The Function of the Secondary Site of RecA Protein During DNA Strand Exchange

Research by A.V. Mazin and S.C. Kowalczykowski, EMBO J. *1998*, 17, 1161

Condensation and commentary by **Andrzej Stasiak**, Université de Lausanne, Switzerland

Condensation of the Research

Purpose of the Study

To investigate the molecular mechanism of RecA protein-promoted homologous pairing and DNA strand exchange and, in particular, to determine the function of the secondary DNA binding site of RecA protein

Background

RecA protein of *Escherichia coli* and its structural and functional homologs in other organisms, like yeast and human Rad51 protein, play a central role in the process of DNA recombination.[1-4] In vitro purified RecA protein promotes several essential steps of DNA recombination including the recognition of homology and the polar exchange of strands between interacting DNA molecules.[5-7] It is known that during a recombination reaction RecA protein polymerizes on single-stranded or partially single-stranded DNA molecules forming right-handed helical filaments with a regular appearance.[8] DNA in these filaments is stretched to ca. 5.1 Å per base as compared to 3.4 Å per base pair in a regular B-DNA structure.[9] These helical filaments are then able to recognize protein-free double-stranded DNA (dsDNA) that is homologous to the single-stranded DNA (ssDNA) resident within the filaments. Upon homologous recognition, both interacting DNA molecules are aligned within the same helical filament and one strand of the duplex progressively switches partners in favor of the strand initially bound within the filament.[10,11]

In 1984, Howard-Flanders et al.[12] proposed that two DNA binding sites within RecA nucleoprotein filaments are involved in the concerted process of homologous recognition and DNA strand exchange. The primary site was defined as the one involved in the assembly of RecA protein filaments on ssDNA. The secondary site was defined as being

involved in the binding of dsDNA during the search for homology. Since then, many independent studies provided strong indications for the existence of the two sites and addressed both structural and functional questions concerning the two sites.[13,14] However, the work reported by Mazin and Kowalczykowski is distinguished by the ingenious simplicity of experimental approach and by the completeness of the resultant characterization of both DNA binding sites of RecA protein.

What Researchers Accomplished

Dissecting multistage enzymatic reactions as complex as homologous recombination is notoriously difficult. By using the slowly hydrolyzable analog of ATP, ATPγS, several groups were able to demonstrate which steps of the reaction do not require the energy of ATP hydrolysis.[15,16] It turned out that in the case of the reaction between completely homologous ssDNA and dsDNA, the reaction proceeds almost to completion and becomes blocked only at the very last stage of the reaction, namely, release of the recombined DNA from the RecA filaments.[17] Because RecA protein-promoted DNA strand exchange reaction performed in the presence of ATPγS is easier to analyze as there are no multiple rounds of reaction,[17] it has become a favorite experimental system. Until recently, it was assumed that RecA–ssDNA complexes formed in the presence of ATPγS are extremely stable. However, in their previous paper, Mazin and Kowalczykowski demonstrated that binding of ssDNA to the secondary site is quite dynamic and that ssDNA with a lower affinity can be partially replaced from the complexes formed in the presence of ATPγS by the ssDNA with a higher affinity to RecA protein.[18]

In the studies described here, Mazin and Kowalczykowski analyzed more closely the DNA binding properties of RecA in the presence of ATPγS. In the first set of experiments, they studied ssDNA binding by RecA and observed that when RecA was incubated with an excess of short single-stranded oligonucleotides, each RecA monomer bound slightly more than five nucleotides of ssDNA. However, about half of the bound ssDNA could be quickly exchanged when an excess of challenging ssDNA with a high affinity to RecA protein was added to the reaction. The fact that about half of bound ssDNA easily dissociated, whereas the other half did not, indicates that RecA has two binding sites with similar stoichiometries but with different affinities for ssDNA. To better differentiate between the two sites, the authors performed the following experiments. They incubated RecA protein with ssDNA, providing initially about three nucleotides of ssDNA per RecA protein monomer, and only then did they add a second portion of ssDNA also at a stoichiometry of three nucleotides per RecA protein monomer. In one experimental protocol, the DNA added first was radioactively labeled; in another, the DNA added second was labeled. Upon mixing with unlabeled competitor DNA having a high affinity to RecA protein, a native gel assay was used to determine if the labeled DNA remained within the complexes. The ssDNA that was added as the first component remained stably associated with the complexes (Fig. 1, left panel), whereas the DNA added as the second component was very effectively exchanged with the unlabeled competitor DNA (Fig. 1, right panel). Thus, at least in the presence of ATPγS, the pri-
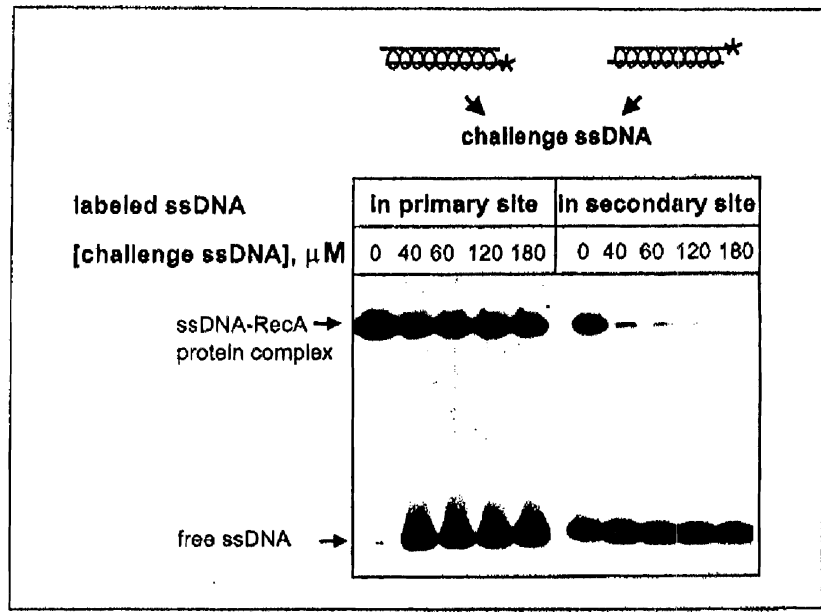
Figure 1. Differences between single-stranded DNA (ssDNA) affinities to primary and secondary DNA binding sites in RecA filaments. (Left panel), Gel retardation assay demonstrates that labeled ssDNA bound to the primary site remains resistant to the challenge with up to 15-fold excess of unlabeled ssDNA showing a high affinity to the secondary site. (A portion of ssDNA dissociates easily, but it does not increase with the amount of challenging ssDNA. This portion corresponds presumably to the ssDNA that was originally bound by the secondary site.) (Right panel), Labeled ssDNA bound by the secondary site completely dissociates upon challenge with an excess of unlabeled competitor ssDNA with a high affinity to the secondary site. (Reproduced, with permission, from Mazin, A.V., Kowalczykowski, S.C. *EMBO J.* 1998, *17*, 1161. Copyright © 1998 by Oxford University Press.)

mary DNA binding site displays irreversible binding (it is the site that governs formation of the active RecA–ssDNA complexes), and the secondary site permits exchange of the bound DNA in favor of the DNA that shows a higher affinity to this site. According to the model of Howard-Flanders et al.,[12] the secondary site is involved in binding the dsDNA; dsDNA that is not homologous to the ssDNA resident in the complex is bound with a lower affinity than the homologous dsDNA.

Being able to differentiate between ssDNA that is bound by the primary and secondary sites, the authors decided to verify the fate of the strand that becomes single-stranded after the reaction between ssDNA and dsDNA. To this aim, they performed DNA strand exchange reactions between homologous single- and double-stranded oligonucleotides. The applied labeling of the substrates was such that radiolabeling was placed on this strand of the dsDNA that becomes displaced from the duplex during the reaction. Gel assays showed that after DNA strand exchange, the displaced strand remained in the postsynaptic complexes even when challenged with low affinity ssDNA (Fig. 2, left panel).
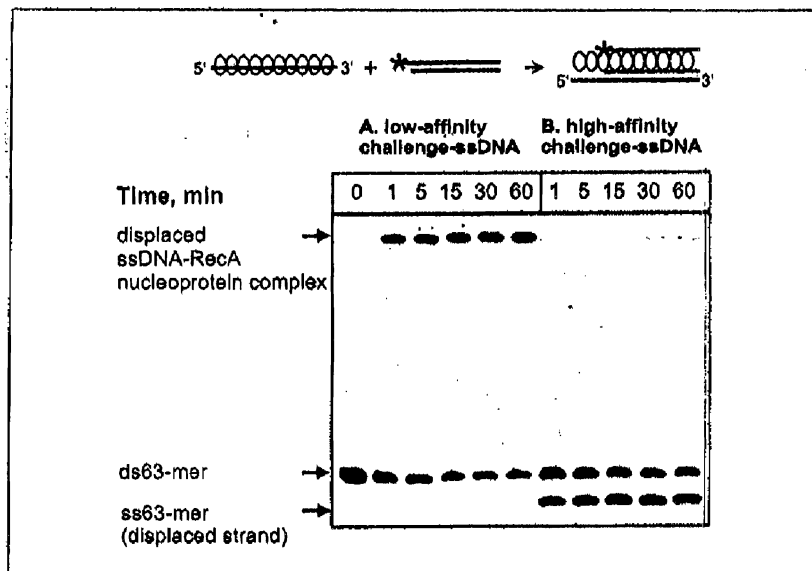
Figure 2. The strand of the duplex DNA that becomes displaced during DNA strand exchange behaves like a single-stranded DNA (ssDNA) bound by the secondary site. Strand exchange reaction was performed using duplex DNA in which the strand that gets displaced in the reaction was radioactively labeled. Upon strand exchange, this strand is bound by the filaments but can be easily chased from the complexes by excess of ssDNA having high affinity to the secondary site (right panel) but not by ssDNA with low affinity to the secondary site (left panel). (Reproduced, with permission, from Mazin, A.V., Kowalczykowski, S.C. *EMBO J.* **1998**, *17*, 1161. Copyright © 1998 by Oxford University Press.)

However, when unlabeled ssDNA with a high affinity to the secondary site was added to the postsynaptic complexes, the labeled displaced strand left the RecA nucleoprotein complexes (Fig. 2, right panel). This demonstrated that the displaced strand was bound to the secondary site within RecA nucleoprotein filaments. Competition between the displaced strand and the excess of ssDNA is probably not a physiological situation and thus has a limited functional significance for understanding the reaction. More interesting, however, is the question of the effect of ssDNA binding protein (SSB) on the ssDNA bound within the secondary site. SSB protein stimulates RecA protein-promoted strand exchange reaction in vitro,[19-22] and genetic analysis demonstrated that SSB protein is also needed for the efficient recombination in vivo.[23,24] Therefore, Mazin and Kowalczykowski examined the effect of SSB protein on ssDNA bound to the primary site of RecA protein and on ssDNA bound to the secondary site. The ssDNA originally bound in the secondary site was found to leave RecA filaments forming complexes with SSB protein, whereas the ssDNA in the primary site was unaffected by the presence of SSB protein. Subsequently, the authors examined the effect of SSB protein on the displaced strand that in the reaction performed in the absence of SSB protein remains bound in the secondary binding site of RecA filaments. Figure 3 shows that in the
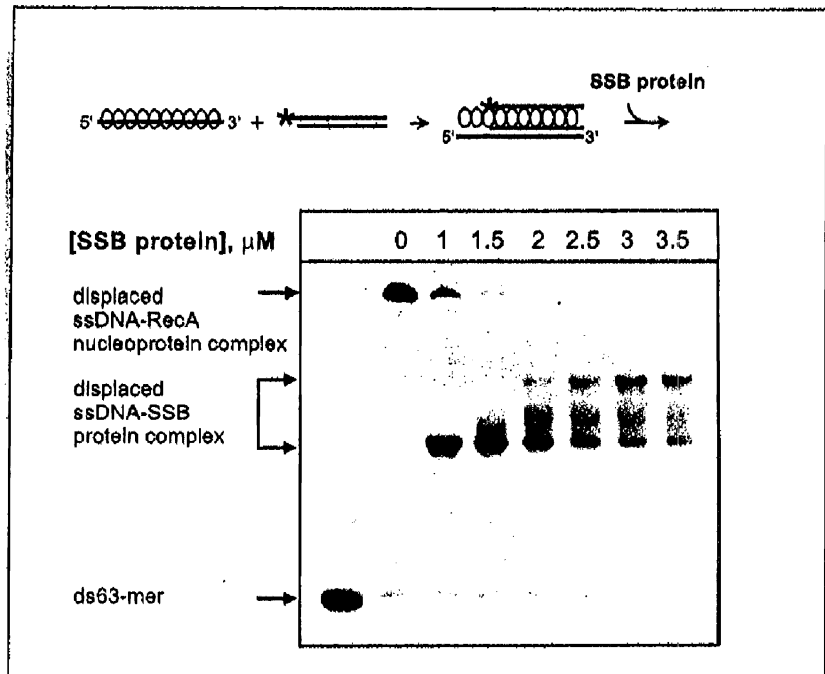
SSB protein

5′ 3′ + → 5′ 3′

[SSB protein], µM	0	1	1.5	2	2.5	3	3.5

displaced
ssDNA-RecA
nucleoprotein complex

displaced
ssDNA-SSB
protein complex

ds63-mer

Figure 3. SSB protein removes the displaced strand from the secondary site in RecA filaments. Strand exchange reaction was performed as analyzed on Figure 2, and then SSB protein was added to the postsynaptic complexes. The labeled single-stranded DNA (ssDNA) that is displaced from the incoming duplex stays bound in the secondary site in the absence of SSB protein. In the presence of SSB protein, the displaced strand becomes bound by SSB protein and leaves the postsynaptic RecA–DNA complex. (Reproduced, with permission, from Mazin, A.V., Kowalczykowski, S.C. *EMBO J.* 1998, *17*, 1161. Copyright © 1998 by Oxford University Press.)

presence of SSB protein the displaced strand left the postsynaptic RecA–DNA filaments and became complexed with SSB protein.

Commentary on the Research

To understand the mechanism of homologous recombination promoted by RecA protein, we need not only to answer the most essential questions related to the actual DNA pairing mechanism (triplex formation or classical annealing) but also to gain insight into the acrobatics of DNA strand switching and into the function of auxiliary factors like SSB protein. Thanks to the paper by Mazin and Kowalczykowski, we better understand the interplay between partner DNA molecules, the DNA binding sites of RecA protein, and SSB protein. The results obtained lead to the model presented in Figure 4. According to the model, RecA nucleoprotein presynaptic filaments containing ssDNA bound within the primary site attract protein-free duplex DNA into the secondary site. For nonhomologous dsDNA, the binding to the secondary
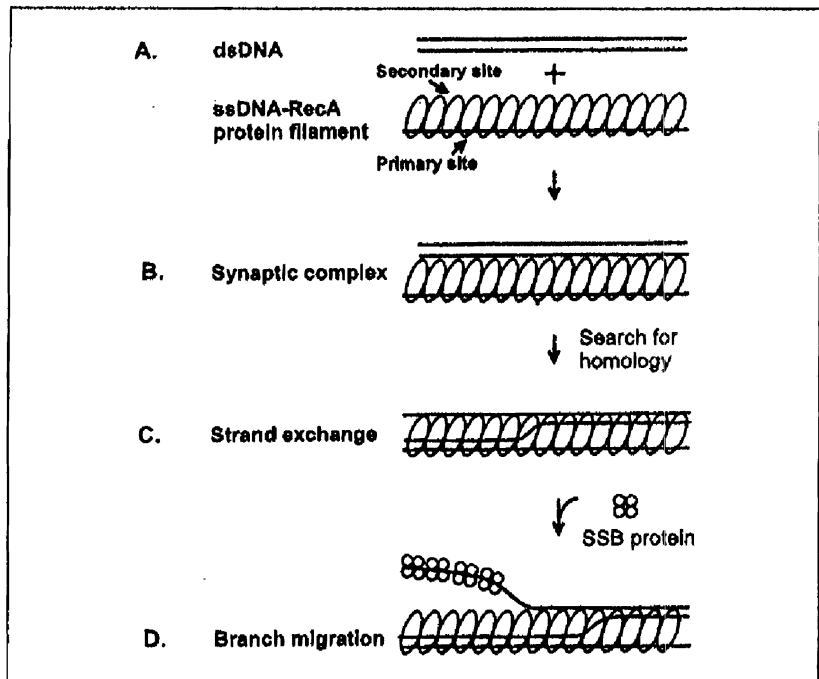
Figure 4. Model presenting the functions of primary and secondary DNA binding sites of RecA in DNA strand exchange reaction. (**A**) RecA polymerizes on single-stranded DNA (ssDNA) forming helical presynaptic complexes where ssDNA is bound in the primary binding sites of consecutive RecA monomers (helicity of the filaments is not shown in this schematic drawing). (**B, C**) Double-stranded DNA (dsDNA) interacts with the secondary DNA binding site in RecA–ssDNA complexes. Upon homologous recognition between dsDNA and ssDNA, the process of strand exchange starts whereby the displaced strand of the incoming duplex DNA stays in the secondary site. (**D**) SSB protein binds the displaced strand thus favoring progression of the strand exchange. (Reproduced, with permission, from Mazin, A.V., Kowalczykowski, S.C. *EMBO J.* 1998, *17*, 1161. Copyright © 1998 by Oxford University Press.)

site is unstable, but, when homologous recognition occurs between ssDNA and dsDNA sequences, the interaction becomes stable and is followed by the process of DNA strand exchange. Strand exchange proceeds in such a fashion that the displaced strand of the incoming duplex DNA stays in the secondary site while the second DNA strand base pairs with the strand that remains bound within the primary site of RecA filaments. In principle, a very similar scenario was proposed by the original model of Howard-Flanders et al.[12] and was supported later by several experimental papers.[17,25-27] However, the studies by Mazin and Kowalczykowski showed more directly where the displaced strand is located in the postsynaptic filaments. It should be stressed perhaps that several alternative scenarios of strand switching are conceivable, for example, the single strand from the primary site could move to the secondary site to pair there with one strand of the duplex

while the displaced strand leaves the complex. The work presented here speaks against such alternative combinations. Considering the role of SSB protein, Mazin and Kowalczykowski demonstrated that SSB does not merely bind to the displaced strand that is excluded from the complexes but that it effectively drives the dissociation of this strand from the postsynaptic complexes (Fig. 4D). Interestingly, earlier electron microscopy studies of the ongoing strand exchange reaction suggested such a role of SSB protein.[28]

Summary

Characterization of the concerted action of the primary and secondary DNA binding site of RecA protein and an elucidation of the role of SSB protein in recombination reactions permitted the authors to clarify existing models of homologous recombination (see Fig. 4). In particular, Mazin and Kowalczykowski established that after DNA strand exchange the displaced strand tends to remain in the secondary DNA binding site of RecA filaments unless it is sequestered by SSB protein. By this activity, SSB protein prevents "reversal" of DNA strand exchange.

Future Research

The efficient action of eukaryotic homologs of RecA protein like Rad51 protein from yeast and humans depends on their cooperation with specific partner proteins including Rad52 protein.[29-32] Investigation of the role of Rad52 protein in the modulation of DNA binding activity of Rad51 protein should help explain the differences that exist in the molecular mechanism of homologous recombination in prokaryotes and eukaryotes.[33]

References

1. Camerini-Otero, R.D., Hsieh, P. *Annu. Rev. Genetics* **1995**, *29*, 509.
2. Dunderdale, H.J., West, S.C. *Curr. Opin. Genet. Dev.* **1994**, *4*, 221.
3. Kowalczykowski, S.C., Eggleston, A.K. *Annu. Rev. Biochem.* **1994**, *63*, 1991.
4. Stasiak, A., Egelman, E.H. *Experientia* **1994**, *50*, 192.
5. Cox, M.M., Lehman, I.R. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 6018.
6. Kahn, R., Cunningham, R.P., DasGupta, C., Radding, C.M. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 4786.
7. West, S.C., Cassuto, E., Howard-Flanders, P. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 6149.
8. Stasiak, A., Di Capua, E. *Nature* **1982**, *299*, 185.
9. Stasiak, A., Di Capua, E., Koller, T. *J. Mol. Biol.* **1981**, *151*, 557.
10. Stasiak, A., Stasiak, A.Z., Koller, T. *Cold Spring Harbor Symp. Quant. Biol.* **1984**, *49*, 561.
11. Register, J.C., Christiansen, G., Griffith, J. *J. Biol. chem.* **1987**, *262*, 12812.
12. Howard-Flanders, P., West, S.C., Stasiak, A.J. *Nature* **1984**, *309*, 215.
13. Takahashi, M., Kubista, M., Norden, B. *J. Mol. Biol.* **1989**, *205*, 137.

14. Müller, B., Koller, T., Stasiak, A. *J. Mol. Biol.* **1990,** *212,* 97.
15. Honigberg, S.M., Gonda, D.K., Flory, J., Radding, C.M. *J. Biol. Chem.* **1985,** *260,* 11845.
16. Menetski, J.P., Bear, D.G., Kowalczykowski, S.C. *Proc. Natl. Acad. Sci. USA* **1990,** *87,* 21.
17. Rosselli, W., Stasiak, A. *J. Mol. Biol.* **1990,** *216,* 335.
18. Mazin, A.V., Kowalczykowki, S.C. *Proc. Natl. Acad. Sci. USA* **1996,** *93,* 10673.
19. Shibata, T., DasGupta, C., Cunningham, R.P., Radding, C.M. *Proc. Natl. Acad. Sci. USA* **1980,** *77,* 2606.
20. West, S.C., Cassuto, E., Howard-Flanders, P. *Mol. Gen. Genet.* **1982,** *186,* 333.
21. Cox, M.M., Lehman, I.R. *J. Biol. Chem.* **1982,** *257,* 8523.
22. Kowalczykowski, S.C., Krupp, R.A. *J. Mol. Biol.* **1987,** *193,* 97.
23. Clark, A.J. *Ann. Rev. Genet.* **1973,** *7,* 67.
24. Vinogradskaya, G.R., Goryshin, I.Y., Lantsov, V.A. *Dokl. Acad. Nauk USSR* **1986,** *290,* 228.
25. Chow, S.A., Honigberg, S.M., Bainton, R.J., Radding, C.M. *J. Biol. Chem.* **1986,** *261,* 6961.
26. Pugh, B.F., Cox, M.M. *J. Biol. Chem.* **1987,** *262,* 1337.
27. Ullsperger, C.J., Cox, M.M. *Biochemistry* **1995,** *34,* 10859.
28. Stasiak, A., Egelman, E.H. In *Genetic Recombination,* Kucherlapati, R., Smith G.R., eds. American Society for Microbiology: Washington, DC, 1988, p. 265.
29. Sung, P. *J. Biol. Chem.* **1997,** *272,* 18996.
30. Benson, F.E., Bauman, P., West, S.C. *Nature* **1998,** *391,* 401.
31. Shinohara, A., Ogawa, T. *Nature* **1998,** *391,* 404.
32. New, J.H., Sugiyama, T., Zaitseva, E., Kowalczykowski, S.C. *Nature* **1998,** *391,* 407.
33. Kanaar, R., Hoeijmakers, J.H.J. *Nature* **1998,** *391,* 335.