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A RecA Mutant, RecA^{730}, Suppresses the Recombination Deficiency of the RecBC^{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 *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, RecA^{730}, that displays enhanced biochemical activity *in vitro* and possesses suppressor function *in vivo*. We show that the recombination deficiency of the RecBC^{1004}D–χ* interaction can be overcome by the enhanced ability of RecA^{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.

Keywords: RecA; RecBCD; recombination hotspot; DNA repair; RecA-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 s, and then resumes unwinding at a rate that is about twofold slower than the rate before 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] 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,[12–14] recent single-molecule studies

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

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showed that the RecD subunit continues to translocate with the holoenzyme after χ recognition.[9] Rather, single-molecule studies,[8,9] 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 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 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 χ 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 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 prokaryotes.[23] 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 isolated originally 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* gene.[30] It was found that a novel sequence, different from the canonical χ sequence, was recognized specifically by one of these mutants, *recC1004*, *in vivo*.[29] Resembling the original χ sequence in its genetic behavior,[31] 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 nt sequence, 5′-GCTGGTGCTCG,[29] and comprises the first seven bases of the *E. coli* χ, plus four novel bases. As for the wild-type pair, the nuclease activity of *the recC1004* mutant protein was attenuated *in vivo* by the χ* sequence.[29] Purified RecBC^{1004}D 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 RecBC^{1004}D 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 RecC^{1004}–χ* 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 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, RecBC^{1004}D 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}D–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 RecBC^{1004}D enzyme processing.

Results

RecA protein is loaded onto χ*-containing ssDNA by RecBC^{1004}D 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 RecBC^{1004}D enzyme produced χ*-dependent joint molecules in response to χ* recognition, but with a lower yield than the wild-type reaction.[30] However, the interaction between RecBC^{1004}D enzyme and χ* did not result in an increased frequency of recombination as measured by bacteriophage λ 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,[10] 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 RecA^{730} 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.[10] 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.[10,39] When RecBC^{1004}D 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.[30,40]

RecA^{730} 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 RecBC^{1004}D enzyme is reduced relative to the wild-type interaction, resulting in a lower yield of RecA nucleoprotein filaments needed for recombination.[30] 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, RecA^{730} protein was examined. The RecA^{730}–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 RecA^{730} protein.[38] RecA^{730} protein also protected ssDNA produced by RecQ, RecB^{1080}CD, or RecB^{2109}CD 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 RecA^{730}

To confirm that the increased protection of all ssDNA is intrinsic to RecA^{730} 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 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 RecA^{730} 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 RecA^{730} 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).[10,39] In agreement, in an

Figure 1. RecBCD and RecBC^{1004}D enzymes load RecA and RecA^{730} 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) RecBC^{1004}D 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 RecA^{730}) 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: RecA^{730} (exp. set 7); and open squares, RecA omitted (exp. set 8).

uncoupled reaction with wild-type RecA protein, all of the ssDNA was digested by exonuclease I, whereas only the χ-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 χ 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 χ-containing ssDNA and full-length ssDNA were more protected (compare to Figure 3(a), experimental set 1 and (c), experiment set

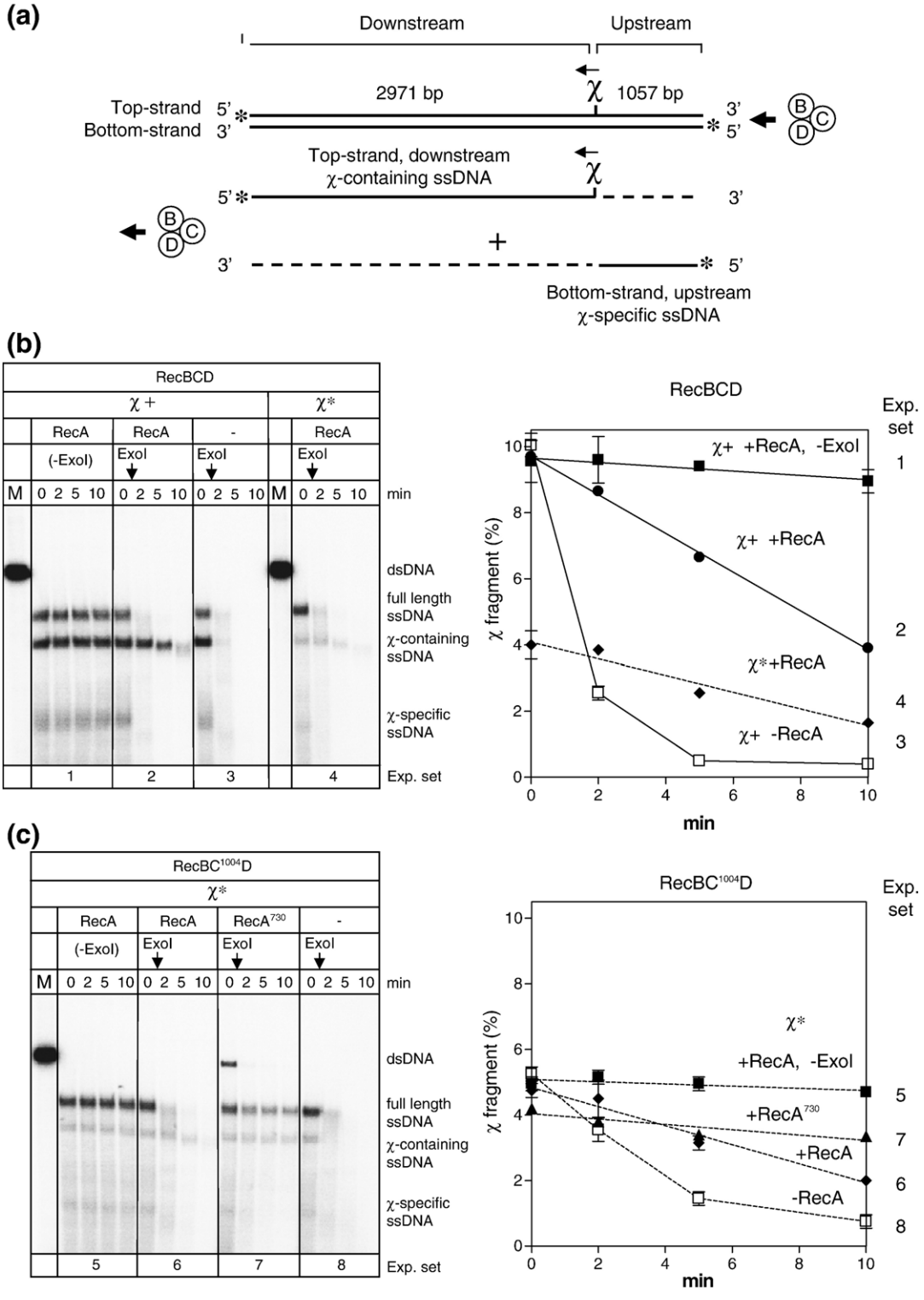

Figure 1 (*legend on opposite page*)

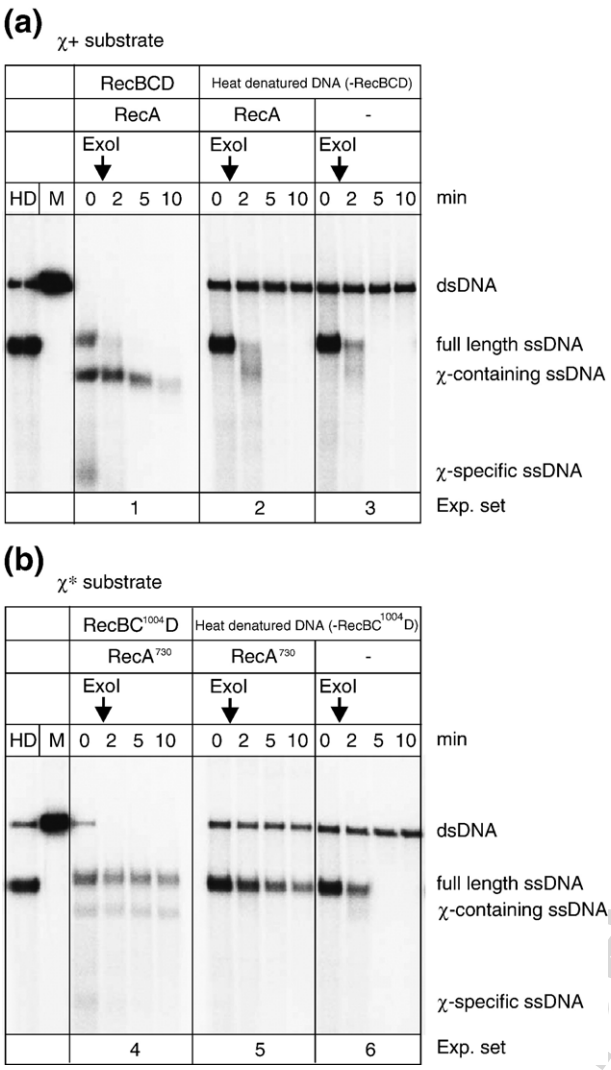

Figure 2. RecA^{730} possesses an enhanced capacity to form stable nucleoprotein filaments on SSB–ssDNA complexes. RecA nucleoprotein filaments were formed on heat-denatured linear dsDNA in the presence of SSB protein for comparison to nucleoprotein filament formation in the coupled RecA and RecBCD reactions. (a) Wild-type RecA protein and χ^+ DNA. (b) RecA^{730} 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.

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 RecA^{730} is more difficult to assess by visual inspection; however, quantification of five independent replicates of Figure 3(b) shows that 76(±7)% of the χ^*-containing ssDNA remained after 10 min in the coupled reaction (experiment set 4), whereas only 38(±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(±2)%) was the same as that of the χ^*-containing ssDNA (38(±2)%). Thus, RecA^{730} 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 RecA^{730} mutation partially suppress the UV sensitivity of recF$^-$ recC1004 strains

To determine whether the recombination deficiency of the RecBC^{1004}D enzyme can be suppressed by RecA^{730} 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 4(a)). Also, as reported,[34,36] *recA730* partially suppressed a *recF* mutation (Figure 4(b)), whereas wild-type *recA* could not. The *recA730* mutation partially suppressed the original *recC1004* strain, which also carried a *recF*$^-$ mutation and consequently showed severe UV-sensitivity (Figure 4(c)).[28] To determine the effect of *recA730* on the RecBCD pathway, a *recF*$^+$ background was investigated. In the *recF*$^+$ 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 *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 4(b)).

RecA^{730} 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 RecA^{730} should also compensate for the lower production of χ^*-containing ssDNA by RecBC^{1004}D enzyme. Recombination between λ phages was investigated to test this hypothesis.[29] 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 5(a)). In the *recF recC1004* background, both the recombination frequency (Figure 5(b), left panel) and stimulation of recombination by χ^+ and χ^* was indistinguishable experimentally (Figure 5(c), left panel), as reported.[29]

However, in the presence of RecA^{730}, the frequency of recombination in *recC1004* strains was increased (Figure 5(b), right panel). Also, and more importantly, χ^* shows significant recombination hotspot activity in the *recC1004* background when RecA^{730} protein was present (Figure 5(c), right panel). Even in the *recF*$^+$ background, a similar suppression of RecBC^{1004}D–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 χ^0 λ 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 5(b) and (c), center panel). These findings suggest that increased concentrations of RecA protein can overcome the deficiency of RecBC^{1004}D–χ* stimulated recombination *in vivo*.

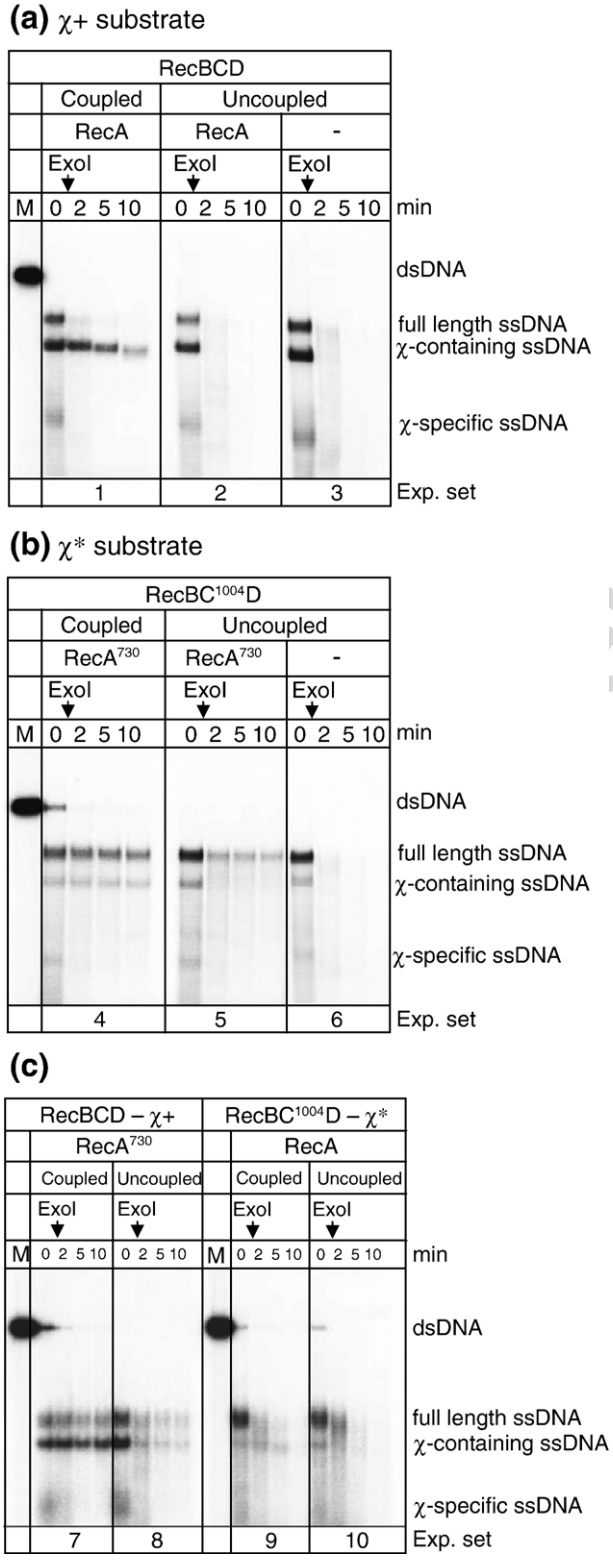

Figure 3. RecA^{730} protein can be loaded onto χ-containing ssDNA to form nucleoprotein filaments that are more stable than those formed by wild-type RecA protein. (a) Wild-type RecBCD enzyme, RecA protein and χ^{+} DNA. Coupled experiments refer to reactions where RecA protein was present at the beginning 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 χ* DNA. (c) Comparison of RecA^{730} with wild-type RecBCD and χ *versus* wild-type RecA with RecBC^{1004}D and χ*; both coupled and uncoupled reactions are shown.

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 χ*-containing ssDNA, produced by the processing of dsDNA with a χ* sequence by RecBC^{1004}D enzyme, is increased. *In vivo*, the frequency of χ*-stimulated recombination is increased by the RecA^{730} protein. Previously, we found that the χ* sequence attenuated the nuclease activity of the RecBC^{1004}D 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 RecBC^{1004}D 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 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–χ* 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.[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 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

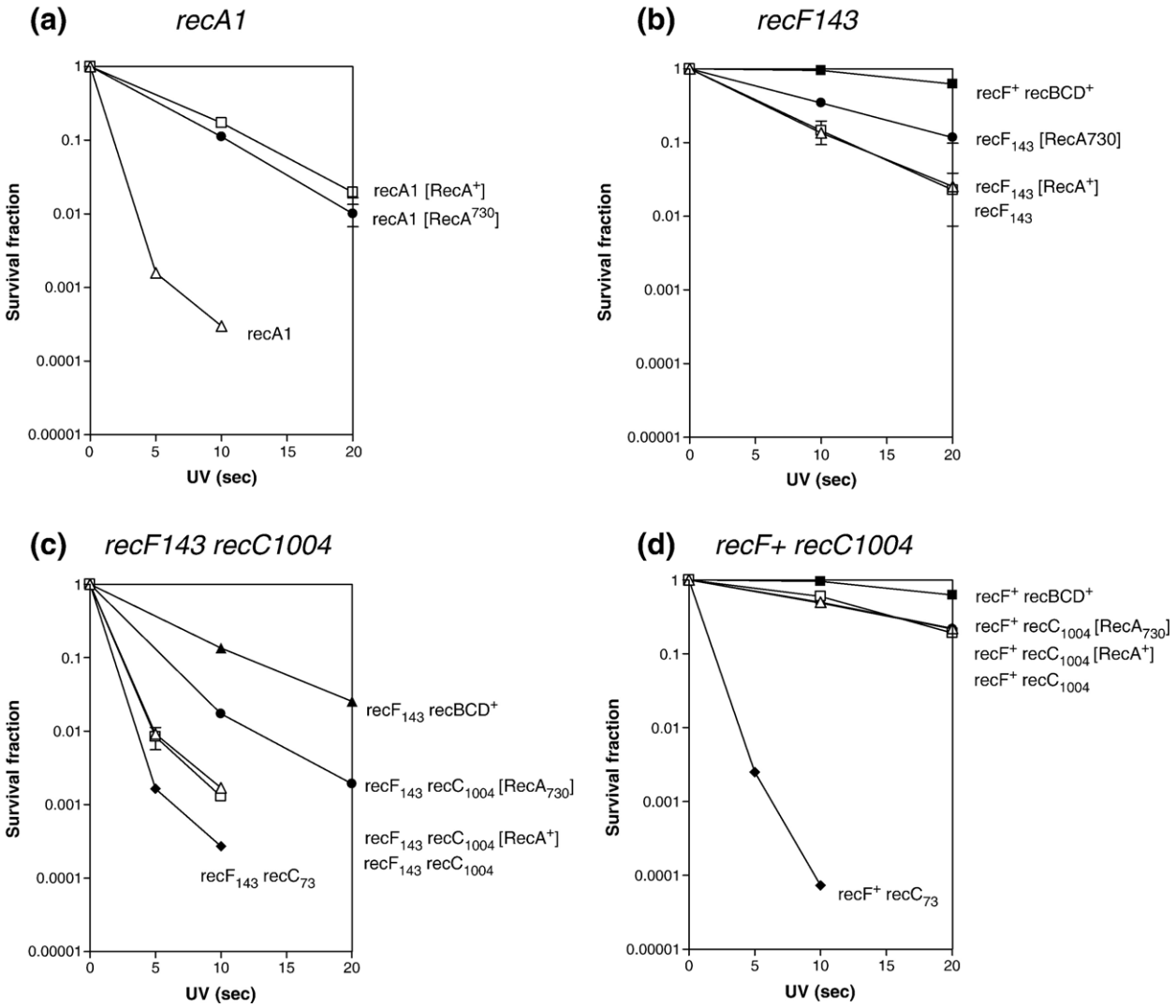

Figure 4. Suppression of UV sensitivity by *recA730*. (a) *recA1* background. (b) *recF143* background. (c) *recF143 recC1004* background. (d) *recC1004* background. Open triangles represent the strains lacking any *recA*-expressing plasmid. Open squares represent the strains expressing wild-type *recA*. 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*.

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 RecA^{730} could suppress the UV-sensitivity of the *recC*^{1004} 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 *recC*^{1004} is phenotypically *Rec*$^+$ in phage λ crosses (Figure 5). Thus, due to the relatively low level of UV-sensitivity of the *recC*^{1004} strain, an effect of RecA^{730} on UV survival could not be detected. However, we could clearly detect suppression of the UV-sensitivity of *recC*^{1004} 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 RecA^{730} 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).[29] Consequently, we conclude that the increased SSB-displacement capability of this mutant protein is responsible for the heightened recombination frequency. Although RecA^{730} suppressed the recombination defect of RecBC^{1004}D 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.

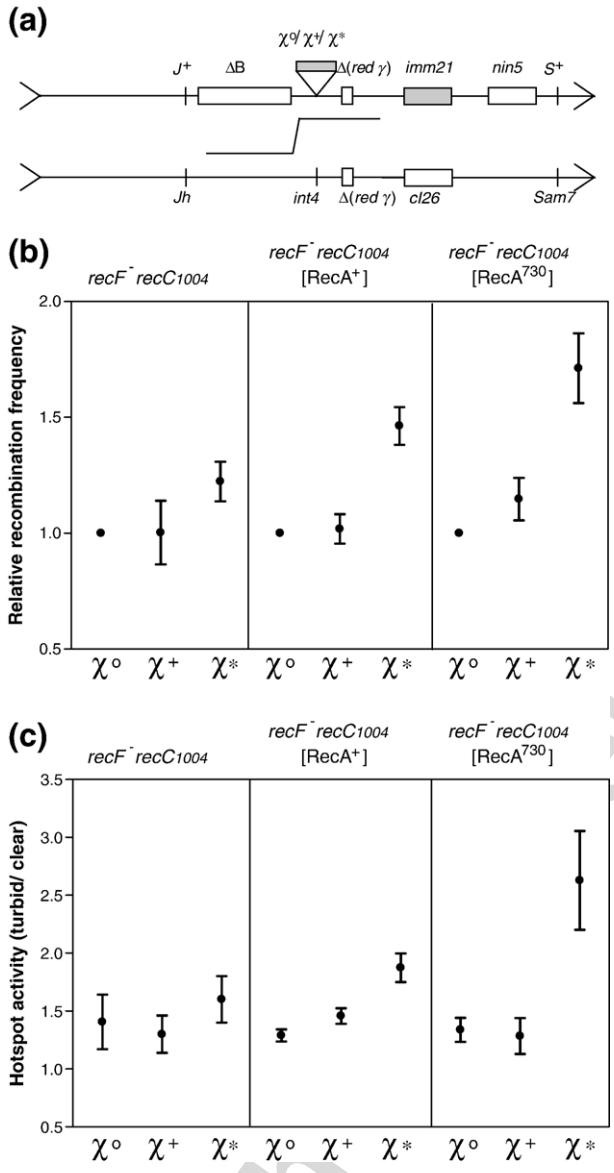

Figure 5. *recA730* restores the recombination hotspot activity of χ* in a *recF⁻ recC1004* strain. (a) Design of the λ recombination crosses.[29] Parental phages are defective for their phage-encoded recombination functions (*red⁻ γ⁻ int⁻*). The *S⁺ Jh* recombinant phages were scored as to whether the 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. 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^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.

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 universality of this concept is supported by recent findings in eukaryotic recombination. The assembly of a *Saccharomyces cerevisiae* Rad51 nucleoprotein filament is facilitated by Rad55/57[51], and this Rad55/57-loading can be bypassed by suppressors in Rad51 protein that acquire an enhanced capacity to displace the SSB protein, RPA.[52] 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 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 Δ*recA srl*::Tn*10* derivative of KK2186[58]; laboratory collection), BIK1291 (= DH10B; *araD139* Δ(*ara, leu*)*7697* Δ*lacX74 galU galK mcrA* Δ(*mrr-hsdRMS-mcrBC*) *rpsL deoR* (ϕ80d*lacZ* ΔM15) *endA1 nupG recA1*; Dr Ichizo Kobayashi),[59] V66 (= BIK796; *recF143 argA his-4 met rpsL31* λ⁻ F⁻; Dr. Ichizo Kobayashi),[28] BIK1288 (as V66, but *recF⁺ zic*::Tn*10*; Dr Ichizo Kobayashi),[29] V72 (= BIK1274; as V66, but *recC1004*; Dr Ichizo Kobayashi),[28] BIK1284 (as V72, but *recF⁺ zic*::Tn*10*; 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^r *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] which is 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 residue polypeptide, comprising 646 residues of the wild-type sequence, followed by an additional 17 residues. Plasmid pMS421 carrying the *lacI^q* gene has been described.[60] Multicopy plasmids pKM100, containing a *lacUV5*-controlled *recA* gene, and pKM300, containing a phage T7-promoter-controlled *recA* gene, as well as bacteriophage M13-KM2 have been described.[61] The *recA730* derivative plasmids, pSNH50 and pSNH60, were made by site-directed mutagenesis (Stratagene) using two synthetic oligonucleotides:

5′-GGGTGAAGACCGTTCTATGGATGTGAAAACCATCTCTACCG
5′-CGGTAGAGATGGTTTTCACATCCATAGAACGGTCTTCACCC

from pKM100 and pKM300, respectively. Sequencing of the entire *recA* gene in these plasmids confirmed that no other mutation was present (data not shown).

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 $MgSO_4$, and 10 μg/ml of vitamin B1. Antibiotics were added at the following concentrations 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, RecBC^{1004}D, SSB, and wild-type RecA proteins were purified as described.[40,62–64] RecA^{730} protein was purified as described.[65] Plasmid pSNH60, 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 (A_{600}=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, re-suspended 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 A_{260} 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 $(NH_4)_2SO_4$ and 5 mM β-mercaptoethanol, stirred for 2 h, and then centrifuged. After the centrifugation, the supernatant was made 60% saturated by adding solid $(NH_4)_2SO_4$ and centrifuged. The pellet was suspended in a dialysis buffer (20 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10% (v/v) glycerol) and dialyzed overnight against the same buffer at 40 °C. The precipitate was dissolved in 20 mM Tris–HCl (pH 7.5), 5 mM EDTA, 1 M NaCl, 5 mM β-mercaptoethanol, 10% glycerol and 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). RecA^{730} protein, identified by ssDNA-dependent ATPase activity and SDS-PAGE analysis, eluted as a single peak. The pooled peak fractions from the S-300HR column were loaded onto an EconoPack Q column (Bio-Rad; 5 ml; flow-rate of 0.5 ml/min) after dialysis against TEM+50 mM NaCl; the RecA^{730} protein eluted in the flow-through. The pool was dialyzed against 50 mM Tris–HCl (pH 8), 1 mM EDTA, 5 mM β-mercaptoethanol, solid $(NH_4)_2SO_4$ was added to 70% saturation, and the solution was centrifuged. The pellet was suspended in 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 μm pore size filter, and then loaded onto a MonoQ HR10/10 column (Pharmacia Biotech; 8 ml; flow-rate of 3.0 ml/min). RecA^{730} 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 RecA^{730} protein was determined spectrophotometrically using an extinction coefficient of 2.15×10^4 M^{-1} cm^{-1} at 278 nm.

Exonuclease I, restriction endonucleases, and phage 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. ATP (Sigma) was dissolved in water at pH 7.5 and the concentration was determined spectrophotometrically using an extinction coefficient of 1.54×10^4 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[30] were purified using a Qiagen kit and digested by the 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 phage T4 polynucleotide kinase with ^{32}P, unincorporated [γ-^{32}P] 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,[10,39] except that ATPγS 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 RecA^{730} protein, 4 μM SSB protein, and either 0.1 nM RecBCD or 0.2 nM RecBC^{1004}D 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 sample 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/μl of proteinase K and 0.04% bromophenol blue) at the indicated times after the addition of exonuclease I, and were analyzed by 1.0% (w/v) agarose gel 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 ($\sqrt{[\Sigma(y_i-y_{mean})^2/N-1]}$) 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,[66] 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.[29] 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^{+}-*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, for the recombinant phages plated on BIK808.

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