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A RecA mutant, RecA⁷³⁰, suppresses the recombination deficiency of the RecBC¹⁰⁰⁴D- χ^* interaction *in vitro* and *in vivo*

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Abstract

In *Escherichia coli*, homologous recombination initiated at double-stranded DNA breaks requires the RecBCD enzyme, a multifunctional heterotrimeric complex that possesses processive helicase and exonuclease activities. Upon encountering the DNA regulatory sequence, χ , the enzymatic properties of RecBCD enzyme are altered. Its helicase activity is reduced, the 3'→5' nuclease activity is attenuated, the 5'→3' nuclease activity is up-regulated, and it manifests an ability to load RecA protein onto single-stranded DNA. The net result of these changes is the production of a highly recombinogenic structure known as the presynaptic filament. Previously, we found that the *recC1004* mutation alters χ -recognition so that this mutant enzyme recognizes an altered χ sequence, χ^* , which comprises seven of the original nucleotides in χ , plus four novel nucleotides. Although some consequences of this mutant enzyme-mutant χ interaction could be detected *in vivo* and *in vitro*, stimulation of recombination *in vivo* could not. To resolve this seemingly contradictory observation, we examined the behavior of a RecA mutant, RecA⁷³⁰, that displays enhanced biochemical activity *in vitro* and possesses suppressor function *in vivo*. We show that the recombination deficiency of the RecBC¹⁰⁰⁴D- χ^* interaction can be overcome by the enhanced ability of RecA⁷³⁰ to assemble on single-stranded DNA *in vitro* and *in vivo*. These data are consistent with the previous findings showing that the loading of RecA protein by RecBCD is necessary *in vivo*, and they also show that RecA proteins with enhanced single-stranded DNA binding capacity can partially bypass the need for RecBCD-mediated loading.

Introduction

RecBCD enzyme is a helicase/nuclease that functions at the initiation step of recombinational DNA repair in *Escherichia coli* ^{1,2}. From a nearly blunt double-stranded DNA (dsDNA) end, RecBCD enzyme unwinds and degrades the dsDNA, powered by the two motor subunits, RecB and RecD ³. When the enzyme encounters the recombination hotspot sequence, χ (5'-GCTGGTGG), from the 3' side on one of the DNA strands ⁴, its 5' to 3' nuclease activity is up-regulated and its 3' to 5' nuclease activity is down-regulated ^{5–7}. The consequence of these changes is the production of single-stranded DNA (ssDNA) with the χ sequence at the 3'-terminus; this product is referred to as the " χ -containing ssDNA". In addition, RecBCD enzyme pauses at χ for approximately 5 seconds, and then resumes unwinding at a rate that is about 2-fold slower than the rate prior to χ recognition ^{8,9}. Perhaps the most biologically important property of the χ -modified RecBCD enzyme is its ability to load RecA onto ssDNA ^{10,11}.

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Although the molecular details of this regulatory process remain to be fully understood, the changes elicited by χ -recognition are not the result of RecD-ejection at χ because, even though the RecD subunit regulates RecBCD enzyme activities^{12–14}, recent single-molecule studies showed that the RecD subunit continues to translocate with the holoenzyme after χ recognition⁹. Rather, single-molecule studies^{8,9}, the x-ray crystallographic structure of the enzyme-DNA complex¹⁵, and analysis of a RecBCD enzyme homolog (the *Bacillus subtilis* AddAB enzyme)¹⁶ suggest that binding of the χ sequence itself to RecBCD enzyme elicits the allosteric changes that underlie the enzymatic alterations.

Many studies suggest that the degradative capacity of RecBCD enzyme is part of an antiviral system of *E. coli* that works in conjunction with the restriction-modification system^{17,18}; the χ sequence allows RecBCD enzyme to recognize chromosomal DNA and to thereby protect it from degradation. Consequently, in *recBC*-deficient cells, DNA double-strand breaks accumulate^{19–21}. The χ sequence is over-represented in the *E. coli* genome²², whereas it is absent from or under-represented in bacteriophages (N. H., unpublished observations). Therefore, the χ sequence is an identification marker of the *E. coli* genome. Interestingly, this relationship between a nuclease and its cognate attenuation sequence is conserved in many prokaryotes²³. In *Salmonella typhimurium*, the same eight-nucleotide sequence functions as χ ²⁴, whereas in *Lactococcus lactis*, *Bacillus subtilis* and *Haemophilus influenzae*, the cognate χ sequences are 5'-GCGCGTG, 5'-AGCGG, and 5'-GNTGGTGG, respectively^{25–27}.

Mutant alleles of *recBCD* that recovered all the activities of RecBCD enzyme, except for χ recognition, were originally isolated as pseudo-revertants of a mutant with a *recC* null phenotype. This class of mutants, called RecC*, showed almost the same basal level of recombination as wild type, but this recombination was not stimulated by χ ^{28,29}. Sequencing revealed that, due to a frameshift, all of the analyzed alleles possess substitutions of amino acid residues between positions 647 and 655 in the *recC* gene³⁰. It was found that a novel sequence, different from the canonical χ sequence, was specifically recognized by one of these mutants, *recC1004*, *in vivo*²⁹. Resembling the original χ sequence in its genetic behavior³¹, this novel sequence conferred an increased growth rate to *red⁻ gam⁻* bacteriophage λ on the *recC1004* strain. This novel sequence, which was named χ^* , is the 11 nucleotide sequence, 5'-GCTGGTGCTCG²⁹ and comprises the first seven bases of the *E. coli* χ , plus 4 additional novel bases. As for the wild type pair, the nuclease activity of the *recC1004* mutant protein was attenuated *in vivo* by the χ^* sequence²⁹. Purified RecBC¹⁰⁰⁴D enzyme possessed wild type levels of dsDNA exonuclease and helicase activities, but displayed reduced recognition of wild type χ *in vitro*³⁰. However, this mutant RecBCD enzyme recognized the mutant χ sequence more efficiently than wild type χ , albeit with a lower efficiency than the wild-type enzyme recognized wild type χ . Furthermore, χ^* -dependent joint molecule formation was stimulated by the RecBC¹⁰⁰⁴D enzyme, demonstrating that RecA-loading activity was preserved but, again, the yield was lower than for the fully wild type reaction. Despite these biochemical results, stimulation of recombination *in vivo* using bacteriophage λ crosses could not be detected for this RecC¹⁰⁰⁴ - χ^* interaction²⁹. This inconsistency was explained by a greater detection sensitivity of the *in vitro* assays relative the *in vivo* assay³⁰.

E. coli possesses a second recombination pathway, called the RecF pathway². In the absence of RecBCD function, the RecF pathway can be activated to function at double-stranded DNA breaks by mutation of *sbcb*, which is in the gene encoding exonuclease I³². RecF protein works together with RecO and RecR proteins to load RecA protein onto ssDNA complexed with ssDNA-binding protein (SSB) protein at 5'-end of dsDNA gaps³³. Mutations in the *recF* gene can be suppressed by alleles of *recA* that display enhanced functionality^{34–36}. These mutant RecA proteins (e.g., RecA⁸⁰³ and RecA⁷³⁰) nucleate onto ssDNA more rapidly than wild type RecA protein^{37,38}. Biochemical analysis further established that these enhanced RecA proteins displaced SSB protein from ssDNA faster and more completely than

wild type protein, and the resulting filaments were kinetically more resistant to subsequent displacement by SSB protein.

The relationship between RecA-loading *in vitro* and recombination activity *in vivo* is relatively unstudied. Toward this end, we examined the effect of the *recA730* allele on the recombination phenotype of cells defective in *recBCD* function. We found that, in response to χ^* recognition, RecBC¹⁰⁰⁴D enzyme loaded RecA⁷³⁰ protein onto ssDNA to form nucleoprotein filaments that were more stable than those formed by wild type RecA protein. Furthermore, we found that *recA730* suppressed the recombination deficiency of the mutant RecBC¹⁰⁰⁴D- χ^* interaction *in vivo*, showing that an intrinsic increased propensity to nucleate on ssDNA and to form more stable nucleoprotein filaments can compensate for the lower yield of χ^* -containing ssDNA produced by RecBC¹⁰⁰⁴D enzyme processing.

Results

RecA protein is loaded onto χ^* -containing ssDNA by RecBC¹⁰⁰⁴D enzyme

Previously, it was shown that the *recC1004* mutation changed the specificity of χ recognition from the canonical sequence to a novel sequence, χ^* ²⁹. The purified RecBC¹⁰⁰⁴D enzyme produced χ^* -dependent joint molecules in response to χ^* recognition, but with a lower yield than the wild type reaction³⁰. However, the interaction between RecBC¹⁰⁰⁴D enzyme and χ^* did not result in an increased frequency of recombination as measured by bacteriophage λ crosses²⁹, suggesting that the *in vitro* assay was more sensitive than the *in vivo* assay. To analyze the mutant interaction in more detail, RecA-loading assays were performed as described previously¹⁰, but with one significant difference: ATP γ S was not added to stabilize the RecA nucleoprotein filaments. By omitting the ATP γ S, the resulting ATP-RecA nucleoprotein filaments were kinetically less stable, permitting experimental distinction between wild type and RecA⁷³⁰ proteins (see below).

As shown in Figure 1, in response to χ -recognition, wild type RecBCD enzyme produced full-length ssDNA and two χ -specific ssDNA fragments (Figure 1B, experiment set 1). To determine whether RecA protein is bound to the 3'-end of any of these ssDNA products, exonuclease I, a 3'-specific ssDNA exonuclease, was added prior to deproteinization¹⁰. As reported previously, when exonuclease I was added to the processing products that were formed in the absence of RecA protein, both the full length ssDNA and the χ -specific ssDNA were degraded within a few minutes because exonuclease I rapidly degrades ssDNA that is complexed with SSB protein (Figures 1B, experiment set 3, and 1C, experiment set 8). On the other hand, when RecA protein was present, the χ -containing ssDNA, but not the full length ssDNA nor the other χ -specific ssDNA, was protected by the RecA protein from exonuclease digestion (Figure 1B, experiment set 2) because exonuclease I digests the RecA-coated ssDNA slowly than the SSB-ssDNA complex^{10,39}. When RecBC¹⁰⁰⁴D enzyme and a χ^* -containing dsDNA were examined in the same reactions (Figure 1C), protection of the χ^* -containing ssDNA by RecA protein was also detected (compare Figure 1C, experiment sets 5 and 6), even though production of the χ^* -containing ssDNA was reduced by approximately half relative to production of the χ -specific ssDNA as was reported previously^{30,40}.

RecA⁷³⁰ protein is loaded onto χ -containing fragments more rapidly and produces more stable nucleoprotein filaments than wild type protein

The yield of χ^* -containing ssDNA produced by the RecBC¹⁰⁰⁴D enzyme is reduced relative to the wild type interaction, resulting in a lower yield of RecA nucleoprotein filaments needed for recombination³⁰. However, we reasoned that perhaps a mutant RecA protein which had an intrinsically greater SSB-displacement activity might increase the observed yield of active nucleoprotein filaments; consequently, recombination might be increased *in vivo*. Therefore,

RecA⁷³⁰ protein was examined. The RecA⁷³⁰-ssDNA complex was found to produce nucleoprotein filaments that were more resistant to exonuclease I (Figure 1C, experiment set 7). Full length ssDNA was also protected by the mutant RecA protein, as expected from the enhanced SSB-displacement ability of RecA⁷³⁰ protein³⁸. RecA⁷³⁰ protein also protected ssDNA produced by RecQ, RecB¹⁰⁸⁰CD, or RecB²¹⁰⁹CD helicases from degradation (data not shown), showing that the increased protection is not specific to any DNA helicase, but rather it is consistent with its enhanced filament nucleation capability.

Enhanced assembly of nucleoprotein filaments is an intrinsic property of RecA⁷³⁰

To confirm that the increased protection of all ssDNA is intrinsic to RecA⁷³⁰ protein, reactions where RecA nucleoprotein filament assembly was coupled to ssDNA production by RecBCD enzyme were compared to reactions where RecA protein was assembled on heat-denatured DNA in the absence of RecBCD enzyme^{10,39}. As previously reported, wild type RecA protein did not protect ssDNA produced by heat-denaturation from exonuclease I degradation, because it cannot displace SSB protein efficiently (Figure 2A, experiment set 2). In contrast, there was greater protection by RecA⁷³⁰ than by wild type protein of the full length ssDNA produced either by heat denaturation (Figure 2B, experiment set 5) or by RecBCD enzyme (Figure 2B, experiment set 4). This observation supports our conclusion that the higher nucleation frequency of RecA⁷³⁰ protein, which results in increased displacement of SSB protein, is responsible for the enhanced protection of any ssDNA produced.

Previously, it was also shown that RecA protein was loaded onto χ -containing SSB-complexed ssDNA by RecBCD enzyme only when the RecA protein was present during DNA unwinding (a coupled reaction); in contrast, RecA protein was not loaded onto the χ -containing ssDNA when it was added subsequent to DNA processing by RecBCD enzyme (an uncoupled reaction)^{10,39}. In agreement, in an uncoupled reaction with wild type RecA protein, all of the ssDNA was digested by exonuclease I whereas, in a coupled reaction, only the χ -containing ssDNA was protected (Figure 3A, compare experiment sets 1 and 2). In contrast, but consistent with Figures 1 and 2, RecA⁷³⁰ protein afforded better protection to all ssDNA in both the coupled and uncoupled reactions, with both mutant and wild type RecBCD enzyme and both mutant and wild type χ sequences (Figures 3B and 3C). The enhanced ability of RecA⁷³⁰ protein, relative to wild type, to displace SSB protein is apparent in the coupled reactions (Figure 3B, experiment set 4 and Figure 3C, experiment set 7): both χ -containing ssDNA and full length ssDNA were more protected (compare to Figure 3A, experimental set 1 and Figure 3C, experiment set 9, respectively). Because the yield of χ^* -containing ssDNA is always lower than that of the wild-type χ -containing ssDNA, the protection afforded by RecA⁷³⁰ is more difficult to assess by visual inspection; however, quantification of 5 independent replicates of Figure 3B shows that $76 \pm 7\%$ of the χ^* -containing ssDNA remained after 10 minutes in the coupled reaction (experiment set 4), whereas only $38 \pm 2\%$ (two independent replicates) remained in the uncoupled reaction (experiment set 5). As expected, due to the absence of loading by RecBCD enzyme, in the uncoupled reaction, protection of the full length ssDNA ($34 \pm 2\%$) was the same as that of the χ^* -containing ssDNA ($38 \pm 2\%$). Thus, RecA⁷³⁰ protein can assemble more quickly on any ssDNA by an enhanced intrinsic polymerization capacity; in addition, it can be loaded onto χ -containing ssDNA by both wild type and mutant RecBCD enzyme in response to wild-type and mutant χ sequences.

The RecA⁷³⁰ mutation partially suppress the UV sensitivity of recF recC1004 strains

To determine whether the recombination deficiency of the RecBC¹⁰⁰⁴D enzyme can be suppressed by RecA⁷³⁰ protein *in vivo*, the UV sensitivity of *recC1004* mutants harboring RecA expression plasmids was measured. Expression of both *recA730* and wild type *recA* suppressed the UV sensitivity of the *recA*⁻ strain (Figure 4A). Also, as reported previously^{34,36}, *recA730* partially suppressed a *recF* mutation (Figure 4B), whereas wild type *recA* could

not. The *recA730* mutation also partially suppressed the original *recC1004* strain, which also carried a *recF* mutation and consequently showed severe UV sensitivity²⁸ (Figure 4C). To determine the effect of *recA730* on the RecBCD pathway, a *recF*⁺ background was also investigated. In the *recF*⁺ background, however, the *recC1004* mutation did not show a severe UV sensitivity (Figure 4D), and there was no detectable suppression of the modest UV sensitivity of the strain by *recA730*. Therefore, it is most likely that the partial suppression observed in *recF*[−] background by *recA730* is due to suppression of the *recF* mutation, rather than the *recC1004* mutation (Figure 4B).

RecA⁷³⁰ restores the recombination deficiency of the RecC1004 - χ^* interaction

Partial suppression of the UV sensitivity of *recF* by *recA730* was detected, but our *in vitro* findings suggested that RecA⁷³⁰ should also compensate for the lower production of χ^* -containing ssDNA by RecBC¹⁰⁰⁴D enzyme. To test this hypothesis, recombination between λ phages was investigated²⁹. The parental λ phages have either a *Sam7* or *Jh* mutation, and the products of recombination crossover, recombinant phage possessing *S*⁺ and *Jh*, were selected (Figure 5A). In the *recF recC1004* background, both the recombination frequency (Figure 5B left panel) and stimulation of recombination by χ^+ and χ^* was experimentally indistinguishable (Figure 5C left panel), as previously reported²⁹.

However, in the presence of RecA⁷³⁰, the frequency of recombination in *recC1004* strains was increased (Figure 5B, right panel). Also, and more importantly, χ^* shows significant recombination hotspot activity in the *recC1004* background when RecA⁷³⁰ protein was present (Figure 5C, right panel). Even in the *recF*⁺ background, a similar suppression of RecBC¹⁰⁰⁴D- χ^* recombination was observed (data not shown). Using this identical assay, wild type RecBCD and χ^+ showed a 4.2-fold increase in recombination frequency (the recombination frequency for $\chi^0 \lambda$ phage was 0.50 ± 0.18), and a 6.5-fold increase for hotspot activity assay (data not shown;²⁹). Finally, it is worth noting that when wild type RecA protein was over-expressed, a partial stimulation of both recombination (1.46 ± 0.13 fold) and hotspot activity (1.87 ± 0.22) was observed only for χ^* (Figure 5B and C, center panel). These findings suggest that increased concentrations of RecA protein can overcome the deficiency of RecBC¹⁰⁰⁴D- χ^* stimulated recombination *in vivo*.

Discussion

Here we show that a mutant RecA protein, RecA⁷³⁰, which has an enhanced capacity to nucleate on ssDNA, can rescue deficiencies of a mutant RecBCD enzyme. *In vitro*, the yield of RecA nucleoprotein filaments assembled on χ^* -containing ssDNA, produced by the processing of dsDNA with a χ^* sequence by RecBC¹⁰⁰⁴D enzyme, is increased. *In vivo*, the frequency of χ^* -stimulated recombination is increased by the RecA⁷³⁰ protein. Previously, we found that the χ^* sequence attenuated the nuclease activity of the RecBC¹⁰⁰⁴D enzyme both *in vivo* and *in vitro*^{29,30}, and that χ^* -dependent joint molecules were produced *in vitro*³⁰. However, stimulation of recombination was not detected *in vivo*²⁹. Because DNA pairing *in vitro* coordinated by RecBC¹⁰⁰⁴D enzyme and χ^* was lower than for wild type enzyme, we concluded that the failure to detect recombination *in vivo* resulted from the lower yield of χ^* -containing nucleoprotein filaments³⁰. Since the RecA⁷³⁰ protein both nucleates faster on ssDNA and more efficiently displaces SSB protein^{38,41}, we reasoned that this mutant RecA protein might suppress the recombination deficiency displayed by the RecBC¹⁰⁰⁴D - χ^* interaction. Indeed, RecA⁷³⁰ protein did so.

Suppressors of mutations in the RecF pathway were discovered that mapped in *recA*^{34–36}. Subsequently, it was established that these mutant RecA proteins assembled on ssDNA faster due to an increased frequency of spontaneous nucleation; as a consequence, these mutant RecA proteins more rapidly and more fully displace SSB protein from ssDNA^{38,42–47}. Recently,

it was shown that components of the RecF pathway can contribute to RecBCD pathway if the RecA-loading activity of the RecBCD enzyme was inactivated^{11,48–50}. This suppression is not restricted to the *recB1080* allele, because the UV sensitivity of *recB2154*, *recB2155*, *recC2145*, *recC1002*, and *recC1004*, which had been measured previously in a *recF*[−] background, was also corrected by *recF*⁺ (Figure 4 and N.H. and Ichizo Kobayashi (unpublished observations)). The partial suppression of UV sensitivity by the RecF pathway can be explained by RecA-loading ability of the RecFOR complex which compensates for the lost RecA-loading capacity of certain mutant RecBCD enzymes^{11,50}. Consequently, we could not determine whether RecA⁷³⁰ could suppress the UV sensitivity of the *recC1004* mutation because this mutant showed little sensitivity to UV in a *recF*⁺ background, showing that the RecF pathway makes a significant contribution to UV resistance in these cells. This result is also consistent with the original finding that *recC1004* is phenotypically *Rec*⁺ in phage λ crosses (Figure 5). Thus, due to the relatively low UV sensitivity of the *recC1004* strain, an effect of RecA⁷³⁰ on UV survival could not be detected. However, we could clearly detect suppression of the UV sensitivity of *recC1004* in a *recF*[−] background. Collectively, these results suggest that the basal level of recombination in *recC1004* is sufficiently high in otherwise wild type cells for most χ^* and χ -like sequence-stimulated recombinational DNA repair. However, this level of recombinational repair is clearly less than that of the wild-type RecBCD enzyme, which is apparent in a *recF*[−] background. We suggest that this sensitivity arises from the reduced yield of χ (-like) ssDNA that is needed for efficient repair. This sub-optimal level of repair can be suppressed by RecA⁷³⁰ protein or by over-expression of wild type RecA protein, either of which results in more effective utilization of the limited χ -containing ssDNA produced.

However, the suppression of recombination in a *recC1004* background by *recA730* that we observed in lambda crosses involving χ^* (Figure 5) cannot be due to suppression by the RecF pathway, because χ^* did not stimulate recombination even in a *recF*⁺ background²⁹ (data not shown). Consequently, we conclude that the increased SSB-displacement capability of this mutant protein is responsible for the heightened recombination frequency. Although RecA⁷³⁰ suppressed the recombination defect of RecBC¹⁰⁰⁴D enzyme and χ^* , the suppressed level was still below that of the wild type RecBCD-canonical χ interaction *in vivo* simply because the yield of the processed χ^* -containing ssDNA is reduced.

Extending previous studies, here we demonstrated that the assembly of a RecA nucleoprotein filament, either intrinsic or loaded by RecBCD enzyme after χ recognition, is an important aspect of genetic recombination. These findings further enforce the idea that the loading of a DNA strand exchange protein by recombination mediators is a crucial aspect of recombinational DNA repair. The universality of this concept is supported by recent findings in eukaryotic recombination. The assembly of a *Saccharomyces cerevisiae* Rad51 nucleoprotein filament is facilitated by Rad55/57⁵¹, and this Rad55/57-loading can be bypassed by suppressors in Rad51 protein that acquire an enhanced capacity to displace the ssDNA binding protein, RPA⁵². Also, both Rad51 nucleoprotein filament assembly and RPA-displacement are mediated by Rad52 protein^{53–55}. Finally, the fungal homolog of BRCA2, *Ustilago maydis* Brh2 protein⁵⁶, also facilitates loading of Rad51 protein onto complexes of RPA and ssDNA⁵⁷. Thus, catalysis of RecA/Rad51 nucleoprotein filament formation is an essential aspect of recombinational DNA repair.

Materials & Methods

Bacterial strains, phages and plasmids

Escherichia coli strains used were: SCK303 (a $\Delta recA$ srl::Tn10 derivative of KK2186⁵⁸; laboratory collection), BIK1291 (= DH10B; *araD139* $\Delta(ara, leu)$ 7697 $\Delta lacX74$ *galU galK mcrA* Δ (*mrr-hsdRMS-mcrBC*) *rpsL deoR* (ϕ 80 $\Delta lacZ$ Δ M15) *endA1 nupG recA1*; Dr. Ichizo Kobayashi)⁵⁹, V66 (= BIK796; *recF143 argA his-4 met rpsL31* $\lambda^- F^-$; Dr. Ichizo Kobayashi)

²⁸, BIK1288 (as V66, but *recF*⁺ *zic::Tn10*; Dr. Ichizo Kobayashi) ²⁹, V72 (= BIK1274; as V66, but *recC1004*; Dr. Ichizo Kobayashi) ²⁸, BIK1284 (as V72, but *recF*⁺ *zic::Tn10*; Dr. Ichizo Kobayashi) ²⁹, V68 (= BIK2411; as V66, but *recC73*; Dr. Ichizo Kobayashi) ²⁸ and BIK3738 (as BIK1288, but *recC73*; Dr. Ichizo Kobayashi) ²⁹, BIK808 (= FS620; C600 λ *recB21 supE*; Dr. Ichizo Kobayashi) ²⁹, JM1 (= FS611; *recB21 recC22 sbcA20 supF*; Dr. Ichizo Kobayashi) ²⁹. Bacteriophage λ strains, LIK916 (χ^0), LIK950 (χ^+), LIK907 (χ^*) and LIK1068, were used for the recombination crosses (Dr. Ichizo Kobayashi) ²⁹. The *recC73* mutation displays a null phenotype ²⁸ due to truncation by a frameshift mutation at position 1938 in the *recC* gene ³⁰. The mutant gene product should produce a 663 amino acid polypeptide, comprising 646 amino acids of the wild type sequence, followed by an additional 17 amino acids. Plasmid pMS421 carrying *lacI^q* gene was described ⁶⁰. Multicopy plasmids pKM100, containing a *lacUV5*-controlled *recA* gene, and pKM300, containin a T7-promoter-controlled *recA* gene, as well as bacteriophage M13-KM2 were described previously ⁶¹. The *recA730* derivative plasmids, pSNH50 and pSNH60, were made by site-directed mutagenesis (Stratagene) using 2 synthetic oligonucleotides (5'-GGGTGAAGACCGTTCTATGGATGTGAAAACCATCTCTACCG and 5'-CGGTAGAGATGGTTTTTCACATCCATAGAACGGTCTTCACCC) from pKM100 and pKM300, respectively. Sequencing of the entire *recA* gene in these plasmids confirmed that no the other mutations were present (data not shown).

Media

E. coli cells were grown in L broth (1.0% Bacto-tryptone, 0.5% yeast extract and 1.0% sodium chloride (NaCl)), or tryptone broth (1.0% Bacto-tryptone and 0.5% NaCl) supplemented with 0.2% maltose, 10 mM MgSO₄, and 10 μ g/ml vitamin B1. Antibiotics at the following concentrations were added when required: ampicillin (amp) at 100 μ g/ml, chloramphenicol (cam) at 25 μ g/ml, tetracycline (tet) at 10 μ g/ml, and spectinomycin (spc) at 30 μ g/ml.

Proteins and reagents

RecBCD, RecBC¹⁰⁰⁴D, SSB, and wild type RecA proteins were purified as described previously ^{40,62–64}. RecA⁷³⁰ protein was purified using a procedure as described ⁶⁵: Plasmid pSNH60 that carries the *recA730* gene downstream of the T7 promoter was introduced into SCK303. The transformant was cultured at 37 °C in L broth containing amp to mid-log phase (OD₆₀₀ = 0.3). RecA protein synthesis was induced by adding M13 phage (multiplicity of infection (moi) = 10) that expressed T7 RNA polymerase, M13-KM2, together with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 3 hr. Cells were harvested, re-suspended in ice-cold buffer (50 mM Tris-HCl (pH 8), 5 mM EDTA, 25% sucrose and 5 mM β -mercaptoethanol) and frozen at –80 °C. After thawing, cells were lysed with 0.5 mg/ml lysozyme followed by sonication. The lysate was mixed with 0.31% Brij-58 and centrifuged with 25 krpm for 45 min. The cleared lysate was diluted with the same buffer to adjust the OD₂₆₀ to 160. Polyethyleneimine (pH 8) was added to 0.5% to precipitate the nucleic acids, and then centrifuged at 10 krpm for 20 min. The pellet was suspended in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.3 M ammonium sulphate (Am₂SO₄) and 5 mM β -mercaptoethanol, stirred for 2 hr, and then centrifuged. After the centrifugation, the supernatant was made 60% saturating by adding solid Am₂SO₄ and centrifuged. The pellet was suspended in a dialysis buffer (20 mM Tris-HCl (pH 7.5), 20 mM magnesium chloride (MgCl₂), 5 mM β -mercaptoethanol and 10% glycerol) and dialyzed over night. The precipitate was dissolved in buffer (20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 M NaCl, 5 mM β -mercaptoethanol and 10% glycerol) and centrifuged. The supernatant was loaded onto a S-300HR column (Pharmacia Biotech; 300 ml; flow rate of 3 ml/min) equilibrated with TEM + 1M NaCl buffer (20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM β -mercaptoethanol, 10% glycerol and 1 M NaCl). RecA⁷³⁰ protein, identified by ssDNA-dependent ATPase activity and SDS-PAGE analysis, eluted as a single peak. The pooled peak fractions from the S-300HR were loaded onto

EconoPack Q column (Bio-Rad; 5 ml; flow rate of 0.5 ml/min) after dialysis against TEM + 50 mM NaCl buffer; the RecA⁷³⁰ protein eluted in the flow through. The pool was dialyzed against buffer (50 mM Tris-HCl (pH 8), 1 mM EDTA, 5 mM β -mercaptoethanol), solid Am₂SO₄ was added to 70% saturating, and the solution was centrifuged. The pellet was suspended into TEDS + 100 mM NaCl (20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT) and 100 mM NaCl) and dialyzed against the same buffer. The solution was filtered through a 0.2 μ m filter, and then loaded onto MonoQ HR10/10 column (Pharmacia Biotech; 8 ml; flow rate of 3.0 ml/min). RecA⁷³⁰ protein eluted at approximately 480 mM NaCl in a 360 ml linear gradient (100 – 1000 mM NaCl). The pooled protein was concentrated by dialysis against storage buffer (50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM DTT, 150 mM NaCl, and 10% glycerol). The concentration of RecA⁷³⁰ protein was determined spectrophotometrically using an extinction coefficient of $2.15 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 278 nm.

Exonuclease I, restriction endonucleases, and T4 polynucleotide kinase were products of New England Biolabs. Shrimp alkaline phosphatase was purchased from United States Biochemical Corp. Proteinase K was purchased from Roche Molecular Biochemicals. Adenosine triphosphate (ATP) was purchased from Sigma. ATP was dissolved in water at pH 7.5 and the concentration was determined spectrophotometrically using an extinction coefficient of $1.54 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 260 nm. All chemicals were reagent grade and solutions were prepared with NanoPure Water.

Substrate DNA for biochemical analysis

Plasmids pBR322, pNH92 and pNH94³⁰ were purified using a Qiagen kit and digested by a restriction endonuclease, AvaI, following a reaction with shrimp alkaline phosphatase for removal of phosphoryl groups. After the 5'-end of the linear dsDNA was labeled by T4 polynucleotide kinase with ³²P, unincorporated [γ -³²P]ATP was removed by passage through a MicroSpin S-200 HR column (Amersham Pharmacia Biotech).

Exonuclease I protection assay and quantification of χ -specific fragment production

The procedure described previously was followed^{10,39} except that ATP γ -S was omitted. Reactions contained 25 mM Tris-acetate (pH 7.5), 8 mM magnesium acetate, 5 mM ATP, 1 mM DTT, 10 μ M nucleotide linear dsDNA, 5 μ M either wild-type RecA or RecA⁷³⁰ protein, 4 μ M SSB protein, and either 0.1 nM RecBCD or 0.2 nM RecBC¹⁰⁰⁴D enzyme. Reactions (37 °C) were started by addition of RecBCD enzyme. After 3 min, poly(dT) (50 μ M nucleotide) was added to sequester the free RecA protein. After 2 min of further incubation, an aliquot was taken (representing time zero) and then exonuclease I was added to a final concentration of 100 U/ml and incubated at room temperature for 10 min. Control reactions contained heat-denatured DNA instead of dsDNA processed by RecBCD enzyme or, in the case of the uncoupled reactions, RecA protein was added 3 min after addition of RecBCD enzyme. Aliquots were added to stop solution (40 mM EDTA, 0.8% SDS, 1.5 μ g/ μ l proteinase K and 0.04% BPB) at the indicated times after exonuclease I addition, and were analyzed by agarose gel (1.0 % (w/v)) electrophoresis. Production of χ -specific fragments was quantified by using a Molecular Dynamics STORM 870 PhosphorImager and ImageQuant software (Molecular Dynamics). The percentages were calculated relative to the initial amount of the substrate.

Standard deviations (S.D. = $\sqrt{\frac{\sum (y_i - y_{\text{mean}})^2}{(N - 1)}}$) were performed using GraphPad Prism version

4.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. In all graphs, points represent the mean and the error bars are the standard deviation.

UV sensitivity measurement

Exponentially growing cultures (in L broth with amp and spc for selection of plasmid and IPTG to express the *recA* gene) were diluted into M9 medium⁶⁶ and spread on L agar plates. The plates were irradiated with UV light (254 nm) for various doses (times). Colonies were scored after incubation at 37 °C for 20 hr in the dark.

Lambda phage recombination assay

The experimental design is shown in Figure 5A. The procedure described previously was followed²⁹: Parental phages (both LIK916, 950 or 907 and LIK1068) were mixed together before infection of pre-warmed *E. coli* host cells. Infection was carried out at $\text{moi} = 5$ for each phage. After a cycle, S^+ - *Jh* recombinant phages were counted by plating on BIK808 and total phages were measured by plating on JM1. The recombination frequency (%) was calculated as $[\text{recombinant phage titer}/\text{total phage titer}] \times 100$ and the hotspot activity was assessed by the ratio, turbid plaque number/clear plaque number, four the recombinant phages plated on BIK808.

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Figure 1. RecBCD and RecBC¹⁰⁰⁴D enzymes load RecA and RecA⁷³⁰ proteins onto χ^* -containing ssDNA

A. Illustration of the substrate used and the major products produced by the helicase/nuclease activities of RecBCD enzyme upon χ recognition. **B.** Wild type RecBCD enzyme. *Left:* Gel showing RecA-loading onto χ -containing ssDNA, assayed by protection from exonuclease I digestion (present in all lanes except “-ExoI” controls). The proteins and linear dsDNA (χ^+ or χ^*) present are indicated at the top of the gel. The vertical arrows indicate the time of exonuclease I addition. *Right:* Quantification of χ -containing ssDNA protection. The amount of χ -containing ssDNA remaining is expressed relative to the initial amount of dsDNA (lane M): filled squares: RecA in the absence of exonuclease I (exp. set 1); filled circles: RecA (exp. set 2); open squares: RecA omitted (exp. set 3); and filled diamonds: RecA and χ^* instead of

χ (exp. set 4). C. RecBC¹⁰⁰⁴D enzyme and χ^* . *Left*: Gel showing RecA-loading onto χ^* containing ssDNA, assayed by protection from exonuclease I digestion. The proteins (wild type RecA or RecA⁷³⁰) and linear dsDNA (χ^*) present are indicated at the top of the gel. *Right*: Quantification of χ^* -containing ssDNA protection: filled squares: RecA in the absence of exonuclease (exp. set 5); filled diamonds: RecA (exp. set 6); filled triangles: RecA⁷³⁰ (exp. set 7); and open squares: RecA omitted (exp. set 8). .

A. χ^+ substrate **B. χ^* substrate**

Figure 2. RecA⁷³⁰ possesses an enhanced capacity to form stable nucleoprotein filaments on SSB-ssDNA complexes

RecA nucleoprotein filaments were formed on heat-denatured linear dsDNA in the presence of SSB protein for comparison to nucleoprotein filament formation in the coupled RecA and RecBCD reactions. **A.** Wild type RecA protein and χ^+ DNA. **B.** RecA⁷³⁰ protein and χ^* DNA. The RecA proteins used in each experiment are indicated. The vertical arrows indicate the time of exonuclease I addition. Lanes marked "HD" and "M" represent heat-denatured and linear dsDNA, respectively. For comparison, experiments 1 and 4 are coupled reactions.

A. χ^+ substrate

B. χ^* substrate

C.

Figure 3. RecA⁷³⁰ protein can be loaded onto χ -containing ssDNA to form nucleoprotein filaments that are more stable than those formed by wild type RecA protein
A. Wild type RecBCD enzyme, RecA protein and χ^+ DNA. “Coupled” experiments referred to reactions where RecA protein was present at the beginning of the DNA processing reaction by RecBCD enzyme. In “uncoupled” experiment, RecA protein was added after DNA processing by RecBCD enzyme. The vertical arrows indicate the time of exonuclease I addition. B. RecA⁷³⁰ protein, RecBC¹⁰⁰⁴D enzyme, and χ^* DNA. C. Comparison of RecA⁷³⁰ with wild type RecBCD and χ versus wild type RecA with RecBC¹⁰⁰⁴D and χ^* ; both coupled and uncoupled reactions are shown.

Figure 4. Suppression of UV sensitivity by *recA730*

A. *recA1* background. **B.** *recF143* background. **C.** *recF143 recC1004* background. **D.** *recC1004* background. Open triangles represent the strains lacking any *recA*-expressing plasmid. Open squares represent the strains expressing wild type *recA*. Closed circles represent the strains expressing *recA730*; closed squares represent the wild type *rec+* strains; closed diamonds represent the *recC73* strains; and the closed triangles in panel C are *recF143*.

Figure 5. *recA730* restores the recombination hotspot activity of χ^* in a *recF recC1004* strain
A. Design of the λ recombination crosses²⁹. Parental phages are defective for their phage-encoded recombination functions (*red⁻ γ⁻ int⁻*). The *S⁺ Jh* recombinant phages were scored as to whether the plaque was turbid (*cI⁺*; crossover before the immunity region) or clear (*cI⁻*; crossover beyond the region). **B.** Recombination frequency. The relative value, normalized to the wild type (χ^0) strain, of the ratio [*S⁺ Jh* recombinants/total phage] is plotted. Left panel is the *recF143 recC1004* strain without plasmid (N = 3). Center panel is the same strain with the wild type *recA* expressing plasmid (N = 3). Right panel is the same strain with the *recA730* expressing plasmid (N = 4). The recombination frequency and standard deviation for χ^0 was 0.43 ± 0.08 , 0.41 ± 0.14 , and 0.43 ± 0.11 for the parental strain, wild type *recA*, and

recA730, respectively. **C.** Hotspot activity. The ratio of turbid plaques to clear plaques is plotted.