A RecA Mutant, RecA\textsuperscript{730}, Suppresses the Recombination Deficiency of the RecBC\textsuperscript{1004} D–χ* Interaction in Vitro and in Vivo

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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 recC\textsuperscript{1004} 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, RecA\textsuperscript{730}, that displays enhanced biochemical activity in vitro and possesses suppressor function in vivo. We show that the recombination deficiency of the RecBC\textsuperscript{1004} D–χ* interaction can be overcome by the enhanced ability of RecA\textsuperscript{730} to assemble on single-stranded DNA in vitro and in vivo. These data are consistent with findings showing that the loading of RecA protein by RecBCD is necessary in vivo, and they show that RecA proteins with enhanced single-stranded DNA-binding capacity can partially bypass the need for RecBCD-mediated loading.

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Introduction

RecBCD enzyme is a helicase/nuclease that functions at the initiation step of recombinational DNA repair in *Escherichia coli*. 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. When the enzyme encounters the recombination

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Abbreviations used: ds, double-stranded; ss, single-stranded.

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hotspot sequence, χ (5′-GCTGGTG), from the 3′ side on one of the DNA strands, its 5′ to 3′ nuclease activity is up-regulated and its 3′ to 5′ nuclease activity is down-regulated. 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 s, and then resumes unwinding at a rate that is about twofold slower than the rate before χ recognition. Perhaps the most biologically important property of the χ-modified RecBCD enzyme is its ability to load RecA onto ssDNA. Although the molecular details of this regulatory process are not 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, recent single-molecule studies...
showed that the RecA subunit continues to translocate with the holoenzyme after \( \chi \) recognition.\(^9\) Rather, single-molecule studies,\(^6,9\) the X-ray crystallographic structure of the enzyme–DNA complex,\(^15\) and analysis of a RecBCD enzyme homolog (the *Bacillus subtilis* AddAB enzyme)\(^16\) suggest that binding of the \( \chi \) 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;\(^7,18\) the \( \chi \) sequence allows the 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 \( \chi \) sequence is over-represented in the *E. coli* genome,\(^22\) whereas it is absent from or under-represented in bacteriophages (N. H. and Ichizo Kobayashi, unpublished results). Therefore, the \( \chi \) 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 prokaryotes.\(^23\) In *Salmonella typhimurium*, the same eight-nucleotide sequence functions as \( \chi \),\(^24\) whereas in *Lactococcus lactis*, *Bacillus subtilis* and *Haemophilus influenzae*, the cognate \( \chi \) sequences are 5ʼ-GGGCCCTG, 5ʼ-AGCGG, and 5ʼ-GNTGTGG, respectively.\(^25-27\)

Mutant alleles of recBCD that recovered all the activities of RecBCD enzyme, except for \( \chi \) recognition, were isolated originally as pseudo-revertants of a mutant with a recC null phenotype. This class of mutants, called RecC\(\ast\), showed almost the same basal level of recombination as wild-type, but this recombination was not stimulated by \( \chi \).\(^28,29\) 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 *recG* gene.\(^30\) It was found that a novel sequence, different from the canonical \( \chi \) sequence, was recognized specifically by one of these mutants, \( \chi \) in *in vivo*.\(^29\) This original \( \chi \) sequence in its genetic behavior,\(^31\) this novel sequence conferred an increased growth rate to *red*–*gam*– bacteriophage \( \lambda \) on the *recC1004* strain. This novel sequence, which was named \( \chi \), is the 11 nt sequence, 5ʼ-GCTGGTGG,\(^29\) and comprises the first seven bases of the *E. coli* \( \chi \) plus four novel bases. As for the wild-type pair, the nuclease activity of the *recC1004* mutant protein was attenuated in *in vivo* by the \( \chi \) sequence.\(^29\) Purified RecBC\(1004\) enzyme possessed wild-type levels of dsDNA exonuclease and helicase activities, but displayed reduced recognition of wild-type \( \chi \) in *in vitro*.\(^30\) However, this mutant RecBCD enzyme recognized the mutant \( \chi \) sequence more efficiently than wild-type \( \chi \), albeit with a lower efficiency than the wild-type enzyme recognized wild-type \( \chi \). Furthermore, \( \chi \)-dependent joint molecule formation was stimulated by the RecBC\(1004\) 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 *in vivo* using bacteriophage \( \lambda \) crosses could not be detected for this RecC\(1004\)-\(\chi\) interaction.\(^29\) This inconsistency was explained by a greater detection sensitivity of the *in vitro* assays relative to the *in vivo* assay.\(^30\)

*E. coli* possesses a second recombination pathway, called the RecF pathway.\(^2\) In the absence of RecBCD function, the RecF pathway can be activated to function at dsDNA breaks by mutation of *sbcB*, which is in the gene encoding exonuclease I.\(^32\) RecF protein works together with RecO and RecR proteins to load RecA protein onto ssDNA complexed with ssDNA-binding (SSB) protein at the 5′ end of dsDNA gaps.\(^33\) Mutations in the *recF* gene can be suppressed by alleles of *recA* that display enhanced functionality.\(^34,36\) These mutant RecA proteins (e.g. RecA\(803\) and RecA\(730\)) nucleate on ssDNA more rapidly than wild-type RecA protein.\(^37,28\) 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 *in vitro* and recombination activity in *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 \( \chi \)-dependent joint molecules in response to *sbcB* mutation, RecBC\(1004\) enzyme loaded RecA\(730\) 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 RecBC\(1004\)-\(\chi\) interaction in *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 \( \chi \)-containing ssDNA produced by RecBC\(1004\) enzyme processing.

### Results

RecA protein is loaded onto \( \chi \)-containing ssDNA by RecBC\(1004\) enzyme

Previously, it was shown that the *recC1004* mutation changed the specificity of \( \chi \) recognition from the canonical sequence to a novel sequence, \( \chi \).\(^29\) The purified RecBC\(1004\) enzyme produced \( \chi \)-dependent joint molecules in response to \( \chi \) recognition, but with a lower yield than the wild-type reaction.\(^30\) However, the interaction between RecBC\(1004\) enzyme and \( \chi \) did not result in an increased frequency of recombination as measured by bacteriophage \( \lambda \) crosses,\(^29\) 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,\(^1\) but with one significant
difference: ATPγS was not added to stabilize the RecA nucleoprotein filaments. By omitting the ATPγS, 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 1(b), 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 before deproteinization. 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 (Figure 1(b), experiment set 3, and (c), 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 1(b), experiment set 2) because exonuclease I digests the RecA-coated ssDNA more slowly than the SSB-ssDNA complex. When RecBC1004D enzyme and a χ*-containing dsDNA were examined in the same reactions (Figure 1(c)), protection of the χ*-containing ssDNA by RecA protein was also detected (compare Figure 1(c), 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 reported previously.

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 recombination. However, we reasoned that perhaps a mutant RecA protein that 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 1(c), experiment set 7). Full-length ssDNA was also protected by the mutant RecA protein, as expected from the enhanced SSB-displacement ability of RecA730 protein. 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. As 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 2(a), 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 2(b), experiment set 5) or by RecBCD enzyme (Figure 2(b), 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.

It has been 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). In agreement, in an
uncoupled reaction with wild-type RecA protein, all of the ssDNA was digested by exonuclease I, whereas only the \( \chi \)-containing ssDNA was protected in a coupled reaction (Figure 3(a), compare experiment sets 1 and 2). In contrast, but consistent with Figures 1 and 2, RecA\(^{730} \) 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 \( \chi \) sequences (Figure 3(b) and (c)). The enhanced ability of RecA\(^{730} \) protein, relative to wild-type, to displace SSB protein is apparent in the coupled reactions (Figure 3(b), experiment set 4 and (c), experiment set 7): both \( \chi \)-containing ssDNA and full-length ssDNA were more protected (compare to Figure 3(a), experimental set 1 and (c), experiment set...
The RecA<sup>730</sup> mutation partially suppress the UV sensitivity of recF<sup>−</sup> recC1004 strains

To determine whether the recombination deficiency of the RecBC<sup>1004D</sup> enzyme can be suppressed by RecA<sup>730</sup> protein in vivo, the UV sensitivity of recC1004 mutants harboring RecA expression plasmids was measured. Expression of both recA<sup>730</sup> and wild-type recA suppressed the UV sensitivity of the recC<sup>−</sup> strain (Figure 4(a)). Also, as reported, recA<sup>730</sup> partially suppressed a recF<sup>−</sup> mutation (Figure 4(b)), whereas wild-type recA could not. The recA<sup>730</sup> mutation partially suppressed the original recC1004 strain, which also carried a recF<sup>−</sup> mutation and consequently showed severe UV-sensitivity (Figure 4(c)). To determine the effect of recA<sup>730</sup> on the RecBCD pathway, a recF<sup>+</sup> background was investigated. In the recF<sup>+</sup> background, however, the recC1004 mutation did not show severe UV-sensitivity (Figure 4(d)), and there was no detectable suppression of the modest UV sensitivity of the strain by recA<sup>730</sup>. Therefore, it is most likely that the partial suppression observed in recF<sup>−</sup> background by recA<sup>730</sup> is due to suppression of the recF mutation, rather than the recC1004 mutation (Figure 4(b)).

**RecA<sup>730</sup> restores the recombination deficiency of the RecC1004−χ<sup>+</sup> interaction**

Partial suppression of the UV-sensitivity of recF by recA<sup>730</sup> was detected, but our in vitro findings suggested that RecA<sup>730</sup> should also compensate for the lower production of χ<sup>−</sup>-containing ssDNA by RecBC<sup>1004D</sup> enzyme. Recombination between λ phages was investigated to test this hypothesis. The parental λ phages have either a Sam7 or Jh<sup>+</sup> mutation, and the products of recombination crossover, recombinant phage possessing S<sup>+</sup> and Jh<sup>−</sup>, were selected (Figure 5(a)). In the recF<sup>−</sup> recC1004 background, both the recombination frequency (Figure 5(b), left panel) and stimulation of recombination by χ<sup>+</sup> and χ<sup>+</sup> was indistinguishable experimentally (Figure 5(c), left panel), as reported.

However, in the presence of RecA<sup>730</sup>, the frequency of recombination in recC1004 strains was increased (Figure 5(b), right panel). Also, and more importantly, χ<sup>+</sup> shows significant recombination hotspot activity in the recC1004 background when RecA<sup>730</sup> protein was present (Figure 5(c), right panel). Even in the recF<sup>+</sup> background, a similar suppression of RecBC<sup>1004D−χ<sup>+</sup></sup> recombination was observed (data not shown). Using this identical assay, wild-type RecBCD and χ<sup>+</sup> showed a 4.2-fold increase in recombination frequency (the recombination frequency for χ<sup>+</sup>λ phage was 0.50±0.18), and a
6.5-fold increase for hotspot activity assay (data not shown). 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 $\chi^*$ (Figure 5(b) and (c), center panel). These findings suggest that increased concentrations of RecA protein can overcome the deficiency of RecBC$^{1004}D$–$\chi^*$ stimulated recombination in vivo.

**Discussion**

Here, we show that RecA$^{730}$, a mutant RecA protein that 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 $\chi^*$-containing ssDNA, produced by the processing of dsDNA with a $\chi^*$ sequence by RecBC$^{1004}D$ enzyme, is increased. In *vitro*, the frequency of $\chi^*$-stimulated recombination is increased by the RecA$^{730}$ protein. Previously, we found that the $\chi^*$ sequence attenuated the nuclease activity of the RecBC$^{1004}D$ enzyme both in *vitro* and in *vivo* $^{29,30}$ and that $\chi^*$-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 RecBC$^{1004}D$ enzyme and $\chi^*$ was lower than for wild-type enzyme, we concluded that the failure to detect recombination in *vivo* resulted from the lower yield of $\chi^*$-containing nucleoprotein filaments. $^{30}$ Since the RecA$^{730}$ protein nucleates faster on ssDNA and displaces SSB protein more efficiently, $^{38,41}$ we reasoned that this mutant RecA protein might suppress the recombination deficiency displayed by the RecBC$^{1004}D$–$\chi^*$ interaction and, indeed, RecA$^{730}$ 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 displace SSB protein from ssDNA more rapidly and more fully, $^{39,42-47}$ 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 $^{11,14-36}$ This suppression is not restricted to the recB1080 allele, because the UV sensitivity of recB2154, recB2155, recC2145, recC1002, and recC1004, which had been measured in a recF$^+$ background, was also corrected by recF$^+$ (Figure 4; N.H. and Ichizo Kobayashi, unpublished results). The partial suppression of UV sensitivity by the RecF pathway can be explained by

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**Figure 3.** RecA$^{730}$ protein can be loaded onto $\chi$-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 $\chi^+$ DNA. Coupled experiments refer to reactions where RecA protein was present at the beginning of the DNA processing reaction by enzyme RecBCD. In the uncoupled experiment, RecA protein was added after DNA processing by RecBCD enzyme. The vertical arrows indicate the time of exonuclease I addition. (b) RecA$^{730}$ protein, RecBC$^{1004}D$ enzyme, and $\chi^*$ DNA. (c) Comparison of RecA$^{730}$ with wild-type RecBCD and $\chi$ versus wild-type RecA with RecBC$^{1004}D$ and $\chi^*$; both coupled and uncoupled reactions are shown.
RecA-loading ability of the RecFOR complex, which compensates for the lost RecA-loading capacity of certain mutant RecBCD enzymes. 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 consistent also with the original finding that recC1004 is phenotypically Rec+ in phage λ crosses (Figure 5). Thus, due to the relatively low level of 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 λ crosses involving χ+ (Figure 5) cannot be due to suppression by the RecF pathway, because χ+ did not stimulate recombination even in a recF- background (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

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. Filled circles represent the strains expressing recA730; filled squares represent the wild-type rec+ strains; filled diamonds represent the recC73 strains; and the filled triangles in (c) are recF143.
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, we demonstrate here
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 univers-
ality of this concept is supported by recent findings
in eukaryotic recombination. The assembly of a
Saccharomyces cerevisiae Rad51 nucleoprotein fil-
ament is facilitated by Rad55/57, and this Rad55/57-loading can be bypassed by suppressors in Rad51
protein that acquire an enhanced capacity to displace
the SSB protein, RPA.56 Also, both Rad51 nucleo-
protein filament assembly and RPA-displacement
are mediated by Rad52 protein.53–55 Finally, the fungal homolog of BRCA2, Ustilago maydis Brh2 protein,56 also facilitates loading of Rad51 protein onto complexes of RPA and ssDNA.57 Thus, catalysis of RecA/Rad51 nucleoprotein filament formation is
an essential aspect of recombinational DNA repair.

Materials and Methods

Bacterial strains, phages and plasmids

The Escherichia coli strains used were: SCK303 (a ΔrecA
str) Tn10 derivative of K2186,58; laboratory collection,
BIK1291 (= DH10B; araD139 Δ(ara, leu)7697 ΔlacX74 gallU
galK mcrA Δ(nrr-hsdRMS-ncrbC) rpsL deoR (Δ80lacZ
ΔM15) endA1 supG recA1; Dr Ichizo Kobayashi),29 V66
(= BIK796; recF143 argA his-4 met rpsL31 λ F−; Dr Ichizo
Kobayashi),29 BIK1288 (as V66, but recF−::Tn10; Dr Ichizo
Kobayashi),29 V72 (= BIK1274; as V66, but recC1004; Dr
Ichizo Kobayashi),29 BIK1284 (as V72, but recF−::Tn10;
Dr Ichizo Kobayashi),29 V68 (= BIK2411; as V66, but recC73; Dr
Ichizo Kobayashi),29 and BIK3738 (as BIK1288, but recC73;
Dr Ichizo Kobayashi),29 BIK3808 (= F6620; C600 λ recB21
supE; Dr Ichizo Kobayashi),29 JM1 (= F6611; recB21 recC22
sbcA20 supF; Dr Ichizo Kobayashi).29 Bacteriophage λ
strains LIK916 (χ0), LIK950 (χ−), LIK907 (χ+), and
LIK1068, were used for the recombination crosses (Dr Ichizo
Kobayashi).29 The recC73 mutation displays a null phen-
type,65 which is due to truncation by a frameshift muta-
tion at position 1938 in the recC gene.50 The mutant gene
product should produce a 663 amino acid residue polypep-
id, comprising 646 residues of the wild-type sequence,
with 17 residues of the recC73 mutation.65 The recC73
mutant strain, recC73 Δ recF− recC22 recB21 Δ recA−
recB−ΔrpsLΔdeoRΔgalKΔmcrAΔnrr-hsdRMS-ncrbCΔ80lacZ
ΔM15 endA1 supG recA1; Dr Ichizo Kobayashi),29 V66
to BIK796, recF143 argA his-4 met rpsL31 λ F−; Dr Ichizo
Kobayashi),29 BIK1288 (as V66, but recF−::Tn10; Dr Ichizo
Kobayashi),29 V72 (= BIK1274; as V66, but recC1004; Dr
Ichizo Kobayashi),29 BIK1284 (as V72, but recF−::Tn10;
Dr Ichizo Kobayashi),29 V68 (= BIK2411; as V66, but recC73; Dr
Ichizo Kobayashi),29 and BIK3738 (as BIK1288, but recC73;
Dr Ichizo Kobayashi),29 BIK3808 (= F6620; C600 λ recB21
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with 17 residues of the recC73 mutation.65 The recC73
mutant strain, recC73 Δ recF− recC22 recB21 Δ recA−
recB−ΔrpsLΔdeoRΔgalKΔmcrAΔnrr-hsdRMS-ncrbCΔ80lacZ
ΔM15 endA1 supG recA1; Dr Ichizo Kobayashi),29 V66

Figure 5. recA730 restores the recombination hotspot
activity of χ+ in a recF− recC1004 strain. (a) Design of the λ
recombination crosses.28 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 (χ+; crossover before the immunity
region) or clear (χ−; crossover beyond the region). (b)
Recombination frequency. The relative value, normalized
to the wild-type (χ−) strain, of the ratio S+ Jh recombi-
nants/total phage is plotted. The left panel is the recF143
recC1004 strain without plasmid (N = 3). The center panel
is the same strain with the wild-type recA expressing plasmid
(N = 3). The right panel is the same strain with the recA730
expressing plasmid (N = 4). The recombination frequency
and standard deviation for χ− 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.
media

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

Proteins and reagents

RecBCD, RecBC1004D, SSB, and wild-type RecA proteins
were purified as described.66 RecA730 protein was
purified as described.55 Plasmid pSNH160, which 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 (A600 = 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-β-d-thiogalactopyranoside (IPTG)
for 3 h. Cells were harvested, resuspended in ice-cold
buffer (50 mM Tris–HCl (pH 8), 5 mM EDTA, 25% (w/v)
sucrose and 5 mM β-mercaptoethanol) and frozen at
−80 °C. After thawing, cells were lysed with 0.5 mg/ml
of lysozyme followed by sonication. The lysate was
mixed with 0.31% (w/v) Brij-58 and centrifuged at
25,000 rpm in a JA-25 rotor (Beckman-Coulter) for
45 min. The cleared lysate was diluted with the same
buffer to adjust the A260 to 160. Polyethyleneimine (pH 8)
was added to 0.5% (w/v) to precipitate the nucleic acids,
and then centrifuged at 10,000 rpm in a JA-25 rotor for
20 min. The pellet was suspended in 50 mM Tris–HCl
(pH 7.5), 1 mM EDTA, 0.3 M (NH4)2SO4 and 5 mM β-
mercaptoethanol, stirred for 2 h, and then centrifuged.
After the centrifugation, the supernatant was made 60%
saturated by adding solid (NH4)2SO4 and centrifuged.
The pellet was suspended in dialysis buffer (20 mM
Tris–HCl (pH 7.5), 20 mM MgCl2, 5 mM β-mercapto-
ethanol, 10% (v/v) glycerol) and dialyzed overnight against
the same buffer at 40 °C. The precipitate was dissolved in
50 mM Tris–HCl (pH 8), 5 mM EDTA, 5 mM β-mercapto-
ethanol, solid (NH4)2SO4 was added to
70% saturation, and the solution was centrifuged. The
supernatant was loaded onto an S-300HR column
(Pharmacia Biotech; 300 ml; flow-rate of 3 ml/min)
equilibrated with TEM + 1 M 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 pool was
fractionated with 0.2 μm pore
size filter, and then loaded onto a 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 of 100 mM–1 M 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, 10% glycerol). The concentration of RecA730 protein was determined spec-
trophotometrically using an extinction coefficient of
2.15×104 M−1 cm−1 at 280 nm. Exonuclease I, restriction endonucleases, and phage T4
poly nucleotide 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. ATP
(Sigma) was dissolved in water at pH 7.5 and the
concentration was determined spectrophotometrically
using an extinction coefficient of 1.54×103 M−1 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 pNH94 were purified using a Qiagen kit and digested by the restriction endo-
nuclease Avai, following a reaction with shrimp alkaline
phosphatase for removal of phosphoryl groups. After the
5'-end of the linear dsDNA was labeled by phage T4
poly nucleotide 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 was as described, except that ATPγ-S
was omitted. Reactions contained 25 mM Tris-acetate
(pH 7.5), 8 mM magnesium acetate, 5 mM ATP, 1 mM DTT,
10 μM nucleotide linear dsDNA, 5 μM either wild-type
RecA or RecA730 protein, 4 μM SSB protein, and either
0.1 mM RecBCD or 0.2 mM RecBC1004D enzyme. Reactions
(37 °C) were started by addition of RecBCD enzyme. After
3 min, poly(dT) (50 μM nucleotide) was added to sequester
the free RecA protein. After 2 min of further incubation, a
cycle 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. Samples were added to
stop solution (40 mM EDTA, 0.8% (w/v) SDS, 1.5 μg/ml of
proteinase K and 0.04% bromophenol blue) at the
indicated times after the addition of exonuclease I, and
were analyzed by 10% (w/v) agarose gel electrophoresis.
Production of χ-specific fragments was quantified by using
a Molecular Dynamics STORM 870 PhosphorImager
and ImageQuant software (Molecular Dyna mics). The
percentages were calculated relative to the initial amount
of the substrate. Standard deviations (√Σ(y−ymean)2/N−1)
were calculated using GraphPad Prism version 4.02 for
Windows, GraphPad Software, San Diego, CA. In all
graphs, points represent the mean and the error bars are
the standard deviations.

UV-sensitivity measurement

Exponentially growing cultures (in L broth with amp
and spc for selection of plasmid and IPTG to express the
† http://www.graphpad.com
recA gene) were diluted into M9 medium, 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 h in the dark.

**Lambda phage recombination assay**

The experimental design is shown in Figure 5(a). The procedure was as described. Parental phages (both LIK916, 950 or 907 and LIK1068) were mixed together before infection of warmed *E. coli* host cells. Infection was carried out at moi = 5 for each phage. After a cycle, S-<i>lh</i> recombinant phages were counted by plating on BIK808, and total phages were measured by plating on JM1. The recombination frequency (%) was calculated as:

\[
\text{(recombinant phage titer/total phage titer)} \times 100
\]

and the hotspot activity was assessed by the ratio, turbid plaque number/ clear plaque number, for the recombinant phages plated on BIK808.

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