Supplemental Data

The RecA Binding Locus of RecBCD Is a General Domain for Recruitment of DNA Strand Exchange Proteins

Maria Spies and Stephen C. Kowalczykowski

Supplemental Experimental Procedures

Pyruvate kinase, lactate dehydrogenase, and phosphoenol pyruvate were purchased from Sigma. Restriction endonucleases, shrimp alkaline phosphatase (SAP), and T4 polynucleotide kinase were from New England Biolabs. Covalently closed circular χ^{+}-3F3H dsDNA (a pBR322 derivative, containing tandem χ-sequences (Anderson et al., 1999)) was purified using a QIAGEN "Maxi kit". M13mp7 replicative form was purified by cesium chloride gradient centrifugation. χ^{+}-3F3H dsDNA, linearized with *Nde*I restriction endonuclease, and M13mp7 dsDNA, linearized with *Bgl*II enzyme, were dephosphorylated with SAP, and 5'-end labeled with T4 polynucleotide kinase and [γ^{-32}P]ATP. Hydroxyapatite (Bio-Gel HTP Gel) was from Bio-Rad; and Sepharose Fast-flow and HiLoad^{TM} 16/60 Superdex^{TM} 200 columns were from Amersham Pharmacia.

The 6xHis RecB^{nuc} was affinity purified as reported by (Zhang and Julin, 1999) with the following changes. Cells (BL21(DE3)) were disrupted by sonication in lysis buffer (50 mM TrisHCl (pH 7.5), 0.5 M NaCl, 50 mM imidazole, 0.5% triton X-100, and 1 mM PMSF). The cleared lysate was applied to a Sepharose Fast-flow column charged with 0.2 M nickel sulphate, and equilibrated with Ni-A buffer containing 50 mM TrisHCl (pH 7.5), 0.5 M NaCl, and 50 mM imidazole. Fractions that eluted with Ni-B buffer which contained 150 mM TrisHCl (pH 7.5), 0.5 M NaCl, and 300 mM imidazole, were dialyzed against HA-A buffer containing 20 mM potassium phosphate (pH 7.0), 1 mM EDTA, 0.1 mM DTT and 1 mM PMSF, and were applied to the hydroxyapatite column.

RecB^{nuc} protein was eluted with a 20 – 200 mM gradient of potassium phosphate (pH 7.0). Fractions containing RecB^{nuc} protein were pooled, dialyzed against B^{200} buffer (20 mM Tris HCl (pH 7.5), 200 mM NaCl, 0.1 mM EDTA, and 0.1 mM DTT), and concentrated against dry PEG (20K) to 20 mg/ml. Large molecular weight impurities, RecB^{nuc} degradation products and aggregates were removed by gel filtration on Superdex 200 column equilibrated with B^{200} buffer.

Supplemental References

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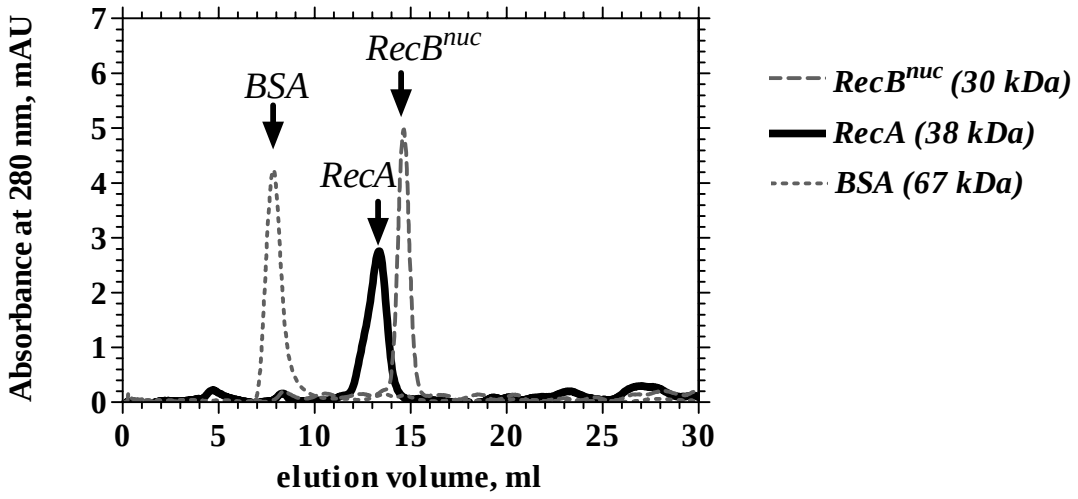


Figure S1. RecA exists predominantly as a monomer in 1 M NaCl.

Proteins **(**BSA (67 kDa), $RecB^{nuc}$ (30 kDa), and RecA (38 kDa)) were analyzed by gel filtration using FPLC: 0.5 ml of each protein (1 μM) was run on a Superose-12 column in buffer consisting of 20 mM Tris acetate (pH 7.5), 1 M NaCl, 0.1 mM DTT, and 0.1 mM EDTA.

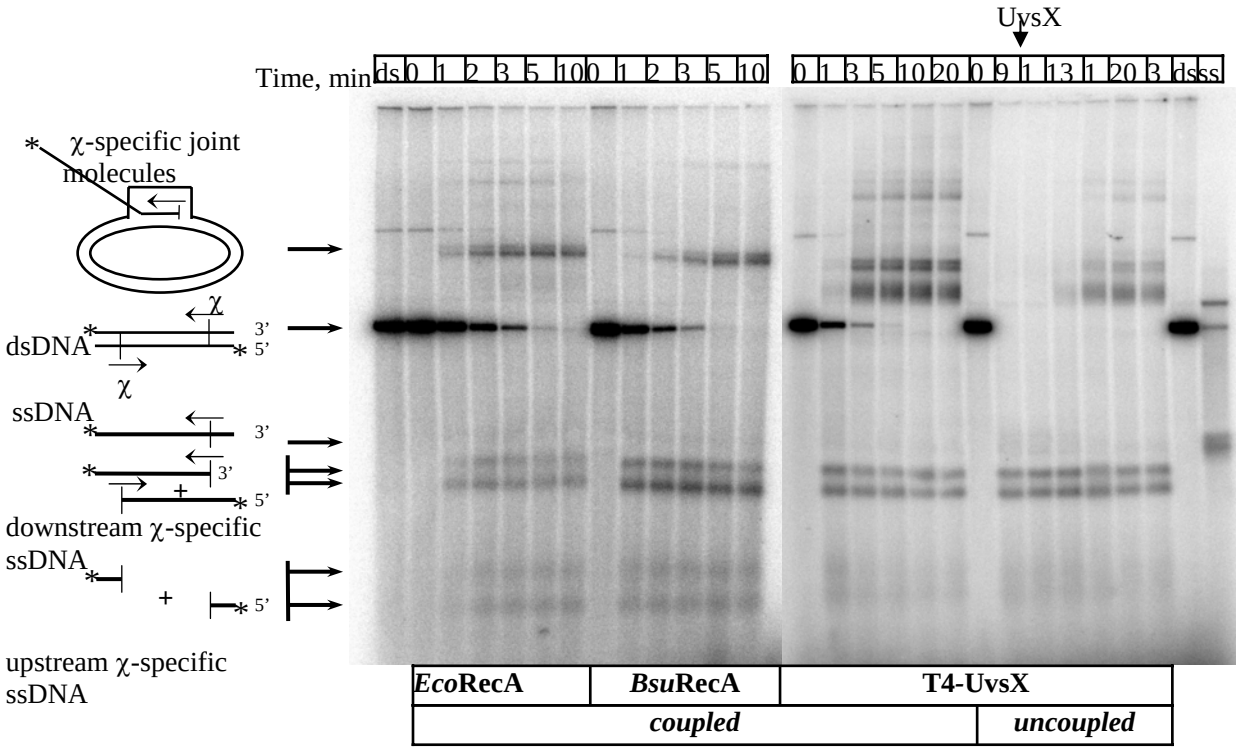


Figure S2. RecBCD facilitates the loading of non-cognate DNA strand exchange proteins onto the χ-containing ssDNA product of its helicase/nuclease activity.

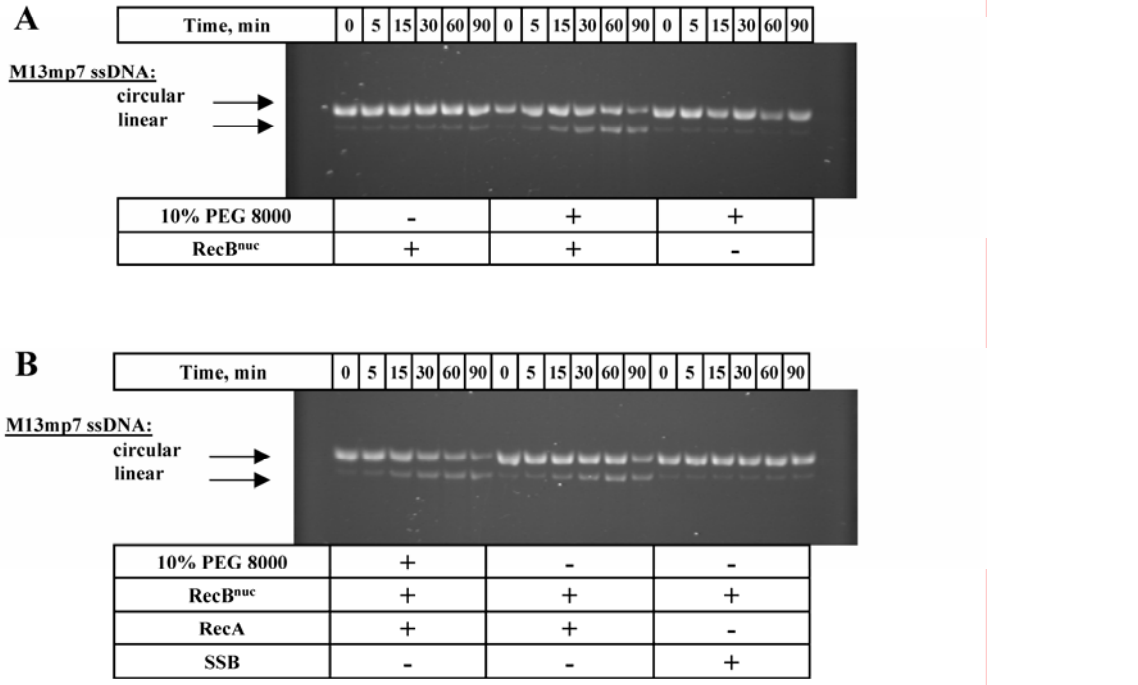
Coupled pairing reactions were performed as described (Anderson and Kowalczykowski, 1997; Dixon and Kowalczykowski, 1991), using χ^+-3F3H DNA. The reaction consisted of 25 mM Tris-acetate (pH 7.5), 8 mM magnesium acetate, 1 mM DTT, 1.5 mM PEP, 4 U/ml of pyruvate kinase, 20 μM (nucleotides) ^{32}P-labeled linear dsDNA, 40 μM supercoiled DNA, 0.2 nM RecBCD, 1 μM SSB, and 7.5 μM of RecA. Because UvsX has higher affinity for DNA compared to RecA (Ando and Morrical, 1998), the reaction for the RecBCD-facilitated loading of UvsX was changed to contain 4 mM magnesium acetate, 2.5 μM SSB, and 5 μM of UvsX, to ensure that D-loop formation was due predominantly to the RecBCD-facilitated loading of the UvsX, rather than to the greater intrinsic ability of UvsX to displace an SSB. The RecA- and UvsX-RecBCD coupled pairing reactions were

started with addition of, respectively, 3 and 2 mM ATP upon pre-incubation of all other components at 37 °C for 5 minutes. In uncoupled reactions, the DNA-pairing protein was added 10 minutes after ATP addition. Assays were stopped by addition of proteinase K to a final concentration of 0.5 mg/ml (in 250 mM EDTA, 20% Ficoll 400, 5% SDS, 0.25% bromphenol blue, 0.25% xylene cyanol). After a 5 min incubation with proteinase K, the reaction products were separated on a 1% (w/v) TAE-agarose gel at 700 V·hr, visualized and then quantified using a Molecular Dynamics STORM 840 PhosphorImager and ImageQuaNT software.

The substrate, as well as products and intermediates of the RecABCD reactions (full length ssDNA, χ-specific ssDNA, and joint molecules), is indicated at the left of Figure S2. The DNA strand exchange protein used is indicated under the gel. In the uncoupled reactions, the DNA strand exchange protein was added 10 min after the addition of ATP. The lanes marked “ds” and “ss” represent the reaction mixture before addition of proteins and heat-denatured linear χ^{+}-3F3H dsDNA, respectively. Rad51 protein could not be examined in the coupled assays, because of both its limited ability to mediate joint molecules formation and its high affinity for dsDNA, which inhibited DNA unwinding by RecBCD helicase.

In conjunction with RecBCD, *E. coli* and *B. subtilis* RecA converted approximately 12% of the linear dsDNA to χ-specific joint molecules and UvsX produced approximately 18%. In the uncoupled reactions (Anderson and Kowalczykowski, 1997), joint molecules formation was less than 3%. Thus, consistent with the specificity of the binding assays, the functional assay confirmed that RecBCD can load other DNA strand exchange proteins. The lower affinities for $RecB^{nuc}$ observed in the pull-down assays were not reflected in lower amounts of χ-specific joint molecules in the coupled reactions likely because the RecBCD-mediated loading of these DNA strand

exchange proteins is not, under these conditions, the limiting step in the sequence of reactions leading to the production of joint molecules.



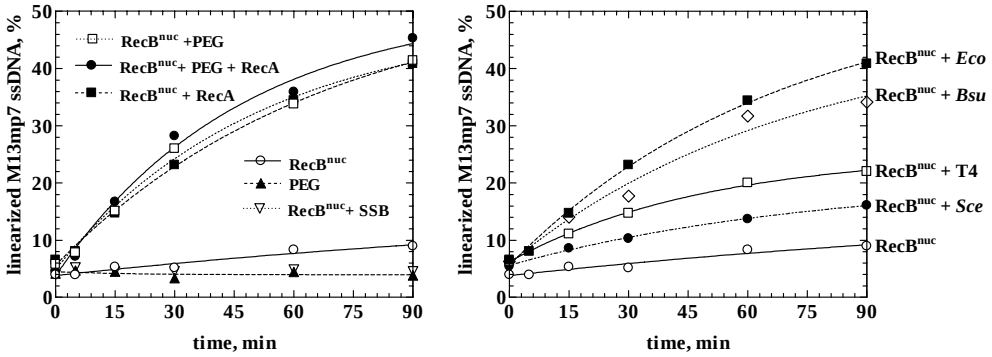


Figure S3. DNA strand exchange proteins stimulate the endonuclease activity of RecB^{nuc}.

Endonuclease activity was assayed using circular M13mp7 ssDNA as described (Zhang and Julin, 1999). Reactions contained 20 mM Tris-acetate (pH 7.5), 1 mM DTT, 20 mM magnesium acetate, 3

mM ATP-γ-S, and 6 nM (43 μM nucleotides) M13mp7 ssDNA. at 37°C Where indicated, 10% PEG (8K), 1.8 μM 6xHis-RecB^{nuc}, 7 μM RecA (which is half-saturating relative to the ssDNA), UvsX, or Rad51 protein, or 3 μM SSB were added. Aliquots (3 μl) were removed at specified times and mixed with 3 μl of stop solution (250 mM EDTA, 20% Ficoll 400, 5 %SDS, 0.25% bromphenol blue, and 0.25% xylene cyanol). Circular and linear forms of M13mp7 ssDNA were resolved by electrophoresis in 0.8% TBE-agarose at 700 V·hr. The gels were stained with ethidium bromide and photographed. The images were analyzed using Gel-Pro Analyzer software.

A and **B)** The endonuclease activity of the RecB^{nuc} in the presence of proteins and additives indicated under the gels. Times at which assays were stopped are indicated above the lanes.

C) Quantification of the RecB^{nuc} nuclease activity shown in panels A and B. An increase in the RecB^{nuc}-mediated cleavage of circular M13mp7 ssDNA was observed when *E. coli* RecA was present in the reaction mixture at a sub-saturating concentration (1 RecA per 6 nucleotides of ssDNA). No increase of the RecB^{nuc} nuclease activity was detected in the presence of SSB, indicating that the RecA-dependent stimulation of RecB^{nuc} nuclease was not due to molecular crowding (panel C).

D) Quantification of the nuclease assays carried out in the presence of various RecA-like proteins (gel not shown). All reaction mixtures contained 1.8 μM RecB^{nuc} and 7 μM of the RecA-like protein indicated to the right of each curve. Other non-cognate strand exchange proteins also stimulated RecB^{nuc} nuclease activity; the stimulation, however, was not as great as for the *E. coli* RecA (7-fold): 6-fold for *B. subtilis* RecA, 3-fold for UvsX, and 2-fold for Rad51. This pattern of stimulation approximately parallels the direct binding results, and provides further evidence that the interactions are specific.

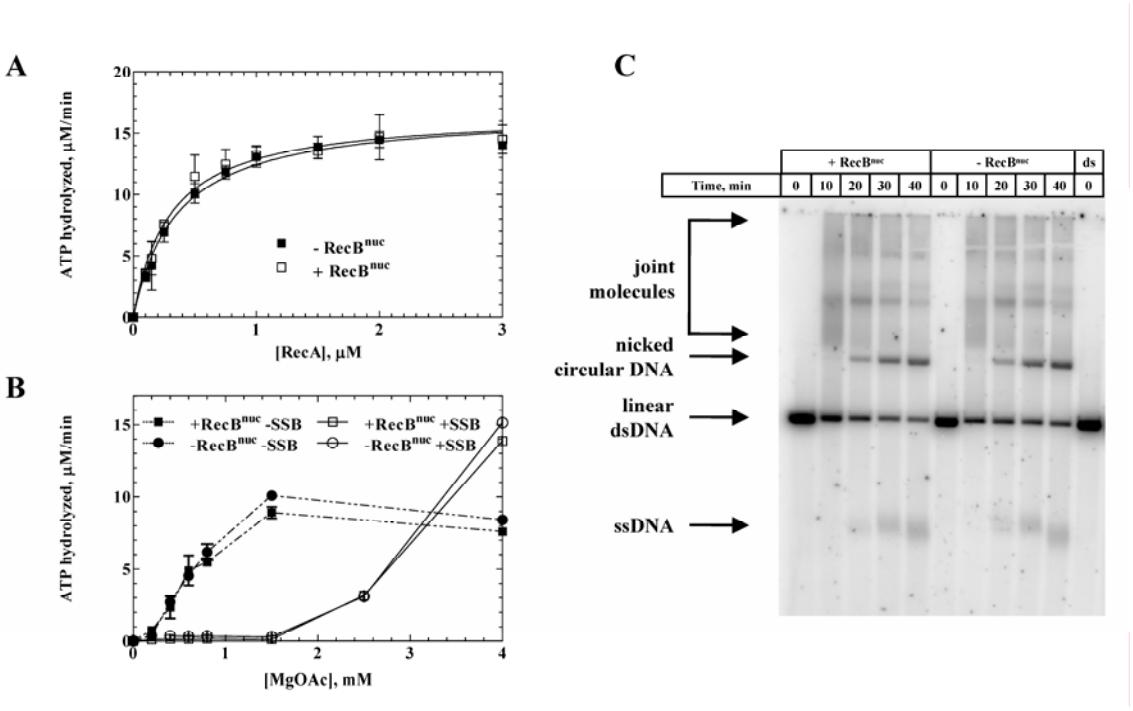


Figure S4. **Interaction with RecB^{nuc} has no effect on the activities of the RecA.**

ATP hydrolysis served as an indicator of RecA nucleoprotein formation. Assays were carried out using Hewlett-Packard Model 8452A diode array spectrophotometer. The ssDNA-dependent ATP hydrolysis was monitored at 37 °C as described (Kowalczykowski and Krupp, 1987). Reaction mixtures contained 25 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate (unless otherwise indicated), 0.1 mM DTT, 1 mM ATP, 1.5 mM PEP, 0.2 mg/ml NADH, 3 μM (nucleotides) M13mp7 ssDNA, 30 U/ml each of pyruvate kinase and lactate dehydrogenase, SSB (0.2 μM where indicated) and the indicated concentrations of RecA and RecB^{nuc} proteins. The rate of ATP hydrolysis was calculated from the rate of change in absorbance at 340 nm due to oxidation of NADH using the following formula: rate of A_{340} decrease (s^{-1}) x 9820 = rate of ATP hydrolysis (μM/min). Where indicated, the error bars represent standard deviation for three independent experiments.

A) RecA nucleoprotein filament formation in the presence of RecB^{nuc}. ATP hydrolysis was measured at the indicated concentrations of RecA in the presence (open squares) or absence (closed

squares) of 1 μM $RecB^{nuc}$ protein. The results show that RecA nucleoprotein filament formation is not influenced by interaction with $RecB^{nuc}$.

B) RecA nucleoprotein filament formation in the presence of $RecB^{nuc}$ and SSB. Because both the assembly of RecA (1 μM) on ssDNA and its ability to compete with SSB for ssDNA increase with increasing magnesium ion concentration, the effect of $RecB^{nuc}$ was tested at various concentrations of magnesium acetate. ATPase assays in the presence (squares) and absence (circles) of 1 μM $RecB^{nuc}$ protein were carried out. $RecB^{nuc}$ did not alter the RecA-mediated ATP hydrolysis.

C) DNA strand exchange in the presence of $RecB^{nuc}$. RecA-mediated DNA strand exchange was performed as follows. Reaction mixtures contained 25 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 1 mM DTT, 1 mM ATP, 7.5 mM PEP, 10 U/ml pyruvate kinase, 3 μM RecA, 1 μM of $RecB^{nuc}$ (if indicated), and 10 μM (nucleotides) M13mp7 ssDNA. Reaction mixtures were pre-incubated at 37°C for 5 min to allow binding of $RecB^{nuc}$ and RecA, another 5 min upon addition of ssDNA, and another 2 min after addition of 1 μM SSB. Reactions were started with addition of 20 μM (nucleotides) linear P^{32}-labeled M13mp7 dsDNA, incubated for the indicated times, and then terminated by addition of proteinase K. Products were analyzed by electrophoresis in 1% TAE-agarose at 700 V·hr, and quantified using a Molecular Dynamics STORM 840 PhosphorImager and ImageQuaNT software. The substrate, as well as products (displaced linear ssDNA and nicked circular dsDNA) and the joint molecule intermediates are indicated at the left of the panel. $RecB^{nuc}$ affected neither synapsis (*i.e.*, formation of the joint molecule intermediate), nor DNA heteroduplex extension (assessed as formation of the final product, nicked circular dsDNA). Thus, the RecA-$RecB^{nuc}$ interaction does not alter activities of RecA that are important for DNA strand exchange.

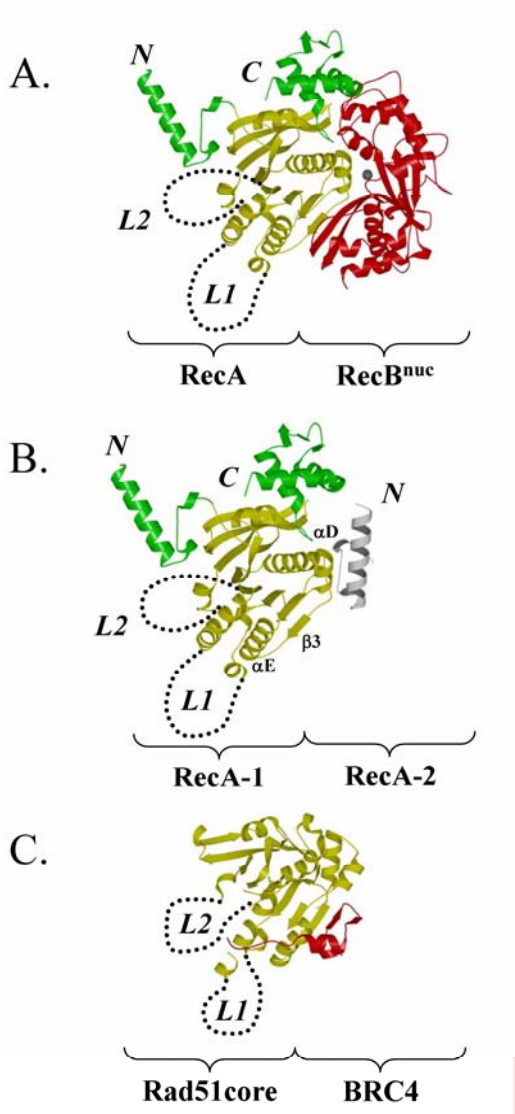


Figure S5. A universal structural can serve as the basis for recruitment of DNA strand exchange proteins.

A) The proposed RecB^{nuc}-RecA complex. The conserved core of RecA is shown in yellow; the N- and C-terminal domains are in green; and the nuclease domain of RecB is in red.

B) The predicted interaction interface involves a surface of RecA that participates in the interaction between adjacent RecA monomers within RecA filament. In the structure of RecA filament obtained by image reconstruction from electron microscopy (Yu and Egelman, 1997), the N-terminus of an adjacent RecA monomer (shown in gray) contacts α-helix D, which is a part of the proposed RecB^{nuc}-RecA interface. Other structural elements of the RecB^{nuc}-RecA interface are β-sheet 3 and α-helix E.

C) Similar structural elements are involved in the interaction between conserved core of human Rad51 protein (shown in yellow) and BRC4 peptide (shown in red) (Pellegrini et al., 2002). Structures were oriented by superimposing structurally conserved cores of RecA and Rad51 proteins using "Deep View/Swiss-pdb Viewer" (www.expasy.org/spdbv/). Figures were generated with MolScript (Esnouf, 1997).

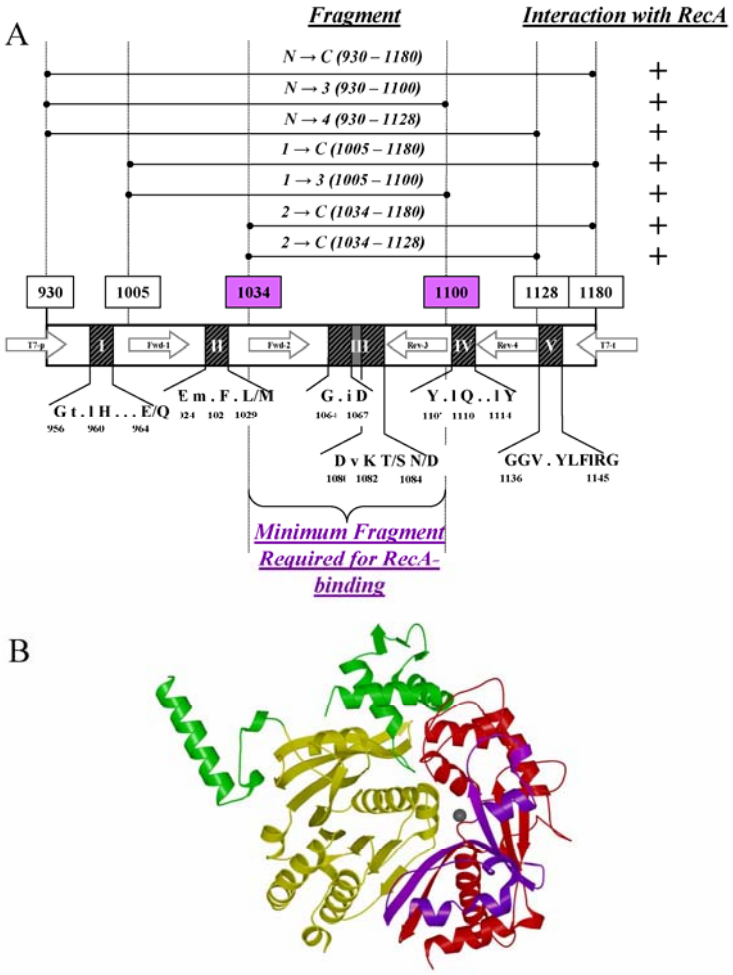


Figure S6. Truncated RecB^{nuc} peptides define a minimal core needed for the interaction between RecB and RecA.

A) Alignment of the nuclease domains from RecB homologs reveals the presence of five conserved regions. His-tagged peptides lacking one or several of the conserved motifs were constructed as shown in the text above the horizontal lines. The binding of these peptides to RecA was determined from pull-down experiments using crude cell extracts to which purified RecA was added; uninduced cell extracts were used as the negative controls. Complexes were detected for all six soluble truncated peptides; the smaller truncated peptides were insoluble (unpublished observations). Region III (amino acids 1034 and 1100) is common to each binding peptide, indicating that this region is necessary for RecA binding,

B) The predicted structure of RecA and RecB^{nuc} showing the location of region III. Color-coding is as per Figure 4A, except that motif III of RecB^{nuc}, which comprises the region that was common to all peptides that bound RecA, is indicated in purple.

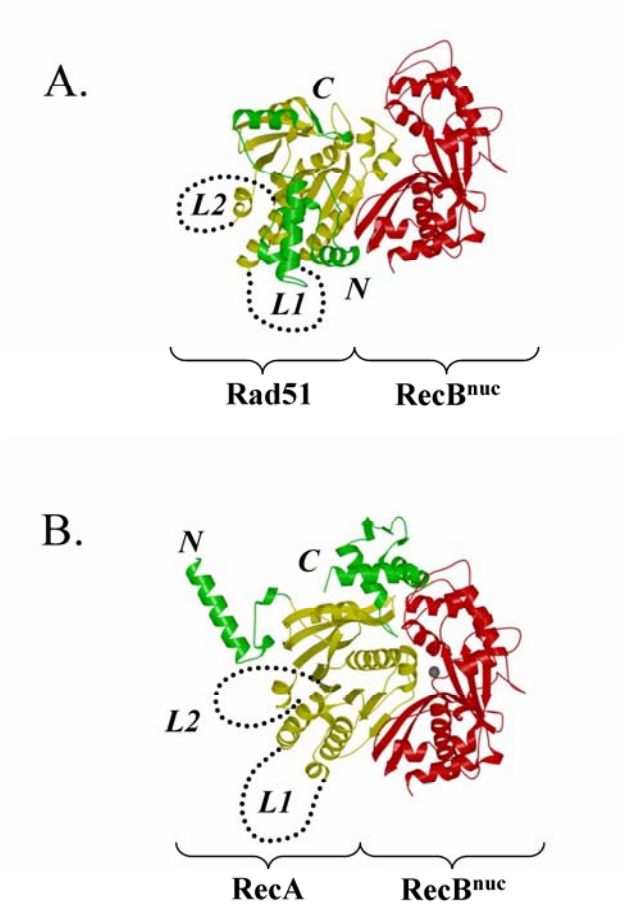


Figure S7. Control docking simulations.

A) The predicted structure of the Rad51-RecB^{nuc} complex. Because of the observed interaction between RecB^{nuc} and Rad51 proteins, another molecular interaction simulation was conducted by docking RecB^{nuc} (probe) and Rad51 protein (Conway et al., 2004) as the target. Two solutions similar to the proposed RecA-RecB^{nuc} complex were found among the top 15 solutions (solution 8 and 13). Other top scoring solutions involved regions that are occupied by the DNA-binding loops but do not appear in the Rad51 protein structure because they are disordered, as well as one solution that involved the N-terminal domain of Rad51 which is absent

in RecA. These former solutions were discarded as improbable, and the latter was not considered further.

B) The predicted structure of the RecA-RecB^{nuc} complex. To compare the Rad51-RecB^{nuc} and RecA-RecB^{nuc} complexes, RecA and Rad51 proteins were first superimposed using "Deep View/Swiss-pdb Viewer", and then the models of Rad51-RecB^{nuc} and RecA-RecB^{nuc} complex were superimposed on the Rad51 and RecA structures, respectively. The RMS values for the α carbons of RecB^{nuc} in the modeled Rad51-RecB^{nuc} and RecA-RecB^{nuc} (top scoring solution) complexes were 8.93 Å and 9.44 Å for the solutions number 8 and number 13, respectively.

In an additional control docking simulation, RecB^{nuc} (probe) was docked with the region of RecC protein (1-781) with which it interacts in the crystal structure; because this target is so large, the flexible hinge region of RecB (residues 890-927) that connects helicase and nuclease domains was included. In the 6 top-scoring solutions, RecB^{nuc} was correctly positioned and oriented relative to the RecC subunit (data not shown). The RMS values for the α carbons of RecB^{nuc} in the modeled complex and in the RecBCD crystal structure were between 4.16 Å (solution number 5) and 9.37 Å (solution number 3).

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