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How RecBCD Enzyme and Chi Promote DNA Break Repair and Recombination: a Molecular Biologist's View

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INTRODUCTION

For over half a century, the central goal of molecular biology has been to elucidate the myriad of chemical reactions harbored in living cells. Although biochemistry allows one to isolate and study the components of these reactions—substrates, products, and catalysts—biochemistry alone cannot demonstrate the reactions required for events in living cells. Genetics does an excellent job of revealing the genes and proteins required for the reactions, but what they do at the molecular level remains unknown from genetic studies. The successful union of genetics and biochemistry in molecular biology has helped enormously to solve this problem, but the benefits and necessity of this dual approach have not always been appreciated and applied. Here, I describe a long-standing problem in biology, the mechanism of genetic recombination, and discuss how both disciplines have contributed in essential ways to our current understanding. I present evidence from both genetics and biochemistry indicating that for recombination and DNA break repair in *Escherichia coli*, Chi sites regulate the RecBCD enzyme by a mechanism contrary to the currently popular view.

OVERVIEW OF HOW RecBCD ENZYME PROMOTES RECOMBINATION

A beautiful example of a "protein machine" (2), the RecBCD enzyme is a large (330-kDa) complex of three polypeptides with both DNA-unwinding (helicase) and DNA hydrolysis (nuclease) activities. Beginning at a DNA double-strand end, it unwinds DNA and, at a special nucleotide sequence called Chi, makes a new 3′ end at which it begins loading multiple RecA protein molecules onto the single-stranded DNA (ssDNA) generated by continued unwinding. RecA promotes the exchange of this ssDNA for its equal in an intact homologous DNA molecule. Further reactions outlined below result in the repair of the broken DNA containing the initial double-strand end and, if this DNA and the intact DNA are genetically different, the production of one or two genetically recombinant molecules. One RecBCD molecule per end suffices for these reactions. Since there are about 10 to 50 RecBCD molecules per cell, even multiple DNA breaks per cell can be repaired without an increase in the level of gene expression; indeed, no transcriptional regulation of the *recBCD* genes is apparent. Instead, the activity of RecBCD is controlled in two other ways. It acts at a high level only on linear DNA and is essentially inactive as long as the bacterial chromosome remains as an intact or replicating circle. However, when the chromosome is broken or when linear DNA is introduced into the cell, the level of RecBCD activity is high and is controlled in a critical way by Chi sites. This review focuses on the molecular mechanism by which Chi controls the RecBCD enzyme. To put this subject into context, I first describe the observations that led to the discovery of Chi.

A BRIEF HISTORY OF RECOMBINATION IN *ESCHERICHIA COLI* AND PHAGE λ

The finding that certain genetic characters in *Drosophila melanogaster* do not sort independently during meiosis, as Mendel stated, led to the complementary concepts of linkage between genes and recombination between linked pairs (70). Genetic studies of the next 50-odd years revealed the fundamental rules for recombination, but advances in our understanding of the molecular mechanism did not come until the discovery of recombination in *E. coli*

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and its phages (32, 50, 53, 61) and, about 20 years later, the isolation of recombination-deficient mutants and the discovery of the *recA*, *recB*, and *recC* genes of *E. coli* (28, 52). Even then, the discovery of the molecular mechanism was not quick. Nearly 15 more years were required for biochemists to find that RecA promotes the interaction of ssDNA and double-stranded DNA (dsDNA) to form a joint DNA molecule at a point of nucleotide sequence identity or near identity, an essential feature of homologous recombination (66, 74, 86). Quicker, however, was the discovery that the *recB* and *recC* genes encode an ATP-dependent nuclease, called exonuclease V or the RecBC enzyme (13, 21, 75). (The enzyme is now called the RecBCD enzyme, after the later discovery of the RecD subunit by a combination of genetics and biochemistry [5, 22].) The biochemistry of this powerful nuclease, however, presented a profound problem: how could a nuclease that destroys DNA produce intact recombinant DNA? This is the focal point of this article: how is the RecBCD enzyme regulated either to save broken DNA or to destroy it?

A major advance in our understanding of recombination was the demonstration by A. J. Clark and coworkers that there is not one mechanism of recombination but genetically separable pathways of recombination in *E. coli*. By isolating and studying recombination-proficient pseudorevertants of *recB recC* double mutants, those researchers elucidated two pathways, called RecE and RecF, that can substitute for the wild-type ("RecBC") pathway when the RecBCD enzyme is absent (26). This finding of multiple pathways changed how most people approached the problem: to seek a solution, but not a universal solution, for the mechanism of recombination.

In fact, the analysis by A. J. Clark et al. was predated by those of investigators studying phage λ, who found that this phage could recombine in *E. coli recA* mutants (20). Lambdologists soon isolated phage mutants that could not recombine, thereby defining the λ Red pathway, which requires the λ *exo* and *beta* gene products (36, 87). λ *exo* was shown to encode an exonuclease (79) slightly before the discovery of the RecBCD nuclease. From related genetic studies came the additional finding that the λ *gam* gene encodes a protein that inhibits the RecBCD nuclease (82) and allows the phage to circumvent the destructive power of the bacterium. Thus, wild-type λ inhibits the bacterial recombination pathway and relies on its own recombination pathway.

The next step central to this story was the discovery that λ *red gam* mutants cannot grow (i.e., make visible plaques) on a *recA* mutant *E. coli* host and that on wild-type *E. coli*, these mutants make plaques much smaller than those of wild-type λ (125). Evidently, λ *red gam* mutants require recombination to grow, but the wild-type *E. coli* pathway is not highly proficient on λ. Remarkably, however, F. W. Stahl and coworkers found that large-plaque mutants of λ *red gam* arise frequently, as first noted by D. Henderson and J. Weil (49), and that the mutations, called χ, map at four discrete loci (97). A χ mutation stimulates recombination exclusively by the RecBCD pathway (44, 100) and maximally near, and to the left of, the χ mutation tested (101). ("Left" and "right" are defined by the conventional genetic map of λ.)

GENETIC AND BIOCHEMICAL FEATURES OF CHI RECOMBINATION HOT SPOTS AND RecBCD ENZYME

These dramatic results showed that the χ mutations create a site, called Chi, for crossover hot spot instigator (60). Chi sites also exist in λ derivatives that incorporated fragments of the *E. coli* chromosome; analyses of these phages indicated that the *E. coli* chromosome contains about 1,000 Chi sites, or about 1 site per 5 kb (40, 65), roughly the distance over which a Chi site stimulates recombination along the λ chromosome (60). The picture that emerged is that Chi sites are an important feature of wild-type recombination in *E. coli* and, more generally, that although homologous recombination can occur at any point along homologous chromosomes, it is more frequent at and near special sites, called hot spots, than far from them. Slightly earlier genetic studies revealed similar sites in fungi (11, 45), and a comparison between prokaryotic and eukaryotic hot spots was immediately tempting (96).

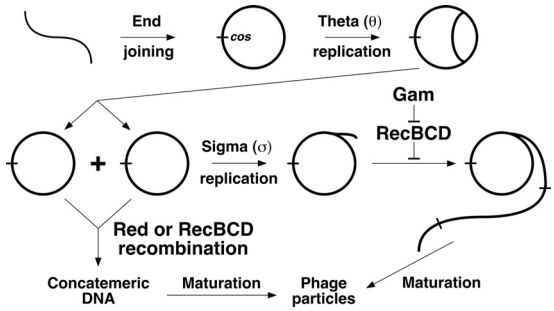

FIG 1 Growth cycle of phage λ. Duplex DNA is represented as a single line. Linear DNA (top left) in the viral particles is injected into cells, where the cohesive ends (*cos*) are ligated to form circular DNA. Early replication in the theta (θ) mode produces monomeric circles, which must be converted to concatemeric DNA to be packaged (matured) into viable phage particles. This can occur by late rolling-circle (σ) replication, but only when the RecBCD nuclease is absent, because of mutation or inhibition by the λ Gam protein. Alternatively, recombination by the λ Red pathway or the *E. coli* RecBCD pathway can convert monomeric circles into dimeric or higher-order concatemers containing two or more *cos* sites required for packaging. RecBCD gains access to a monomeric circle when *cos* is cut during maturation; packaging proteins bound at the left end block access, forcing RecBCD to enter the right end and travel leftward. Chi stimulates RecBCD-promoted recombination, thereby allowing more λ *red gam* mutant DNA to be packaged, with the formation of larger plaques than those formed without Chi. (Modified from reference 90 [copyright 1983, Cold Spring Harbor Laboratory Press].)

The study of *E. coli* Chi-containing DNA fragments inserted into λ *red gam* (here designated simply λ) revealed an astonishing finding: when a Chi-containing fragment was inverted, it did not stimulate recombination to its right, as might have been expected; it barely stimulated recombination at all (40)! Genetic studies of this orientation dependence showed that Chi had to be properly oriented with respect to the λ *cos* site, formed by the ligation of the cohesive ends of λ (54). *cos* is the site at which λ packaging begins and proceeds from left to right. The left end is bound by packaging proteins, but the right end is exposed to cellular nucleases, primarily RecBCD, which can destroy DNA at the right end. Successful packaging thus requires concatemeric λ DNA so that one complete unit of λ DNA from the left end can be packaged before degradation from the right end has hit that unit (Fig. 1). Some entity must travel between *cos* and Chi to communicate their proper or improper orientation. A critical point was to identify that entity.

To identify the entity that recognizes Chi, the identity of Chi itself was important. Since mutations in λ could be mapped genetically with high precision, and since DNA sequencing had been recently developed, our laboratory combined these methods to determine that Chi is the unique, asymmetric sequence, 5′-GCT

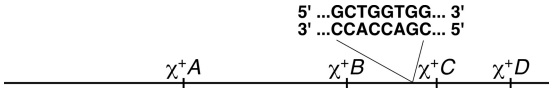

FIG 2 Conventional genetic map of λ. Mutations creating Chi were mapped to four sites (χ^+A to χ^+D) and in plasmid pBR322 inserted into the site indicated between χ^+B and χ^+C. Each of these mutations contains the Chi sequence, 5′-GCTGGTGG-3′, on the top strand, as indicated. (The sequence of χ^+A has not to my knowledge been reported.) The scheme is drawn to scale on the 48.5-kb λ DNA.

GGTGG-3′ (91). All of the mutants sequenced at three χ sites in λ and at three sites in plasmid pBR322 (inserted into λ) contained this sequence on the top strand of λ, as expected from the orientation dependence determined genetically. ("Top" is defined as the strand with the 5′ end at the left, and "bottom" is its complement, as shown in Fig. 2.) The one *E. coli* Chi site sequenced, in *lacZ*, also had this sequence (116). In each case, a single-base-pair change created or, for the *lacZ* Chi, inactivated the Chi site, consistent with the high frequency of Chi sites arising in λ. Later results with chemically synthesized DNA showed that Chi is the sequence 5′-GCTGGTGG-3′ and not its complement (16). Analyses of secondary mutations inactivating a χ site in λ indicated that certain closely related sequences, such as 5′-GCTAGTGG-3′, had partial activity, approximately 10 to 40% of that of wild-type Chi; a few such Chi-like sequences occur in wild-type λ and can account for the low level of RecBCD-promoted recombination in wild-type λ (24).

As noted above, Chi is a site that specifically enhances the RecBCD pathway of recombination (44, 100). This property is akin to that of other chromosomal sites known at that time: origins of replication as well as promoters and operators controlling transcription. In each of these cases, specific proteins act at these sites, and it seemed likely that some protein specific to the RecBCD pathway acts at Chi, especially since it has a unique DNA sequence. The pathway specificity implicated the RecBCD enzyme, since RecA, the only other component of the pathway then known, also acts in the RecE and RecF pathways, at least as they are defined by their activity in *E. coli* conjugational recombination (26). To test this implication, pseudorevertants of *recB* and *recC* recombination-deficient mutants were sought, with the idea that some might have regained recombination proficiency (as initially selected via restored resistance to DNA-damaging agents) but not Chi activation (85). This desired type of mutation was found with the *recC73* mutation: four independent suppressor mutations closely linked to *recC73* restored at least partial recombination proficiency but no detectable Chi hot spot activity. In addition, nuclease activity was restored to nearly the wild-type level, suggesting that these mutants contained functional RecBCD enzymes that lacked an interaction with Chi. A later analysis showed that *recC73* is a frameshift mutation and that the suppressors are compensatory frameshifts altering 6 to 9 amino acids of RecC (12). These results presaged the current view that the RecC subunit "touches" Chi (see Molecular Picture of the RecBCD-Chi Interaction below).

These genetic data were consistent with a direct Chi-RecBCD interaction, but a demonstration of this possibility required biochemical studies of the RecBCD enzyme. In addition to being a potent nuclease, RecBCD is a rapid, highly processive helicase (110): during its degradation of DNA, RecBCD produces long ssDNA intermediates that are subsequently degraded to short oligonucleotides by RecBCD's potent single-stranded exonuclease (64). Electron microscopy of RecBCD-DNA complexes halted during the reaction revealed ssDNA loops and tails produced by RecBCD when the nuclease, which requires Mg^{2+} ions, is strongly inhibited by competing Ca^{2+} ions (Fig. 3) (110, 111). The loops grow in size and move along the DNA, with the DNA lengths and distances being a linear function of the RecBCD reaction time. An analysis of the data showed that RecBCD starts unwinding DNA at an end and proceeds at about 300 bp per s (110). These observations inspired a model (Fig. 4) in which RecBCD is the entity that moves from the unbound right end of λ (the *cos* site), or any other free DNA end, to a properly oriented Chi site, with which it interacts (93).

Since RecBCD is also a nuclease, an obvious possibility was that RecBCD cuts DNA at a properly oriented Chi site during its unwinding of DNA. Using end-labeled DNA fragments with and without Chi, our laboratory observed such Chi-dependent cutting: one strand was cut ("nicked"), about 5 nucleotides to the 3′ (right) side of Chi, but only when RecBCD entered the DNA from the right (Fig. 5, left) (78, 105). Extracts from wild-type cells, but not those from the *recC73* pseudorevertants noted above, made this cut. This observation, plus the ability of purified RecBCD to cut at Chi, showed that Chi is directly recognized by RecBCD, without the need for any other cellular protein. Only the top

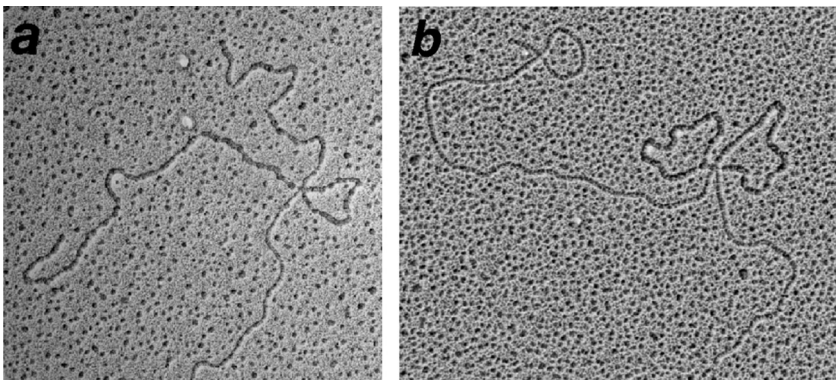

FIG 3 DNA-unwinding intermediates made by RecBCD. Electron micrographs of DNA after a brief reaction with RecBCD enzyme reveal loop-tails (a) and twin loops ("rabbit ears") (b). ssDNA, bound by SSB, is thick, and dsDNA is thin in these preparations. The single-strand tails of loop-tails can anneal to form twin loops (Fig. 4 and 7) (see reference 110).

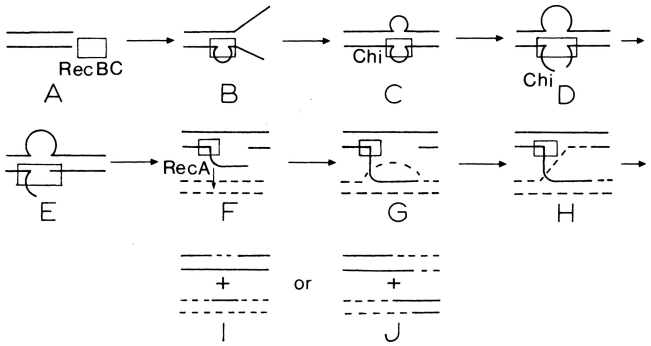

FIG 4 Early model for recombination based on nicking at Chi. Solid lines, DNA of one parent; dashed lines, DNA of the other parent. (A to D) RecBCD, then called RecBC, unwinds DNA (A to C) as shown in Fig. 3 and nicks one strand at Chi (D). (E to G) The newly generated end is elongated by continued unwinding (E) and is bound by RecA protein (F), which forms a D-loop with an intact, homologous duplex (G). (H to J) Cutting of the displaced strand in the D-loop allows that strand to pair with the gap in the Chi-containing parent to form a Holliday junction (H), which is resolved into either a noncrossover (I) or a crossover (J). (Reproduced from reference 93 with permission of the publisher.)

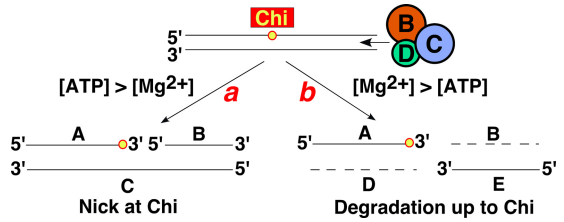

FIG 5 Alternative reactions of purified RecBCD during unwinding of DNA with Chi. With excess ATP (left), RecBCD nicks the top strand containing Chi (5′-GCTGGTGG-3′). With excess Mg^{2+} ions (right), RecBCD endonucleolytically cleaves the top (3′-ended) strand up to Chi, cuts the bottom strand, and endonucleolytically cleaves that strand beyond Chi.

strand was cut, and both the fragment to the right (with a 3′ label) and the fragment to the left (with a 5′ label) were observed in nearly equal yields. The lengths of these fragments indicated that the cut was a simple nick, without the loss of nucleotides (105). Unwinding continued after Chi, since an ssDNA fragment with Chi near its end was generated to the left of Chi. This ssDNA fragment could, with the aid of the RecA protein, invade intact homologous DNA and promote recombination to the left of Chi. These biochemical results fit perfectly with the genetic properties of Chi noted above, and the model that inspired these experiments (Fig. 4) was soon reproduced in many textbooks (e.g., see references 3, 62, 104, and 117).

MECHANISM OF THE CHI-RecBCD INTERACTION

Questions about this model for the Chi-RecBCD interaction arose when the reaction conditions for RecBCD were altered. Upon discovering the Chi-dependent nicking of DNA by RecBCD, we explored conditions that maximized the yield of the Chi fragment, the normal procedure that biochemists undertake when they study a new reaction (78, 84). As expected, Ca^{2+} ions inhibited the reaction, and a nearly neutral pH was optimal. (The double-strand exonuclease reaction of RecBCD is optimal at a much higher pH, about 9 [37, 122].) Most critical was the ratio of ATP and Mg^{2+} ion concentrations: a maximal yield of the Chi fragment was obtained with a slight excess of ATP over Mg^{2+}, such as 5 mM ATP and 3 mM Mg^{2+} ions. These concentrations seemed reasonable, based on the limited available information about their concentrations in *E. coli* (see below).

A serious challenge to the view developed above came from observations in the laboratory of S. C. Kowalczykowski, which used other RecBCD reaction conditions, notably 1 mM ATP and 8 mM Mg^{2+}. Under this condition, those researchers observed the fragment to the left of Chi but not the fragment to the right (34) (Fig. 5, right). DNA to the right of Chi, with the 3′ label, was cut into a set of fragments, visible after gel electrophoresis as a smear rather than a discrete band, as the Chi fragments were previously observed. Those researchers concluded that RecBCD makes frequent endonucleolytic cuts on the strand with a 3′ end up to the Chi site and then ceases degradation but continues unwinding. In this view, Chi blocks the RecBCD nuclease, whereas in the former view, Chi activates the RecBCD nuclease.

In our laboratory, A. F. Taylor then reexamined RecBCD reaction conditions and found that with 1 mM ATP and 8 mM Mg^{2+}, the bottom strand, like the top strand, was cut, in a Chi-dependent manner, within the Chi sequence or a few nucleotides from it (109). He found that the ratio of ATP to Mg^{2+}, rather than the absolute concentrations, determines whether the bottom strand is cut. This is understandable, since ATP chelates Mg^{2+} strongly (121). Excess ATP leaves little free Mg^{2+}, and RecBCD simply nicks at Chi, whereas excess Mg^{2+} activates the nuclease to degrade DNA more rampantly. S. C. Kowalczykowski's laboratory subsequently showed that with excess Mg^{2+}, the bottom strand was degraded to the left of Chi (8); in other words, at Chi, there was a switch in the strand degraded, from the 3′-ended (top) strand before Chi to the 5′-ended (bottom) strand after Chi (Fig. 5, right, and 6). The preservation of the top strand to the left of Chi (fragment A; Fig. 5), which could account for Chi's stimulation to its left, was common to the two reactions.

CONFLICTING VIEWS OF THE CHI-RecBCD INTERACTION DEDUCED FROM BIOCHEMICAL DATA

Which of the two above-described reactions occurs in living cells was left unclear from these biochemical studies, because the effective concentrations of ATP and Mg^{2+} in *E. coli*, or in any other cell, are not clear. In *E. coli* or *Salmonella enterica* serovar Typhimurium, the overall ATP concentration has been estimated to be 3 to 10 mM, that of all nucleoside triphosphates (NTPs) has been estimated to be 5 to 25 mM (14, 19), and that of Mg^{2+} has been estimated to be about 100 mM (69). However, most of the Mg^{2+} is almost certainly bound to macromolecules, primarily rRNA, which is about 200 mM in nucleotides inside cells (69, 73). Indeed, few enzymes are active with 100 mM $MgCl_2$, which has an ionic strength equivalent to that of 300 mM NaCl. The concentration of Mg^{2+} "free in solution in the cellular sap" has been estimated to be 1 to 2 mM (1), and the "free Mg^{2+} concentration" has been estimated to be 0.9 mM (43). Because the free Mg^{2+} and ATP concentrations are similar and because of uncertainties in the assays, it is unclear from these measurements which is in excess and, thus, which reaction of purified RecBCD at Chi most nearly reflects that in living *E. coli* cells. This is a key question in the molecular biology of *E. coli* genetic recombination and one that in principle must be addressed for any question in molecular biology. It is noteworthy that another *E. coli* recombination-promoting protein, RecG, re-

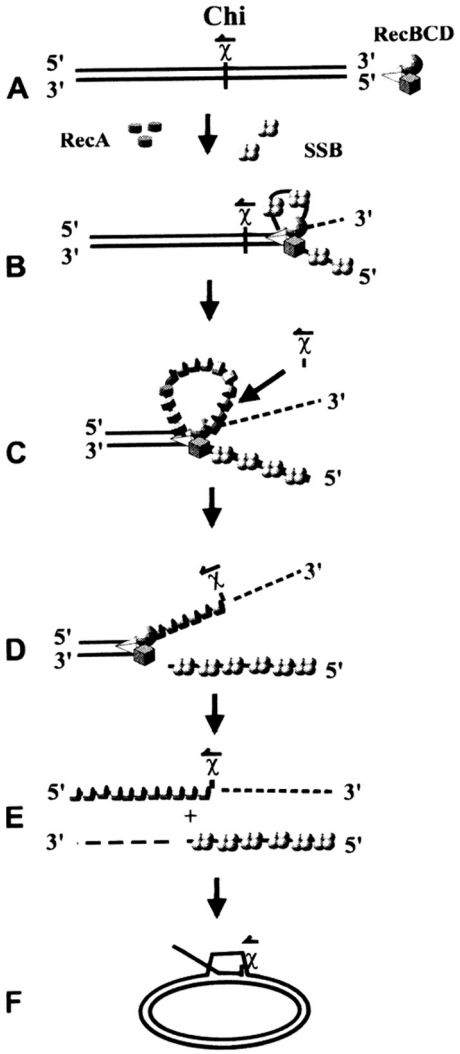

FIG 6 Model for joint molecule formation based on degradation up to Chi (Fig. 5, right). (A and B) RecBCD unwinds DNA, as shown in Fig. 3; SSB binds the single-strand loop and tail. (C) At Chi, degradation switches from the top to the bottom strand, and RecBCD begins to load RecA onto the 3′-ended strand with Chi near its end. (D to F) This RecA-ssDNA filament (D and E) invades intact DNA to form a D-loop (F). (Reprinted from reference 10 with permission of Elsevier.)

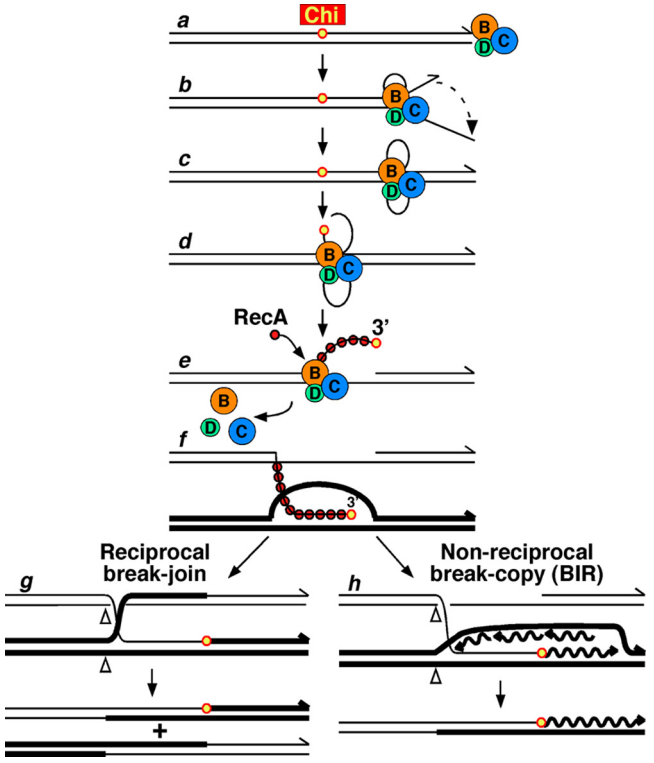

FIG 7 Model for recombination based on nicking at Chi (Fig. 5, left). This is an expanded version of the model shown in Fig. 4, with thick lines representing one parent. The D-loop (f) can be converted into a Holliday junction (g) and resolved into a crossover (shown) or a noncrossover (not shown here). Alternatively, the 3′ end of the invading Chi tail can prime DNA replication (h); the cutting of strands (open arrowheads), swapping of strands, and ligation produce one crossover-type recombinant but not its reciprocal, plus one parental-type molecule (not shown). This mechanism is also called "break-induced replication" (BIR). (Reprinted from reference 6 with permission [copyright 2007, Cold Spring Harbor Laboratory Press].)

quires ATP in excess over Mg^{2+} for its activity, the unwinding of Holliday junctions (118).

Most reviews of Chi and RecBCD and textbooks published in the last 10 years or so have described the reaction with excess Mg^{2+} (switching of the DNA strand degraded at Chi) (e.g., see references 33, 41, 58, and 68), but the basis for this choice is unclear. While it is true that most protocols for nucleic acid enzymes specify excess Mg^{2+}, it is not clear that the reactions require this. I am not aware of enzymes with ATP as a substrate that do not act with ATP in slight excess over Mg^{2+}. If there are any, then their investigation, both in cells and in the purified state, might settle the Chi-RecBCD question. Without definitive evidence of the ratio of ATP to Mg^{2+} in *E. coli*, it seems to me that it is essential to turn to additional genetic and physiological evidence to deduce the reaction of RecBCD at Chi in cells.

GENETICS AND PHYSIOLOGY CLARIFY BIOCHEMICAL DATA

There are numerous arguments indicating that nicking at Chi (Fig. 5, left, and 7) is the physiologically relevant reaction.

Action of Chi "In *Trans*"

The reactions discussed above revealed the effect of Chi only on the DNA molecule containing this site (i.e., in *cis*), but astonishingly, Chi also acts in *trans*, as shown by three reports. Here, "in *trans*" means the ability of Chi on one DNA molecule to influence, positively or negatively, the activity of Chi on another DNA molecule. In the first report (71), circular plasmid DNA in *E. coli* was linearized at a cloned λ *cos* site upon infection with λ, which also allowed the measurement of Chi recombination hot spot activity (the ratio of the recombinant frequency in an interval with Chi to the frequency in the same interval without Chi). If the plasmid did not contain Chi, the λ Chi was fully active (hot spot value of about 6), but if the plasmid did contain Chi, the λ Chi was significantly less active (hot spot value of about 4 [$P < 0.05$]; inactive Chi gives a value of 1). Thus, the linearized plasmid Chi acted in *trans* to reduce the activity of Chi on a separate DNA molecule.

In a second report (56) a plasmid was converted from the usual theta (θ) form of replication to sigma (σ) or rolling-circle replication (Fig. 1) by the activation of an adventitious origin of replica-

tion, and the cells were infected with λ to measure Chi hot spot activity. RecBCD could enter the DNA end on the rolling circle and encounter Chi present, or not, on the plasmid. If Chi was present and properly oriented, Chi hot spot activity was reduced from a value of about 6 to a value of about 4, as in the first report ($P < 0.002$). In a second set of experiments, *E. coli* was treated with bleomycin to linearize the chromosome and expose RecBCD to its hundreds of Chi sites. After such a treatment, the Chi activity in λ crosses was nearly abolished. In addition, the nuclease activity of RecBCD was greatly reduced, as shown by the growth of phage T4 gene *2* mutants (lacking a DNA end-binding protein that protects T4 DNA from RecBCD upon infection), but the cells remained recombination proficient. Although these experiments with bleomycin did not show that the inhibition was due to Chi on the *E. coli* chromosome, it was interpreted that way.

Parallel to this genetic action of Chi in *trans* is the biochemical inactivation of purified RecBCD by Chi, as described in a third report (107). On a DNA molecule with two Chi sites separated by about 0.5 kb, RecBCD nicks DNA about 40% of the time at either the first Chi site or the second Chi site, but if it nicks at the first Chi site, it does not detectably nick at the second (less than 5% of the frequency expected if the first Chi site had no effect). In addition, if RecBCD nicks at Chi on one DNA molecule, it does not detectably nick on a second DNA molecule (in *trans*); indeed, all three subunits disassemble, and the enzyme remains inactive for more than an hour (108). These biochemical effects with purified components are seen with excess ATP but not with excess Mg^{2+}; the addition of Mg^{2+} quickly reactivates the enzyme and allows cutting at Chi in *trans*. With excess Mg^{2+} from the start, the enzyme either does not disassemble or quickly reassembles and acts like a naïve enzyme on a second DNA molecule (i.e., without any detectable effect in *trans*).

The similarity of the genetic results and the biochemical results with excess ATP strongly argue for nicking at Chi as the reaction in *E. coli* cells.

RecC† (Rec$^+$ Chi$^+$ Nuc$^-$) Mutants

Nonnull mutations are particularly informative in deducing the roles of multifunctional enzymes such as RecBCD, as they allow the removal or alteration of one activity at a time. Among the many nonnull *recBCD* mutations isolated, the RecC† class is especially illuminating regarding the reaction at Chi in cells. These mutations include four deletions that truncate translation after codons 790 to 922 out of 1,122 and the nonsense mutation *recC1041* (W841*), which truncates in the same interval (4). These *E. coli* mutants are recombination proficient and have robust, though not full, Chi activity. Most critically, they lack intracellular double-strand exonuclease activity, as measured by the ability of T4 gene *2* mutants to grow in RecC† mutants (see above). This phenotype indicates that the double-strand exonuclease activity, responsible for the degradation up to Chi under conditions of excess Mg^{2+}, is not required for Chi hot spot activity in cells.

Although the RecBCD enzyme purified from RecC† cells is unstable (most of the RecD subunit is dissociated from the RecBC complex, and the level of activity is low), about 40% of the DNA that is unwound is nicked at Chi, the same as that observed for the wild-type enzyme (4). The Chi hot spot activity in these cells requires RecD, as it does in wild-type cells and with purified enzyme. The simplest interpretation of these data is that RecD is associated with RecBC in RecC† cells and that the mutant enzyme, as the RecBCD heterotrimer, promotes recombination with strong Chi hot spot activity but without double-strand exonuclease activity. In other words, the RecC† phenotype is most consistent with the nicking of DNA at Chi in cells.

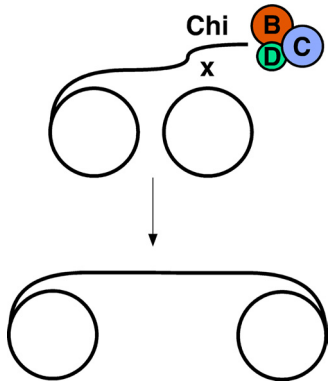

FIG 8 Proposal for formation of high-molecular-weight (HMW) DNA during rolling-circle (σ) replication. RecBCD initiates unwinding at the open end of the rolling circle (Fig. 1). At a properly oriented Chi site, it promotes the recombination of the replicating DNA with an intact circle to form a dumbbell-shaped molecule. Divergent replication from each fork elongates the DNA connecting the circles, which migrates slowly during gel electrophoresis and is measured as HMW DNA (see reference 31).

Chi-Dependent High-Molecular-Weight DNA of Plasmids

The formation of high-molecular-weight (HMW) DNA by certain Chi-containing plasmids in *E. coli* has led some to argue that Chi blocks DNA degradation in cells. When the θ replication of these plasmids is switched to σ or rolling-circle replication (see above), HMW DNA accumulates if the plasmid contains a properly oriented Chi but not if Chi is absent or incorrectly oriented (31). This has been taken as evidence that Chi blocks the degradation of DNA in *E. coli* cells, but the formation of HMW DNA also requires the RecA protein (31), which is not expected if Chi indeed blocks RecBCD's nuclease. (HMW DNA of plasmids with or without Chi accumulates in *recBCD* null mutants, indicating that RecBCD is the only nuclease that blocks HMW DNA formation.) A. Kuzminov et al. (59) showed that HMW DNA formation requires properly oriented Chi, RecA protein, and ssDNA-binding (SSB) protein; these two proteins are needed for efficient Chi-stimulated recombination (38).

The requirement for RecA and SSB proteins suggests an alternative interpretation of HMW DNA formation. The switch to σ replication likely exposes a dsDNA end, at which RecBCD can enter and nick at a properly oriented Chi site to produce a RecA-ssDNA tail (Fig. 1 and 8). This tail could recombine with an intact circular plasmid DNA molecule and form linear DNA with a circle at each end (Fig. 8). This endless (dumbbell-shaped) DNA would be resistant to the RecBCD enzyme, and rolling-circle replication would make it increasingly larger. The σ form of the same plasmid without Chi would be held in the monomeric (θ) form by RecBCD's nuclease or helicase or both, just as λ *gam* mutant DNA is prevented from going from the θ form to the σ form by RecBCD (39). Thus, Chi's stimulation of HMW DNA formation does not show that Chi turns off RecBCD's nuclease activity.

Reciprocality of Chi-Promoted Recombination

Recombination is said to be reciprocal when the two complementary recombinant types are produced in the same event. Recipro-

cality can be readily determined if all of the products of the event can be recovered, as in fungal meiosis, where recombination between distant genes is nearly always reciprocal (i.e., by crossing over) (119). In phage crosses, complete recovery is more difficult or impossible. Nevertheless, one can assay all of the phages emerging from a single cell infected with two genetically different phage types and determine if the reciprocal recombinant types are nearly equal in frequency. This is the case for the recombination of phage λ *red* mutants in wild-type *E. coli* (i.e., by the RecBCD pathway): nearly equal frequencies of recombinants between genes separated by about 10 to 20 kb were recovered in single-burst analyses (83). Although the phage probably did not contain fully active Chi and the phages were *gam*$^+$, it is likely that these crosses were Chi influenced, since there are Chi-like sites with low-level hot spot activity in wild-type λ (see above), and Gam reduces but does not eliminate Chi hot spot activity (100). In contrast, recombination by the λ Red pathway (λ *red*$^+$ in *recA* mutant *E. coli*) is clearly nonreciprocal by the same criterion: reciprocal recombinant types were often recovered in markedly unequal yields (83). This contrast indicates that Chi-stimulated recombination could well be reciprocal.

Reciprocality requires that DNA on both sides of the recombination event be preserved in both parental DNA molecules. The degradation of DNA up to Chi is thus difficult to reconcile with reciprocality, and these observations argue against degradation up to Chi. A nick at Chi would leave one intact strand of DNA on both sides of Chi, facilitating the recovery of reciprocal recombinants (Fig. 7).

Corresponding results with λ *red gam* Chi$^+$ phage are mixed. To my knowledge, no single-burst analyses have been published, but there are several reports of assays of complementary recombinant types in the total infected population. Some reports indicated that Chi-stimulated recombination is reciprocal (55, 98, 102), others indicated that it is nonreciprocal (60, 101), and others indicated that it is both (38, 99, 103). Reciprocality seems to correlate with phage DNA replication not being blocked during infection (i.e., as in a normal wild-type infection). Under replication-blocked conditions, there is extra pressure for the phages to recombine to form the concatemeric DNA required for packaging (Fig. 1). Multiple rounds of recombination, which demonstrably occur under such conditions (99), might obscure the reciprocality of individual events, whose analysis is necessary to assess reciprocality. While these data leave uncertain whether Chi-stimulated recombination is reciprocal, they clearly leave open this possibility, which would argue against degradation up to Chi.

PROPOSAL FOR INTRACELLULAR DNA DEGRADATION BY RecBCD ENZYME

RecBCD clearly degrades various forms of DNA in cells. How might RecBCD degrade these DNAs but not degrade DNA up to Chi?

RecBCD degrades intracellular linear DNA in numerous situations. (Circular dsDNA is totally refractory to RecBCD, either in cells or in the purified state [106, 110, 122].) The first reported degradation by RecBCD was that of the *E. coli* chromosome after the UV irradiation of *recA* mutants; by 2 h after irradiation, about a quarter of the chromosome (i.e., ~1 Mb of DNA) became acid soluble, indicating fragments shorter than one or two dozen nucleotides (27, 29). Because the DNA of irradiated *recA*$^+$ cells is not solubilized, the solubilization in *recA* mutants was called "reckless" degradation (120). This degradation is RecBCD dependent: DNA of *recB* or *recA recB* mutants is not solubilized; *recB* and *recA recB* mutants were thus called "cautious" (120). Phage λ DNA cut into large pieces by the EcoK12 (*hsd*) restriction enzyme (88) and T4 gene *2* mutant DNA (76) (see above) are also solubilized in a RecBCD-dependent manner. Two special classes of *recBCD* mutants do not block the growth of T4 gene *2* mutants: *recD* null mutants, which lack significant nuclease activity but retain unwinding activity (5, 22, 57), and the *recB1080* (D1080A) mutant, which abolishes the sole nuclease active site (124). These results show that DNA degradation is done by the RecBCD nuclease and not another single-strand exonuclease acting in conjunction with RecBCD's unwinding activity. Note that even Chi$^+$ DNA, such as the irradiated *E. coli* chromosome with its thousand Chi sites, is degraded by RecBCD.

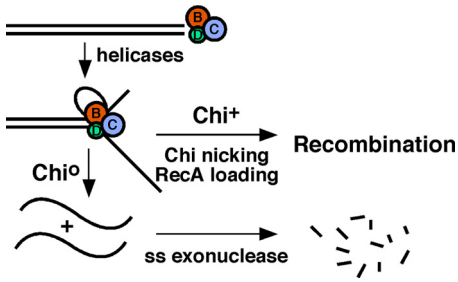

FIG 9 Recombination of Chi$^+$ DNA versus degradation of Chi^o DNA by RecBCD. Linear DNA is unwound by RecBCD (Fig. 3). If the DNA contains a Chi site, RecBCD nicks the DNA and loads RecA protein onto the newly generated 3′ end, which then engages intact DNA and undergoes recombinational repair (Fig. 7). If the DNA does not contain Chi, the ssDNA is degraded to short oligonucleotides by RecBCD's single-strand (ss) exonuclease activity. If the DNA cannot recombine, as in a *recA* mutant or in the absence of sufficient nucleotide sequence identity, Chi$^+$ DNA is also degraded.

In each of the cases noted in this section, the DNA cannot recombine, since in the first case, RecA is missing, and in the other cases, there is often only one phage DNA in each cell, or, for T4, there is no Chi site. I propose that when RecBCD acts on intracellular linear DNA, it unwinds the DNA, nicks it at Chi, and loads RecA protein onto the single-stranded tail; if this tail cannot recombine, RecBCD's single-strand exonuclease subsequently degrades the DNA, even if it has Chi (Fig. 9). The single-strand exonuclease of purified RecBCD is as potent as that of the double-strand exonuclease (37) but does not detectably cut at Chi (78, 105). Since the degradation up to Chi by purified RecBCD produces long (i.e., acid-precipitable) ssDNA (8, 34), this degradation is not sufficient to render the material found in cells acid soluble. An additional nuclease activity must act in cells. Since degradation does not occur in *recBCD* null, *recD*, or *recB1080* mutant cells, as noted above, it apparently occurs by RecBCD's single-strand exonuclease acting in a second round of reactions on DNA that cannot recombine. Thus, the primary cause of DNA degradation in cells appears to be RecBCD's Chi-insensitive single-strand exonuclease and not its double-strand exonuclease before Chi.

BIOLOGICAL ROLE OF CHI

Some authors have proposed that Chi enables *E. coli* to distinguish its DNA from that of foreign DNA and that Chi is the signal for the protection of "self" DNA, versus the degradation of "nonself" DNA by the RecBCD nuclease (9, 33, 47, 48, 72). *Salmonella* Ty-

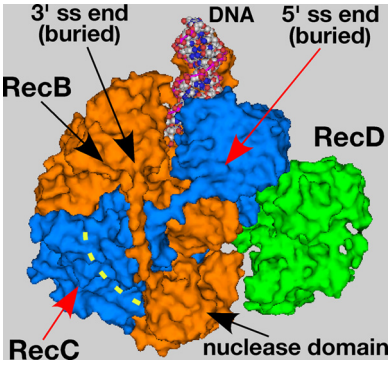

FIG 10 Crystal structure of RecBCD bound to hairpin-shaped DNA (PDB accession number 1W36 [89]). RecB is orange, RecC is blue, and RecD is green. Four base pairs of the DNA (multicolored) are unwound, with the 3′ end in the RecB helicase domain and the 5′ end in RecC headed toward the helicase domain of RecD. During unwinding, the 3′ end may pass through a tunnel (dashed yellow line) in RecC, where Chi may be recognized, and be cut by the RecB nuclease domain.

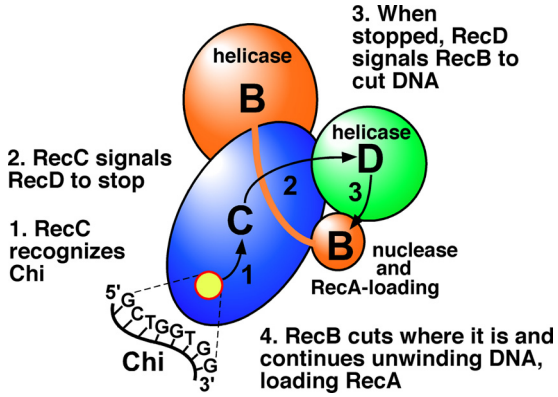

FIG 11 Model for the regulation of RecBCD by Chi. When Chi on the 3′-ended strand passes through the tunnel in RecC (Fig. 10), RecC signals RecD, the faster helicase, to stop. When stopped, RecD signals RecB to cut the DNA at Chi and begin loading RecA protein onto the newly generated 3′ end with Chi (Fig. 7). (Reprinted from reference 6 with permission [copyright 2007, Cold Spring Harbor Laboratory Press].)

phimurium contains 868 Chi sites in its 4.8 Mb of DNA (K. Fowler, personal communication), nearly as many as in *E. coli* DNA (1,009 in 4.6 Mb) (18), and Chi is active in λ crosses in *S.* Typhimurium (92). Nevertheless, *E. coli* DNA recombines with the *S.* Typhimurium chromosome in conjugational crosses about 10^6 times less frequently than does homologous DNA; this frequency can be raised about 10^3-fold by the inactivation of mismatch repair (80). The reduction in the level of recombination of the foreign DNA by at least a factor of 10^3 is likely due to the low (~80%) nucleotide sequence identity between the two species. Regardless, some feature other than Chi must prevent the recombination of this foreign DNA, and Chi cannot be the only, and likely is not the primary, basis of distinguishing self and nonself. That Chi^+ DNA, such as the *E. coli* chromosome after irradiation, is degraded by RecBCD shows that Chi does not protect DNA from degradation, either in cells or with purified components.

It has often been pointed out that Chi sites are more frequent in the *E. coli* chromosome than one might expect and are preferentially oriented to aid in the repair of broken replication forks (e.g., see references 18, 33, 114, and 115). This observation has fostered the idea that Chi sites were enriched during evolution based on their ability to stimulate RecBCD-promoted recombination and DNA repair. The Chi sequence, 5′-GCTGGTGG-3′, contains the most frequent codon (5′-CTG-3′) for the third most abundant amino acid, leucine (73). A consideration of the codon usage in the six possible reading frames covering Chi shows that the frequency and orientation bias of Chi can be fully accounted for by the codon usage of *E. coli* and the preferential orientation of transcription, and hence translation, with the direction of replication of the *E. coli* chromosome (17, 30). The Chi sequence may have been "selected" by RecBCD simply because it is abundant (stemming from its unselected role in translation) and would therefore aid in recombination.

It is not clear why there are hot spots of homologous recombination, although they exist in numerous species (51, 63, 96, 114). Indeed, to my knowledge, no hot spots have been reported for the fully functional *E. coli* RecE and RecF pathways, the *E. coli* ‡ pathway (that in *recD* mutants), and the λ Red pathway, although recombination by these pathways can be elevated near double-strand ends in some situations (e.g., see reference 113). Chi appears to be active throughout enteric bacteria, since their RecBCD enzymes cut at Chi and, when expressed in *E. coli*, activate Chi as a recombination hot spot (67, 84, 92); Chi is demonstrably active in *S.* Typhimurium (92). Short DNA sequences that alter the activity of RecBCD or its analog AddAB in other groups of bacteria appear to act like Chi, at least with respect to the formation of HMW DNA and, for *Bacillus subtilis* AddAB, the generation of a new 3′ end near the sequence (23, 114). Perhaps, further studies of the phylogenetic distribution and properties of RecBCD, AddAB, and the DNA sequences controlling them will shed light on this evolutionary enigma.

MOLECULAR PICTURE OF THE RecBCD-CHI INTERACTION

The crystal structure of RecBCD bound to a DNA end (81, 89) (Fig. 10) confirmed many previous inferences from genetics and conclusions from biochemistry and has led to a remarkably detailed view of how this complex protein machine works. The 5′-ended strand passes through part of RecC and engages the RecD helicase. The 3′-ended strand engages the RecB helicase and heads toward a tunnel in RecC, which likely recognizes Chi. Near the exit of this tunnel is the nuclease domain of RecB, which is connected to the RecB helicase domain by an approximately 30-amino-acid-long tether. The nuclease domain may swing from one position to another in response to Chi, as postulated from biochemical experiments (123). A specific version of this view is based on a special class of RecBCD mutant enzymes altered in the RecB helicase domain; these mutant enzymes nick the 3′-ended strand at a certain fraction of the length of the DNA substrate rather than at Chi (6). In this view (Fig. 11), when Chi passes through the RecC tunnel, RecC signals the RecD helicase to stop; RecD then signals RecB to cut the DNA (i.e., a few nucleotides to the 3′ side of Chi). In accordance with this view, the conformation of RecBCD changes dramatically when the enzyme passes a properly oriented Chi site; this conformational change appears to be the swinging of the RecB nuclease domain from one side of RecC to the other (A. F. Taylor, unpublished data).

In addition to cutting DNA at Chi, RecBCD actively loads RecA protein onto the newly generated 3′ end (10). Wild-type

RecBCD loads only after Chi, but RecBC (lacking RecD) loads RecA onto the double-stranded end, at which it initiates unwinding (25); as noted above, *recD* mutants have no Chi hot spot activity and lack nuclease activity (5, 22). The "constitutive" loading of RecA explains the recombination proficiency but Chi inactivity of *recD* mutants and of *recC1010* (G905D) and C-terminal-deletion *recC* mutants, which do not assemble RecD into the complex (7). The RecB nuclease domain can bind RecA protein (95), and it was hypothesized that the nuclease domain rotates at Chi to exchange the RecA-loading domain for the nuclease active site on the 3′ end (95; Taylor, unpublished). Thus, at Chi, the nuclease domain may swing on its tether to engage the 3′-ended strand, nick it, rotate, and load RecA onto this strand to prepare it for the next step of recombination (Fig. 6 and 7).

Studies of single RecBCD molecules moving along DNA have also enhanced our understanding of this complex protein machine (e.g., see references 15, 35, 42, and 77). Most notable is the reported pausing of DNA unwinding for a few seconds at Chi (94), which may reflect the time for the conformational change noted above or the time that it takes for RecB, the slower helicase, to translocate along the ssDNA loop that accumulates presumably ahead of RecB (Fig. 3). Single-molecule experiments also directly confirmed that the RecD subunit is not released at Chi (46), as had been hypothesized from the cellular phenotype of *recD* mutants and the properties of purified RecBC enzyme (i.e., that lacking the RecD subunit) (112). Much earlier biochemical results had shown that RecBCD that nicked at Chi still cut at a terminal hairpin, which strongly argued that RecD is not released at Chi, since the RecBC enzyme has little or no nuclease activity (5, 22, 107). Single-molecule studies have also shown that RecBCD is exceptionally powerful and can easily displace tightly bound proteins, such as the LacI repressor and RNA polymerase (42), confirming the suspicion that RecBCD overthrows other DNA metabolic functions to repair broken DNA.

CONCLUSION AND PERSPECTIVE

Although this review has focused on the RecBCD-Chi interaction in *E. coli*, the central message—the need to use both genetics and biochemistry to fully understand events in cells—applies to many other situations. The use of both, as illustrated here, advances our understanding but may still not give a complete picture. Ultimately, one would like to become microscopically small and see the events as they happen in cells. New imaging techniques on the horizon, such as intracellular fluorescence resonance energy transfer (FRET) analyses, promise to extend our ability to "see" molecular reactions. Combined with the genetics and biochemistry of purified components, these methods may allow a complete picture of the "molecular biology" of living cells.

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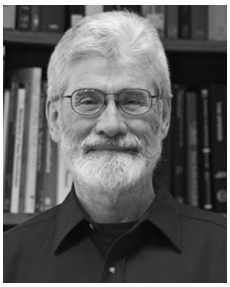

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