THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 16, Issue of April 21, pp. 12261–12265, 2000
© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Facilitated Loading of RecA Protein Is Essential to Recombination by RecBCD Enzyme*

(Received for publication, November 5, 1999)

Deana A. Arnold‡§ and Stephen C. Kowalczykowski‡¶‖

From the ¶Sections of Microbiology and of Molecular and Cellular Biology, and ‡Genetics Graduate Group, University of California, Davis, California 95616

Although the RecB^{2109}CD enzyme retains most of the biochemical functions associated with the wild-type RecBCD enzyme, it is completely defective for genetic recombination. Here, we demonstrate that the mutant enzyme exhibits an aberrant double-stranded DNA exonuclease activity, intrinsically producing a 3′-terminal single-stranded DNA overhang that is an ideal substrate for RecA protein-promoted strand invasion. Thus, the mutant enzyme constitutively processes double-stranded DNA in the same manner as the χ-modified wild-type RecBCD enzyme. However, we further show that the RecB^{2109}CD enzyme is unable to coordinate the loading of RecA protein onto the single-stranded DNA produced, and we conclude that this inability results in the recombination-defective phenotype of the *recB2109* allele. Our findings argue that the facilitated loading of RecA protein by the χ-activated RecBCD enzyme is essential for RecBCD-mediated homologous recombination *in vivo*.

The RecBCD enzyme is a multifunctional protein complex essential to the main pathway of homologous recombination in *Escherichia coli* (1, 2). The holoenzyme, composed of the RecB, RecC, and RecD subunits, possesses DNA-dependent ATPase, helicase, and nuclease activities (3–8). Proper function of RecBCD enzyme in the initiation of recombination is dependent upon its response to a specific DNA sequence, the recombination hotspot, χ (9, 10). Interaction with a χ site results in an attenuation of the 3′ to 5′ nuclease activity and an activation of the 5′ to 3′ nuclease activity (11, 12). This nuclease modification allows the production of a long 3′-terminal ssDNA[1] tail, which is the preferred substrate for RecA protein-promoted DNA strand exchange (13–15). Upon modification of nuclease activity, RecBCD enzyme also promotes the loading of RecA protein onto this ssDNA tail (16). The RecA protein-ssDNA complex, or presynaptic filament, can then initiate strand invasion into a homologous duplex DNA molecule (17).

Although the majority of homologous recombination in wild-type *E. coli* is initiated at χ by the combined actions of RecBCD enzyme and RecA protein (18, 19), there are *recBCD* mutants that promote wild-type levels of recombination, and yet are χ-insensitive. For example, *E. coli* strains that lack a functional RecD subunit are recombination-proficient, but recombination is completely independent of χ (20–22). Biochemical characterization of the RecBC(D$^-$) enzyme revealed that it is nuclease-deficient, but retains helicase activity, and can unwind dsDNA molecules to produce long tracts of ssDNA. Like the RecBCD enzyme after χ recognition, RecBC enzyme also coordinates the loading of RecA protein onto the 3′-terminal ssDNA it produces, further defining the way in which this enzyme promotes χ-independent recombination (23). In contrast to the nuclease-deficient RecBC enzyme, an inability of the nuclease-proficient mutants to respond properly to χ can also manifest itself *in vivo* as a drastic reduction in homologous recombination, as well as a heightened sensitivity to DNA-damaging agents. Strains bearing the *recB2109* mutation are phenotypically similar to a *recB* null strain; both display a severe reduction in conjugal recombination, sensitivity to mitomycin C, and a lack of χ-mediated recombinational hotspot activity (24). Unlike a *recB* null strain, however, *recB2109* cells do not facilitate plaque formation by phage T4 gene *2*$^-$, demonstrating that the RecB2109CD enzyme is functional *in vivo* and that it retains a significant amount of helicase or nuclease activity. Biochemical characterization of the RecB^{2109}CD enzyme demonstrated that it possesses all the biochemical activities of the wild-type enzyme as follows: ATPase, helicase, and ds- and ssDNA nuclease (25). The only significant defect of the RecB^{2109}CD enzyme observed *in vitro* was an absence of nuclease modification at χ. This discovery offered a possible explanation for the failure to promote χ-stimulated recombination; this enzyme was unable to modify 3′ to 5′ nuclease activity and so proceeded through a dsDNA molecule degrading the potentially recombinogenic 3′-terminal ssDNA.

However, here we show that the RecB^{2109}CD enzyme displays an asymmetry of dsDNA degradation that is uncharacteristic of the wild-type enzyme but is, instead, characteristic of the χ-activated RecBCD enzyme. The mutant enzyme degrades dsDNA primarily in the 5′ to 3′ direction, producing processed dsDNA with a 3′-terminal overhang. Although the RecB^{2109}CD nuclease activity produces a substrate suitable for RecA protein action, the RecB^{2109}CD enzyme is unable to facilitate the loading of RecA protein onto these ssDNA products. We conclude that RecA protein loading is an essential function of the RecBCD enzyme and that the generation of a recombinogenic 3′-terminal ssDNA, although necessary, is not sufficient for initiation of recombination by the RecBCD enzyme.

EXPERIMENTAL PROCEDURES

Chemicals and Buffers—All solutions were made using reagent grade chemicals and Barnstead NANOpure water. Radiolabel was pur-

* This work was supported by National Institutes of Health Grant GM-41347. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF179304.

§ Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in genetics at the University of California, Davis (36).

‖ To whom correspondence should be addressed: Section of Microbiology, University of California, One Shields Ave., Davis, CA 95616. Tel.: 530-752-5938; Fax: 530-752-5939; E-mail: sckowalczykowski@ucdavis.edu.

[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; SSB, *Escherichia coli* single-stranded DNA-binding protein; PCR, polymerase chain reaction.

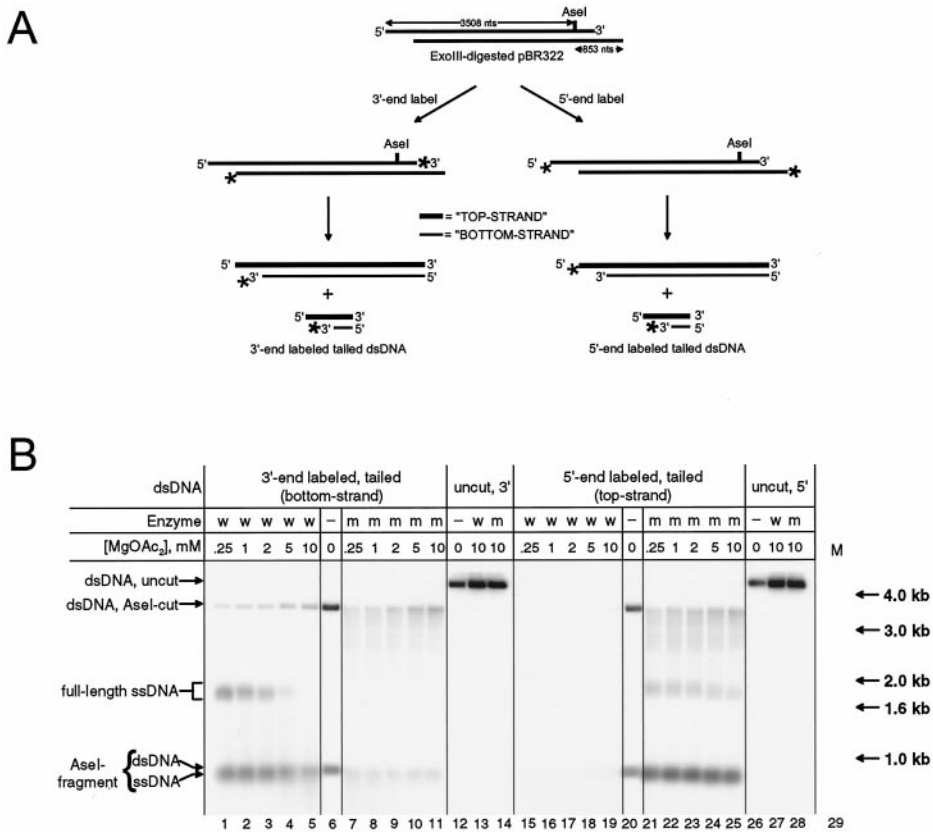

FIG. 1. **RecB^{2109}CD enzyme preferentially degrades linear dsDNA in the 5′ to 3′ direction.** *A,* the construction of the differentially labeled, tailed dsDNA substrates is shown. *B,* wild-type RecBCD (*w*) and mutant RecB^{2109}CD (*m*) enzymes were allowed to act on tailed dsDNA substrates over a range of conditions as described under "Experimental Procedures" as follows: *Ase*I-cut, 3′-end-labeled dsDNA in *lanes 1–11*; uncut, 3′-end-labeled dsDNA in *lanes 12–14*; *Ase*I-cut, 5′-end-labeled dsDNA in *lanes 15–25*; uncut, 5′-end-labeled dsDNA in *lanes 26–28.*

chased from NEN Life Science Products. The 1-kilobase pair DNA ladder was purchased from Life Technologies, Inc. All other chemicals were purchased from vendors as listed and were used as described previously (26).

Enzymes—Wild-type RecBCD, RecB^{2109}CD, SSB, and RecA proteins were purified as described previously (25, 26). Restriction endonucleases, Klenow fragment lacking exonuclease, and T4 polynucleotide kinase were purchased from New England Biolabs. Shrimp alkaline phosphatase and exonuclease I were acquired from United States Biochemical Corp.. Exonuclease III and *Taq* DNA polymerase were obtained from Promega Corp. Proteinase K was purchased from Roche Molecular Biochemicals.

DNA Substrates—Plasmid DNA substrates were purified, linearized, and radioactively end-labeled as described previously (26). All concentrations of DNA are given in nucleotides, unless otherwise indicated. The strain containing the *recB2109* gene that was sequenced, V1000 (27), is the same as that used to purify the RecB^{2109}CD enzyme (25). The *recB2109* gene was sequenced from both a plasmid template purified using a Qiagen midi-prep (Qiagen) and a PCR-amplified 3649-base pair fragment containing the *recB2109* gene. The PCR product was separated from the primers and nonspecific PCR products by electrophoresis through a 1% SeaPlaque gel (FMC BioProducts) and further purified using a Wizard PCR Prep kit (Promega Corp.). The primers for both PCR-amplifying and -sequencing the *recB2109* gene were purchased from Operon Technologies, Inc.

Asymmetry of dsDNA Exonuclease—The substrates for these reactions were constructed as follows (Fig. 1*A*). The pBR322 plasmid devoid of χ was linearized with *Hin*dIII restriction endonuclease and subsequently subjected to degradation by exonuclease III to yield a dsDNA molecule bearing 5′-terminal overhangs (12). The DNA was either 3′- or 5′-end-labeled with [α-^{32}P]dATP or [γ-^{32}P]ATP, respectively, as described previously (26). The tailed, labeled DNA was then treated with *Ase*I restriction endonuclease to produce an unlabeled, nearly blunt (two nucleotide overhang) dsDNA end appropriate for RecBCD or RecB^{2109}CD enzyme loading. Thus, both substrates contain radiolabel only at the end distal from the point of entry of the RecBCD or RecB^{2109}CD enzyme (Fig. 1*A*). A portion of each labeling reaction was not cleaved with *Ase*I as a control to ensure that the tailed ends would block degradation of the substrate by either the wild-type or mutant enzyme. The reaction buffer contained 25 mM Tris acetate (pH 7.5), 1 mM ATP, 1 mM dithiothreitol, 10 μM nucleotides of tailed, labeled DNA, 2 μM SSB protein, and magnesium acetate ranging from 0.25 to 10 mM. The reactions were performed at 37 °C and initiated by addition of 0.46 nM RecBCD or RecB^{2109}CD enzyme (one enzyme per 2.5 accessible dsDNA ends). The 30-μl reactions were stopped after 4 min with 10 μl of 5× stop mix and 14 μg of proteinase K, as described previously (26). The reactions were separated by native gel electrophoresis on 1% agarose in TAE buffer for 550 V-h. The gels were exposed to PhosphorImager screens that were later scanned by a Molecular Dynamics STORM 840 PhosphorImager; the gel images were analyzed using ImageQuaNT version 4.1a software.

Exonuclease I Protection Assays—These reactions were performed as described previously in reaction buffer containing 8 mM magnesium acetate and 5 mM ATP (16). 40 μM nucleotides of plasmid DNA was treated with 0.92 nM functional RecBCD or RecB^{2109}CD enzyme in the presence of 4 μM SSB and 20 μM RecA proteins for 3 min prior to addition of 11 units of exonuclease I. The tailed plasmid was created as described for the dsDNA exonuclease asymmetry assays; the plasmid containing the χ site was linearized with *Ava*I restriction endonuclease and 5′-end-labeled as previously reported (26).

Sequencing of the recB2109 Gene—The *recB2109* gene was sequenced from both a plasmid encoding *recB2109* as well as a pool of PCR fragments (equivalent of 12 individual PCR reactions) amplified from this plasmid. Primer walking was performed on both strands of these dsDNA substrates, and all samples were submitted for sequencing to the Division of Biological Science Automated DNA Sequencing Facility at the University of California, Davis.

RESULTS

RecB^{2109}CD Enzyme Degrades the 5′-Terminal Strand More Extensively Than the 3′-Terminal Strand—The dsDNA exonuclease activity of the wild-type RecBCD enzyme is asymmetric; the 3′ to 5′ nuclease activity is more vigorous than the 5′ to 3′ nuclease activity, which results in a more extensive degradation of the 3′-terminal strand at the entry site of the enzyme (11). To determine if the RecB^{2109}CD enzyme retained the same asymmetry of dsDNA exonuclease as that displayed by the wild-type enzyme, we constructed a linear, asymmetric dsDNA substrate that allowed us to independently monitor the degradation of each strand (Fig. 1*A*). First, linearized plasmid DNA was digested with exonuclease III to produce long (~300 nucleotides), 5′-terminal ssDNA tails that block entry of

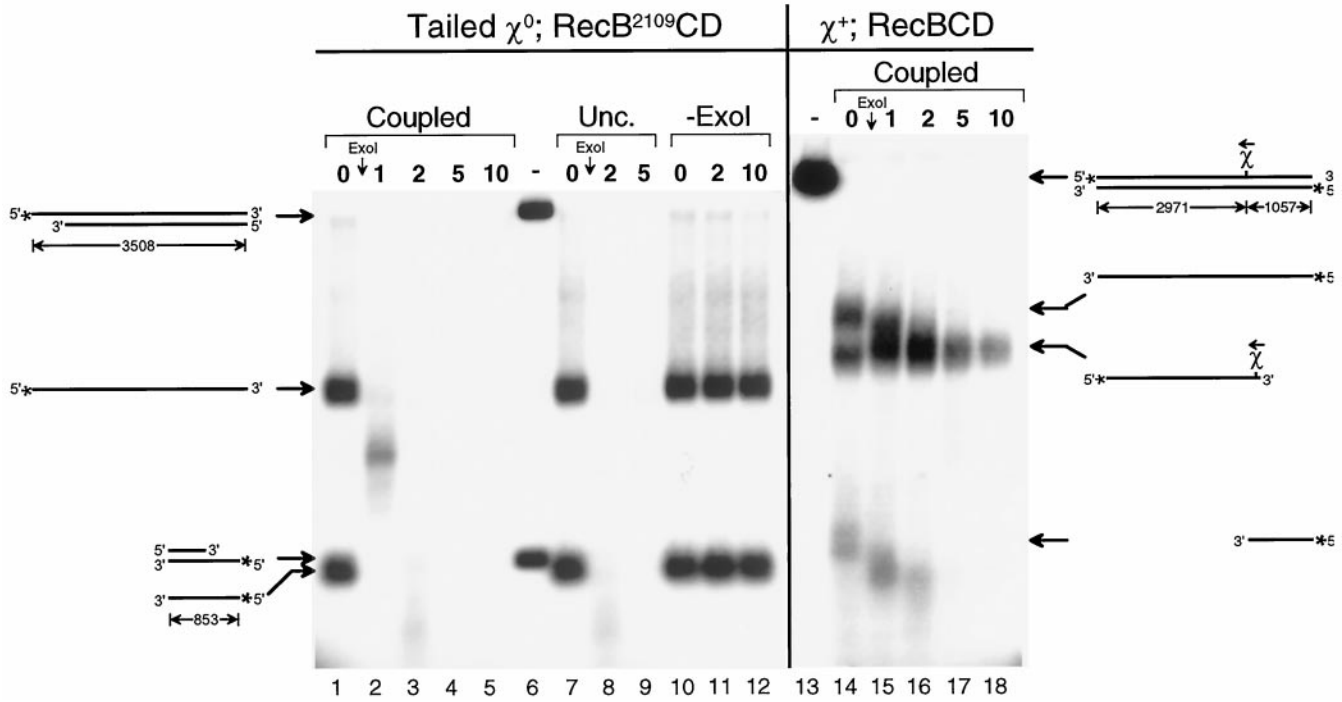

FIG. 2. **RecB^{2109}CD enzyme is unable to facilitate the loading of RecA protein.** Exonuclease I protection assays were carried out as described under "Experimental Procedures." The fragments produced for the tailed, χ^0 DNA and the χ^+ DNA are identified to the *left* and *right*, respectively. The lanes denoted by a − (6, 13) represent the starting dsDNA substrate and contain one-half the reaction volume as do the other lanes. The lanes denoted by a *0* represent the products of the reaction immediately prior to addition of exonuclease I. The RecB^{2109}CD enzyme coupled reaction is shown in *lanes 1–5*; the uncoupled reaction is shown in *lanes 7–9*; and the control reaction lacking exonuclease I is shown in *lanes 10–12*. The wild-type coupled reaction utilizing a χ^+ dsDNA substrate is shown in *lanes 13–18*.

RecBCD and RecB^{2109}CD enzymes at both ends. Next, the substrate was either 3′- or 5′-end-radiolabeled and subsequently digested with *Ase*I restriction endonuclease to provide an unlabeled, blunt dsDNA where the RecBCD or RecB^{2109}CD enzyme can enter. Thus, the resultant asymmetric DNA substrates have both a blunt end that does not contain a radioactive label and a tailed end that carries a radioactive label on either the 5′ or 3′ terminus (Fig. 1*A*). For simplicity, the strand corresponding to the 5′-terminal overhang is defined as the "top strand," and its complement is defined as the "bottom strand." This construct allowed determination of the asymmetry of dsDNA exonuclease activity by revealing whether the 5′-end-labeled top strand or the 3′-end-labeled bottom strand was preferentially degraded by the enzyme. Neither enzyme is able to act on an exonuclease III-tailed, dsDNA substrate that was not digested with *Ase*I restriction endonuclease, demonstrating that the 5′-terminal tails block entry of both the wild-type and mutant enzymes (Fig. 1*B, lanes 12–14*, and *26–28*). A smaller dsDNA fragment is also produced by the *Ase*I digest, which is referred to as the "*Ase*I fragment" in Fig. 1. This molecule is also tailed and labeled in the same orientation as the large fragment, but its degradation was not followed quantitatively.

The nuclease activities of RecBCD enzyme are sensitive to the concentration of free magnesium ions in solution, and this sensitivity is reflected in the frequency of endonucleolytic cleavage on each DNA strand during unwinding. If the magnesium ion concentration is very low, the frequency of nucleolytic cleavages on both strands is infrequent; if the magnesium ion concentration is very high, degradation of both strands is quite extensive. As a result, the ability to detect an asymmetry of dsDNA degradation is affected by the magnesium ion concentration. Therefore, reactions were carried out over a range of magnesium ion concentrations, which was achieved by varying the ratio of magnesium acetate to ATP in the reaction buffer. The magnesium acetate concentration in these experiments ranged from 0.25 to 10 mM, whereas the ATP concentration remained at 1 mM (Fig. 1*B*).

As expected, when the 5′-end-labeled substrate is processed by wild-type RecBCD enzyme, there is sufficient degradation (3′ to 5′) of the top strand to limit detection of a full-length ssDNA over this range of magnesium acetate concentrations (Fig. 1*B, lanes 15–19*). However, when the 3′-end-labeled substrate is processed under low magnesium ion conditions, full-length ssDNA is produced due to the low frequency of nucleolytic cleavage (5′ to 3′) on the bottom strand (Fig. 1*B, lane 1*). As the magnesium acetate concentration increases, less full-length bottom strand ssDNA is produced due to the increased frequency of endonucleolytic cleavage (Fig. 1*B, lanes 2–5*).

The RecB^{2109}CD enzyme displays a different behavior. When the mutant enzyme processes the 5′-end-labeled substrate, full-length top strand ssDNA is produced under all conditions tested (Fig. 1*B, lanes 21–25*), demonstrating a markedly reduced 3′ to 5′ nuclease activity. In contrast, when the 3′-end-labeled DNA is tested (Fig. 1*B, lanes 7–11*), essentially no full-length ssDNA is produced, indicating the presence of a vigorous 5′ to 3′ nuclease activity. The disparate amounts of full-length ssDNA products in *lanes 21–25 versus lanes 7–11* clearly demonstrate that the 5′ to 3′ nuclease activity is significantly more active than the 3′ to 5′ nuclease activity. These results show that RecB^{2109}CD enzyme behaves in a manner opposite that of the wild-type enzyme, by preferentially degrading the bottom strand during unwinding. Due to the low processivity of the RecB^{2109}CD enzyme under these conditions (28), partial duplexes are observed as a smear visible on the gel between the full-length dsDNA substrate and the full-length ssDNA product. These partial duplexes are not detectable in reactions performed at higher concentrations of ATP, which allows the RecB^{2109}CD enzyme to display a higher processivity (Fig. 2).

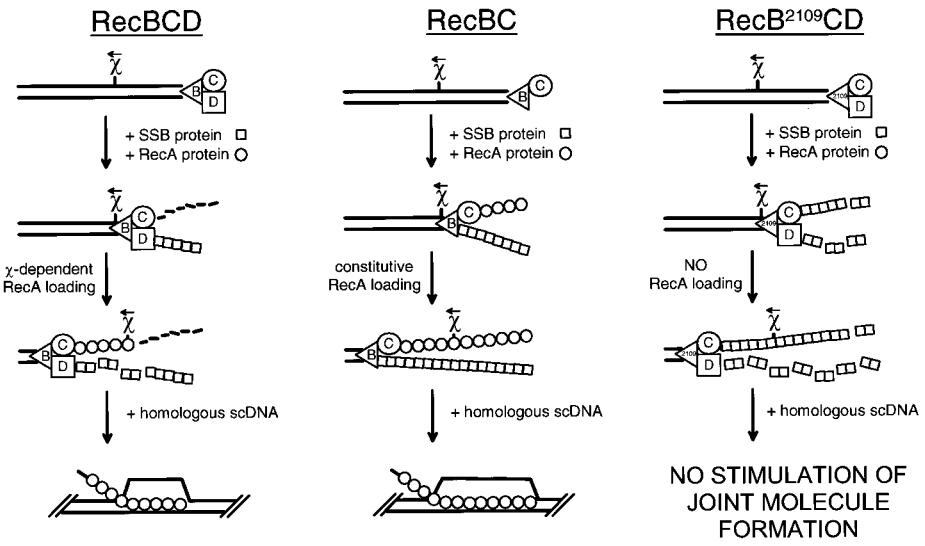

FIG. 3. **Model for recombination deficiency of RecB^{2109}CD enzyme.** The RecBCD, RecBC, and RecB^{2109}CD enzymes all function to generate a 3′-terminal ssDNA tail, but only the recombination-proficient enzymes, RecBCD and RecBC, coordinate the loading of RecA protein (see text for details).

The RecB^{2109}CD enzyme possesses a significantly more active 5′ to 3′ than 3′ to 5′ nuclease activity; thus, processing of a linear, duplex DNA molecule will produce a 3′-ssDNA tailed duplex, which is the appropriate substrate for RecA protein-mediated homologous pairing (29, 30). However, the genetic data clearly demonstrate that the *recB2109* strain is severely deficient for homologous recombination (24). Taken together, these data suggest that merely producing a 3′-terminal ssDNA tail is not sufficient to promote recombination *in vivo*. A second function of the χ-modified RecBCD enzyme is to facilitate the loading of RecA strand exchange protein onto the 3′-terminal χ-containing ssDNA (16). Since the RecB^{2109}CD enzyme can produce a 3′-terminal ssDNA tail, the ability of this enzyme to load RecA protein onto this ssDNA was tested.

RecB^{2109}CD Enzyme Does Not Load RecA Protein onto ssDNA—The loading of RecA protein onto ssDNA by RecBCD enzyme was demonstrated previously by exploiting the ability of RecA protein to protect ssDNA from degradation by exonuclease I (16). When the RecBCD enzyme processes χ-containing dsDNA, three ssDNA products are produced as follows: full-length ssDNA, downstream χ-specific fragments, and upstream χ-specific fragments, all of which are susceptible to degradation by exonuclease I. SSB protein, which binds specifically to ssDNA, cannot protect ssDNA from exonuclease I. On the contrary, if RecA protein is present during processing of the duplex DNA substrate, the downstream χ-specific fragment is specifically bound by RecA protein and protected from degradation. However, this coordinated loading of RecA protein is dependent on neither 3′ to 5′ nuclease activity nor χ recognition. The nuclease-deficient, recombination-proficient RecBC enzyme (31–34) constitutively loads RecA protein on to the 3′-terminal ssDNA (top strand) produced during unwinding, irrespective of the presence or absence of χ (23). Since RecB^{2109}CD enzyme processes duplex DNA to yield a 3′-terminal ssDNA tail, it was of interest to test whether it could also facilitate RecA protein loading like the RecBC and RecBCD enzymes.

Tailed dsDNA substrates that were radioactively labeled at only the 5′ terminus of the top strand were used to test the ability of RecB^{2109}CD enzyme to facilitate the loading of RecA protein (Fig. 2). When the ssDNA products of RecB^{2109}CD enzyme are treated with exonuclease I, the top strand ssDNA fragments are fully degraded, regardless of the presence (Fig. 2, *lanes 1–5*) or absence (Fig. 2, *lanes 7–9*) of RecA protein during processing of the DNA substrate. As a control, coupled reactions (*i.e.* RecA protein present during RecBCD processing of the duplex DNA substrate) using wild-type enzyme with χ-containing dsDNA were performed and, as expected, both full-length ssDNA and upstream χ-specific fragment are quickly degraded upon addition of exonuclease I, whereas the downstream χ-specific fragment is not (Fig. 2, *lanes 13–18*). It was previously shown that RecB^{2109}CD enzyme does not stimulate RecA protein-mediated joint molecule formation in the presence of χ (28). To verify that the RecB^{2109}CD enzyme does not load RecA protein at χ, the exonuclease I-protection assays were performed with the same χ-containing dsDNA as was used with the RecBCD enzyme; no protection was observed.[2] These results show that the RecB^{2109}CD enzyme is incapable of facilitating the loading of RecA protein onto the 3′-terminal ssDNA produced during processing of a duplex DNA substrate. We propose that the inability of the RecB^{2109}CD enzyme to facilitate loading of RecA protein is responsible for the *recB2109* recombination defect observed *in vivo*.

Molecular Nature of the recB2109 Mutation—The *recB2109* gene was sequenced, and a single missense mutation was found. The base pair at position 2420 was changed from C/G to T/A, resulting in a threonine to isoleucine change at amino acid position 807, 121 amino acids upstream of the "hinge region" separating the N-terminal region from the C-terminal region of the RecB subunit (35). The nucleotide sequence for the *recB2109* gene has been deposited in the GenBank™ data base with the accession number AF179304.

DISCUSSION

The ability of RecBCD enzyme to initiate recombination is attributed to two consequences of its interaction with the χ recombination hot spot, nuclease modification and facilitated RecA protein loading (Fig. 3). Prior to χ recognition, the RecBCD enzyme preferentially degrades a dsDNA substrate in the 3′ to 5′ direction. Interaction with χ serves to attenuate this 3′ to 5′ nuclease activity, such that a 3′-terminal ssDNA tail is produced onto which the RecBCD enzyme facilitates the loading of RecA protein (11–14, 16). Although the modification of RecBCD nuclease activity is necessary to preserve a 3′-terminal ssDNA for RecA protein-promoted strand invasion, it was previously unclear whether directed RecA protein loading by RecBCD enzyme is also an essential function of RecBCD enzyme *in vivo*. Here we establish that a recombination-defective mutant RecBCD enzyme, which appropriately processes DNA, fails to load RecA protein onto this 3′-terminal ssDNA strand. Thus, we conclude that RecA protein loading is essential for the

[2] D. A. Arnold and S. C. Kowalczykowski, unpublished observations.

initiation of recombination by RecBCD enzyme.

recB2109 cells are severely defective in homologous recombination. Prior analysis of the RecB^{2109}CD enzyme revealed that this mutant does not attenuate 3′ to 5′ nuclease activity at χ, and it was proposed this defect was responsible for its failure to promote recombination *in vivo* (25, 28). Despite the sensibility of this conclusion, the work presented here demonstrates that the recombination defect of the RecB^{2109}CD enzyme is not so simple. Even though the RecB^{2109}CD enzyme fails to recognize χ, it displays an unexpected pattern of nuclease behavior that essentially mimics that of the χ-activated RecBCD enzyme. This behavior results in the χ-independent generation of a 3′-ssDNA overhang. Thus, if the processing of dsDNA to produce a 3′-terminal ssDNA species were sufficient to promote recombination, then the RecB^{2109}CD enzyme should be recombination-proficient. However, we demonstrate that the RecB^{2109}CD enzyme is also unable to facilitate the loading of RecA protein onto these 3′-terminal tails, perhaps as a consequence of its inability to recognize χ. This finding suggests that the RecBCD recombination pathway is absolutely dependent on the ability of RecBCD enzyme to coordinate the loading of RecA protein onto ssDNA. The corollary to this hypothesis is that enzymes that load RecA onto a 3′-terminal ssDNA, regardless of the presence of χ, would be recombination-proficient. In fact, the RecBC enzyme (lacking the RecD subunit) is a nuclease-free helicase that constitutively loads RecA onto the 3′-terminal ssDNA produced by unwinding (23), and strains expressing functional RecBC enzyme (*recD*$^-$) are recombination-proficient (22, 31, 32). Based on these findings and our current data, we propose that the inability to coordinate the loading of RecA protein is the biochemical defect directly responsible for the severe recombination deficiency of the *recB2109* strain.

Sequencing of the *recB2109* gene revealed a point mutation that results in a single amino acid change at position 807, 121 amino acids upstream of the flexible linker segment (928–933) separating the C-terminal domain from the N-terminal domain of the RecB subunit (35). The 30-kDa C-terminal domain is required for all of the nuclease activities of RecBCD enzyme (35), and although the *recB2109* mutation is not a part of the C-terminal domain, it does affect both 3′ to 5′ and 5′ to 3′ nuclease activities of the RecBCD enzyme, supporting the assertion that RecB plays an important role in the regulation of nuclease activity. Recent work from our laboratory (23) also showed that the C-terminal domain of the RecB subunit is required for RecA protein loading. When the RecB subunit lacking the C-terminal domain is complexed with a wild-type RecC subunit, the truncated RecBC enzyme does not stimulate the loading of RecA protein.[3] These findings, together with the data reported here, further contend that the RecB subunit is intimately involved in both RecBCD nuclease activity and the directed loading of RecA protein.

Interaction of RecBCD enzyme with χ mediates two classes of biochemical events, modification of nuclease activity and the commencement of RecA loading. The RecB^{2109}CD enzyme allowed the examination of one consequence of χ recognition, nuclease modification, in the absence of the other. The model shown in Fig. 3 illustrates the consequences of processing χ-containing duplex DNA by RecBCD, RecBC, and RecB^{2109}CD enzymes. The RecBCD enzyme generates a 3′-terminal ssDNA tail through specific interaction with a χ site, which mediates the attenuation of 3′ to 5′ nuclease activity. The RecBC enzyme lacks nuclease activity, so 3′-terminal (and 5′-terminal) ssDNA is produced by unwinding of any duplex DNA substrate. The altered asymmetry of nuclease activity of the RecB^{2109}CD enzyme also allows preservation of the 3′-terminal ssDNA, since this enzyme preferentially degrades the 5′-terminal strand rather than the 3′-terminal strand. However, only the recombination-proficient enzymes, RecBCD and RecBC, facilitate the loading of RecA protein, allowing preferential invasion of the 3′-terminal ssDNA tail into a homologous supercoiled DNA molecule. RecB^{2109}CD enzyme does not perform this function and does not stimulate the incorporation of the 3′-terminal ssDNA tail into RecA protein-mediated joint molecules (28). The fact that RecB^{2109}CD enzyme produces a 3′-terminal ssDNA tail, but does not promote recombination, supports the conclusion that nuclease modification is necessary, but not sufficient, for initiation of recombination by RecBCD enzyme. The fact that the RecB^{2109}CD enzyme does not mediate the loading of RecA protein strongly suggests that this is a function that is essential for the initiation of homologous recombination by the wild-type RecBCD enzyme.

[3] J. J. Churchill and S. C. Kowalczykowski, submitted for publication.

Acknowledgments—We thank our colleagues in the Kowalczykowski laboratory, Richard Ando, Daniel Anderson, Piero Bianco, Carole Bornarth, Frederic Chedin, Frank Harmon, Julie Kleiman, Jim New, Erica Seitz, and Susan Shetterley, for careful reading of this manuscript.

REFERENCES

1. Kowalczykowski, S. C., Dixon, D. A., Eggleston, A. K., Lauder, S. D., and Rehrauer, W. M. (1994) *Microbiol. Rev.* **58,** 401–465
2. Myers, R. S., and Stahl, F. W. (1994) *Annu. Rev. Genet.* **28,** 49–70
3. Amundsen, S. K., Taylor, A. F., Chaudhury, A. M., and Smith, G. R. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 5558–5562
4. Emmerson, P. T. (1968) *Genetics* **60,** 19–30
5. Eichler, D. C., and Lehman, I. R. (1977) *J. Biol. Chem.* **252,** 499–503
6. Roman, L. J., and Kowalczykowski, S. C. (1989) *Biochemistry* **28,** 2863–2873
7. Roman, L. J., and Kowalczykowski, S. C. (1989) *Biochemistry* **28,** 2873–2881
8. Roman, L. J., Eggleston, A. K., and Kowalczykowski, S. C. (1992) *J. Biol. Chem.* **267,** 4207–4214
9. Smith, G. R., Kunes, S. M., Schultz, D. W., Taylor, A., and Triman, K. L. (1981) *Cell* **24,** 429–436
10. Stahl, F. W., and Stahl, M. M. (1977) *Genetics* **86,** 715–725
11. Dixon, D. A., and Kowalczykowski, S. C. (1993) *Cell* **73,** 87–96
12. Anderson, D. G., and Kowalczykowski, S. C. (1997) *Genes Dev.* **11,** 571–581
13. Dixon, D. A., and Kowalczykowski, S. C. (1991) *Cell* **66,** 361–371
14. Dixon, D. A., and Kowalczykowski, S. C. (1995) *J. Biol. Chem.* **270,** 16360–16370
15. Tracy, R. B., and Kowalczykowski, S. C. (1996) *Genes Dev.* **10,** 1890–1903
16. Anderson, D. G., and Kowalczykowski, S. C. (1997) *Cell* **90,** 77–86
17. Kowalczykowski, S. C. (1991) *Biochimie* (*Paris*) **73,** 289–304
18. Smith, G. R. (1991) *Cell* **64,** 19–27
19. Dower, N. A., and Stahl, F. W. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 7033–7037
20. Biek, D. P., and Cohen, S. N. (1986) *J. Bacteriol.* **167,** 594–603
21. Chaudhury, A. M., and Smith, G. R. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 7850–7854
22. Thaler, D. S., Sampson, E., Siddiqi, I., Rosenberg, S. M., Thomason, L. C., Stahl, F. W., and Stahl, M. M. (1989) *Genome* **31,** 53–67
23. Churchill, J. J., Anderson, D. G., and Kowalczykowski, S. C. (1999) *Genes Dev.* **13,** 901–911
24. Amundsen, S. K., Neiman, A. M., Thibodeaux, S. M., and Smith, G. R. (1990) *Genetics* **126,** 25–40
25. Eggleston, A. K., and Kowalczykowski, S. C. (1993) *J. Mol. Biol.* **231,** 605–620
26. Arnold, D. A., Bianco, P. R., and Kowalczykowski, S. C. (1998) *J. Biol. Chem.* **273,** 16476–16486
27. Ponticelli, A. S., Schultz, D. W., Taylor, A. F., and Smith, G. R. (1985) *Cell* **41,** 145–151
28. Eggleston, A. K., and Kowalczykowski, S. C. (1993) *J. Mol. Biol.* **231,** 621–633
29. Konforti, B. B., and Davis, R. W. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 690–694
30. Konforti, B. B., and Davis, R. W. (1990) *J. Biol. Chem.* **265,** 6916–6920
31. Lovett, S. T., Luisi-DeLuca, C., and Kolodner, R. D. (1988) *Genetics* **120,** 37–45
32. Palas, K. M., and Kushner, S. R. (1990) *J. Biol. Chem.* **265,** 3447–3454
33. Korangy, F., and Julin, D. A. (1993) *Biochemistry* **32,** 4873–4880
34. Anderson, D. G., Churchill, J. J., and Kowalczykowski, S. C. (1997) *Genes Cells* **2,** 117–128
35. Yu, M., Souaya, J., and Julin, D. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 981–986
36. Arnold, D. A. (1999) *Analysis of Two Mutant RecBCD Enzymes Defective in Distinct Aspect of RecBCD-mediated Homologous Recombination.* Ph.D. thesis, University of California, Davis