein)[b]		T4 *2*$^{-}$ eop[c]	DNA unwinding[d]	Chi cutting[d]	Chi hotspot activity[e]
		25 uM ATP	1 mM ATP				
	Δ*recBCD*	<2	ND	1.0	-	-	0.99 ± 0.02
	recBCD+	440, 250	280, 280	<2 x 10^{-8}	+	+	5.1 ± 0.09
2	C2Δ	780	360	<2 x 10^{-8}	+	-	1.5, 1.4
	D2Δ	3.5	<2	1.6 x 10^{-3}	+	-	0.9, 1.0
	C2Δ D2Δ	10	16	1.3 x 10^{-3}	+	-	1.4, 1.1
	C2ala	5.2	12	<2 x 10^{-8}	+	-	3.2, 3.5
	D2ala	8.7	21	<2 x 10^{-8}	+	-	2.4, 2.9
	C2ala D2ala	1.7	1.7	<2 x 10^{-8}	+	-	2.2, 2.3
3	B3Δ	3.5	23	5.1 x 10^{-4}	+	-	0.9, 1.0
	B3ala	400, 610	300, 360	<2 x 10^{-8}	+	+	1.8, 1.6
	D3ala	310, 470	340, 330	<2 x 10^{-8}	+	+	1.9, 1.4
	B3ala D3ala	37, 37	96, 93	<2 x 10^{-8}	+	-	1.5, 1.4
	D3Δ	14, 38	68, 68	3.1 x 10^{-6}	+	-	1.0, 1.2
	B3Δ(10)	400, 370	240, 240	<2 x 10^{-8}	+	+	4.6, 4.2
	B3ala D3Δ	12, 16	47, 35	4.6 x 10^{-4}	+	-	1.1 ± 0.06
4	B4Δ	700	350	<2 x 10^{-8}	+	+	4.2, 4.3
	C4Δ	10	23	<2 x 10^{-8}	+	-	1.1 ± 0.12
	B4Δ C4Δ	12	16	1.9 x 10^{-3}	+	-	1.1, 1.3

[a] The indicated mutation was on a derivative of plasmid pSA607 (*recBC*^{2773}*D*) in strain V2831 (*ΔrecBCD2731*). Alleles are described in Tables 2 – 4 and S1 – S3.

[b] Specific activity in units of ATP-dependent ds DNA nuclease/mg of protein at 25 µM ATP or 1 mM ATP. ND, not determined.

[c] Efficiency of plating (eop) is the phage titer on the indicated strain divided by the titer on strain V2831 (*ΔrecBCD2731*).

[d] Activity in extracts (Figs. 4 and S2). +, activity present. -, activity absent.

[e] Data from Tables 2 – 4 and S1 – S3.