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The function of the secondary DNA-binding site of RecA protein during DNA strand exchange

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RecA protein features two distinct DNA-binding sites. During DNA strand exchange, the primary site binds to single-stranded DNA (ssDNA), forming the helical RecA nucleoprotein filament. The weaker secondary site binds double-stranded DNA (dsDNA) during the homology search process. Here we demonstrate that this site has a second important function. It binds the ssDNA strand that is displaced from homologous duplex DNA during DNA strand exchange, stabilizing the initial heteroduplex DNA product. Although the high affinity of the secondary site for ssDNA is essential for DNA strand exchange, it renders DNA strand exchange sensitive to an excess of ssDNA which competes with dsDNA for binding. We further demonstrate that single-stranded DNA-binding protein can sequester ssDNA, preventing its binding to the secondary site and thereby assisting at two levels: it averts the inhibition caused by an excess of ssDNA and prevents the reversal of DNA strand exchange by removing the displaced strand from the secondary site.
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Introduction

RecA protein promotes the exchange of strands between two homologous DNA molecules, an essential step of homologous recombination in prokaryotes and eukaryotes (Roca and Cox, 1990; Radding, 1991; Dunderdale and West, 1994; Kowalczykowski and Eggleston, 1994; Camerini-Otero and Hsieh, 1995). *In vitro*, RecA protein alone can carry out DNA strand exchange, but the single-stranded DNA-binding (SSB) protein greatly stimulates the reaction (Cox and Lehman, 1982; Muniyappa *et al*., 1984; Kowalczykowski and Krupp, 1987).

DNA strand exchange requires the formation of a RecA nucleoprotein filament. RecA protein can assemble on either single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) to form a helical filament (McEntee *et al*., 1981; Pugh and Cox, 1988); however, ssDNA is the preferred and probably biologically important substrate for RecA protein (Kowalczykowski and Eggleston, 1994). Conventionally, the RecA protein-binding site responsible for this DNA binding is referred to as the 'primary' site.

In the presence of ATP or ATP-γ-S, the RecA protein–ssDNA filament can promote a search for sequence homology within dsDNA. As an initial step of the homology search, the binding of non-homologous dsDNA by the filament is required for the DNA strand exchange (Howard-Flanders *et al*., 1984). Since in the cell, non-homologous genomic sequences are in vast excess, this binding must be weak by necessity to permit a rapid search for sequence homology. The RecA nucleoprotein filament does indeed bind non-homologous dsDNA weakly (Takahashi *et al*., 1989; Muller *et al*., 1990); the resultant complexes hardly survive gel electrophoresis and can be detected only by sensitive, though indirect assays (Chow and Radding, 1985; Tsang *et al*., 1985; Conley and West, 1990). The RecA nucleoprotein filament can also bind non-homologous ssDNA (Menetski and Kowalczykowski, 1987; Takahashi *et al*., 1989; Muller *et al*., 1990; Zlotnick *et al*., 1993). In contrast to the complex with dsDNA, the stability of this complex is sufficiently high to allow detection by gel electrophoresis (Muller *et al*., 1990; Rao and Radding, 1993, 1994; Mazin and Kowalczykowski, 1996). Since ssDNA and dsDNA compete with one another for the binding to the RecA protein–ssDNA filament (Mazin and Kowalczykowski, 1996), it is reasonable to conclude that both ssDNA and dsDNA binding occur at the same site, referred as the 'secondary' site.

Several lines of evidence indicate that the primary site is involved in two types of DNA binding during DNA strand exchange: first, it binds ssDNA to initiate formation of the pre-synaptic filament and, second, after homologous dsDNA is found and DNA strands are exchanged, it accommodates the newly formed heteroduplex DNA within the post-synaptic filament (Chow *et al*., 1986; Pugh and Cox, 1987; Rosselli and Stasiak, 1990; Ullsperger and Cox, 1995). In contrast, the post-synaptic location of the DNA strand displaced from the original duplex DNA is not well determined. The distinct preference of the secondary site for ssDNA prompted us to propose a dual role for this site in DNA strand exchange: it binds the incoming non-homologous dsDNA weakly and then, upon finding homology and promoting local exchange of DNA strands, it binds tightly to the resulting displaced DNA strand, stabilizing the nascent DNA heteroduplex product.

Here, we wanted to test specific features of secondary site function in DNA strand exchange. First, we wanted to ascertain whether the secondary site did indeed bind the displaced DNA strand following DNA strand exchange. Second, although essential for DNA strand exchange, the high affinity of the secondary site for ssDNA also represents a potential liability in that it renders DNA strand exchange sensitive to inhibition by ssDNA; therefore, we sought to determine auxiliary factors and conditions that could alleviate this inhibition. The results obtained demonstrate that, in addition to its role of dsDNA binding during the homology search, the secondary DNA site provides this

Table I. List of oligodeoxyribonucleotides used in this study

No.	Sequence
#1	ACAGCACCAGATTCAGCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAAAGGA
#2	TCCTTTTGATAAGAGGTCATTTTTGCGGATGGCTTAGAGCTTAATTGCTGAATCTGGTGCTGT
#5	CCATCCGCAAAAATGACCTCTTATCAAAAGGA
#21	CCTTCCGCTTTTTTGTCCTCTTTTCTTTTGGT
#25	GCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAAAGGA
#26	TCCTTTTGATAAGAGGTCATTTTTGCGGATGGCTTAGAGCTTAATTGC
#41	TCTGCTCCTGTTTCTGCTTTTTTGCTCTTTGCCTTCCGCTTTTTTGTCCTCTTTTCTTTTGGT
#71	CTTTAGCTGCATATTTACAACATGTTGACCTACAGCACCAGATTCAGCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAAAGGA
htr#1	GTGAGTCGACAAGCCTGACTCAACATTATCCT

important post-pairing function of binding the displaced ssDNA. We further demonstrate that SSB protein, by removing ssDNA from the secondary site, is instrumental in augmenting secondary site function during DNA strand exchange.

Results

Stoichiometry of ssDNA binding to the RecA nucleoprotein filament

The RecA nucleoprotein filament binds ssDNA and displays a base-compositional preference in this binding (Mazin and Kowalczykowski, 1996). We proposed that this binding occurs at a secondary DNA-binding site within the RecA nucleoprotein filament. A distinct secondary DNA-binding site should possess a defined stoichiometry for DNA binding. Therefore, we sought to determine this stoichiometry and to characterize further the function of the secondary DNA-binding site.

When RecA protein, at the indicated concentrations, is incubated with an excess of a ^{32}P-labeled 32mer oligonucleotide (#5); Table I, protein–ssDNA complexes are detected by virtue of their retarded mobility in a 10% polyacrylamide gel (Figure 1A; first lane in each set, devoid of competitor ssDNA). The amount of DNA contained within the slower moving protein–ssDNA complexes is linearly dependent on the concentration of RecA protein (Figure 1B). The slope of Figure 1B (amount of oligonucleotide retained per mole of RecA protein) under these conditions of DNA excess yields an apparent binding stoichiometry of just above five nucleotides per RecA protein monomer.

When RecA–ssDNA complexes are formed, and then incubated with an excess of a challenge 32mer oligonucleotide (#21); which displays a very high affinity for the secondary site (referred to as 'A→T'; Mazin and Kowalczykowski, 1996), half of the labeled ssDNA is displaced; importantly, 42–50% of labeled DNA remains bound at all concentrations of challenge ssDNA (Figure 1). The remaining bound DNA represents a stable plateau level, since it is constant at all concentrations of challenge ssDNA. The kinetics of displacement are rapid, with equilibrium reached within 1 min (data not shown). This result shows that two distinct species of protein-bound ssDNA exist in the presence of ATP-γ-S: one which is stable to dissociation by challenge ssDNA, and another which is sensitive. This differential susceptibility to challenge ssDNA serves as the basic experimental criterion that defines the 'primary' and 'secondary' DNA-binding

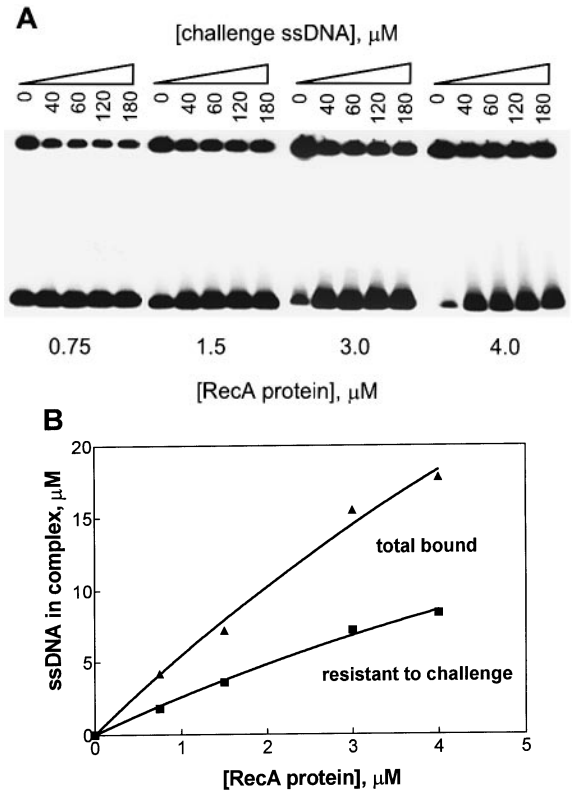

Fig. 1. Stoichiometry of RecA protein binding to ssDNA. RecA protein, at the indicated concentrations, was mixed with a ^{32}P-labeled 32mer oligonucleotide (#5) (24 μM nucleotides) in the standard buffer. The resultant RecA protein–ssDNA filaments were either loaded directly on a 10% polyacrylamide gel or, prior to the loading, challenged with an unlabeled 32mer oligonucleotide (#21). (**A**) An autoradiogram of the gel. Quantitation of these data is presented in (**B**), which shows the total amounts of oligonucleotide bound to RecA protein and the amounts of the complex resistant to the challenge with the 32mer oligonucleotide (#21) (180 μM).

sites of RecA protein, respectively. Given that approximately half of the ssDNA is susceptible to challenge (Figure 1B), the stoichiometry for each of these DNA-binding sites is ~2.5 nucleotides per RecA protein monomer.

RecA protein has two non-equivalent sites for ssDNA binding

If the two distinct ssDNA-binding sites that are defined by the experiments shown in Figure 1 can be attributed to the presence of two unique saturable DNA-binding sites, then the site which binds ssDNA with the higher

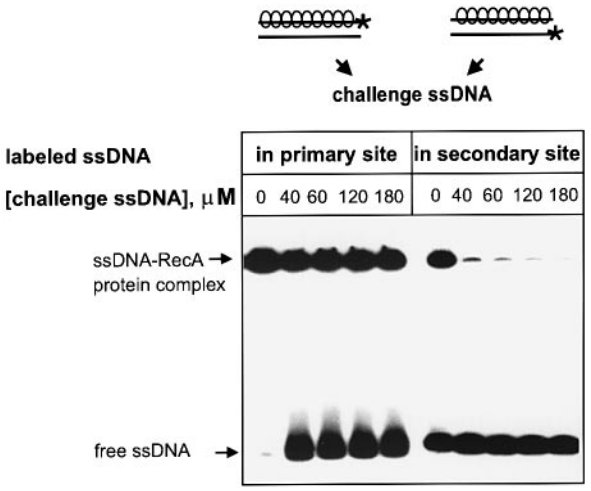

Fig. 2. The existence of two non-equivalent DNA-binding sites within the RecA nucleoprotein filament. RecA protein (4 μM) was mixed with the 32mer oligonucleotide (#5) (12 μM) in standard buffer. After filament formation was completed, another aliquot (12 μM) of the same oligonucleotide was mixed with the filament. To distinguish between these two DNA molecules, either the first or the second DNA was ^{32}P labeled. These two RecA protein–ssDNA filaments were challenged with an unlabeled 32mer oligonucleotide (#21). Complexes were analyzed by electrophoresis in a 10% polyacrylamide gel.

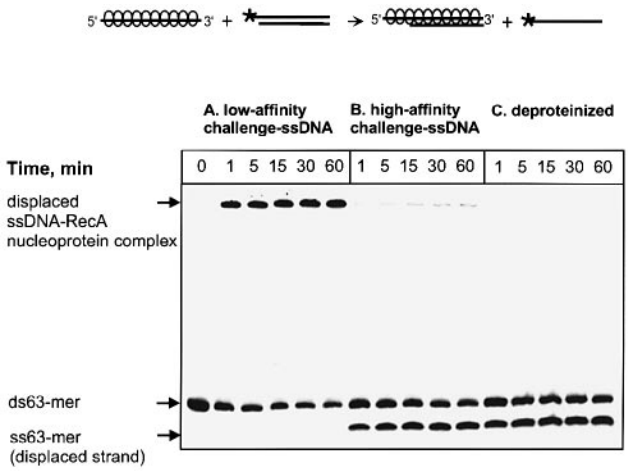

Fig. 3. The DNA strand displaced during DNA strand exchange binds to the secondary site of RecA protein. Nucleoprotein filaments were formed by incubation of RecA protein (4 μM) with the 94mer oligonucleotide (#71) (12 μM). DNA strand exchange was initiated by addition of homologous 63mer dsDNA (#1–2) (16 μM) in which the strand identical to the 3′ region of the 94mer was labeled. After the indicated periods of time, aliquots from the reaction mixture were either challenged with cold ssDNA competitor of (**A**) low affinity (htr#1) (60 μM) or (**B**) high affinity (#41) (60 μM) for the secondary site or, alternatively, deproteinized (**C**). Samples were analyzed by electrophoresis through a 10% polyacrylamide gel.

affinity (i.e. the primary site) should saturate preferentially in the presence of a limiting amount of ssDNA. Subsequent titration with additional ssDNA should result in binding to the secondary site. To establish whether this expectation was correct, two types of labeled ssDNA–RecA protein complexes were formed and then challenged with unlabeled ssDNA. In the first set, RecA nucleoprotein filaments were formed using ^{32}P-labeled 32mer oligonucleotide (#5) at the stoichiometric ratio of one RecA protein monomer per three nucleotides of ssDNA. Based on the data in Figure 1, this concentration of ssDNA would be in slight excess (20%) over the amount required to saturate the primary DNA-binding site of RecA protein. By adding another equivalent of the same, but unlabeled, oligonucleotide, the secondary site should also be filled but with the unlabeled ssDNA. This complex was then challenged with an excess of cold ssDNA 32mer (#21). As shown in the Figure 2 (marked 'labeled DNA in primary site'), the labeled oligonucleotide that was expected to be bound to the primary site was essentially resistant to the ssDNA challenge. About 30% of the labeled 32mer oligonucleotide dissociated from the complex after challenge (based on the concentration used, the 20% excess of the labeled oligonucleotide would have bound to the secondary DNA-binding site, and should have been displaced by the ssDNA challenge), while the remaining labeled complex was resistant to increasing concentrations of the challenger.

In the second set of experiments, complexes were formed just as in the first series, except that the order of addition of labeled and unlabeled oligonucleotide was reversed: the primary site was saturated with unlabeled oligonucleotide and the secondary site with labeled oligonucleotide. In this case, the results show that the labeled oligonucleotide which was bound to the secondary site was completely sensitive to the ssDNA competitor (Figure 2, marked 'labeled DNA in secondary site'). Quantitation of these two experiments showed that the amount of labeled oligonucleotide that was resistant to challenge in the first experiment was approximately equal to the amount of oligonucleotide that was sensitive to the challenge in the second experiment, arguing that the stoichiometric ratio for ssDNA bound to these sites is 1:1.

These data are fully consistent with the presence of two DNA-binding sites in the RecA nucleoprotein filament which bind equal amounts of ssDNA, but show different affinities in this binding.

The DNA strand that is displaced during DNA strand exchange binds to the secondary site

The observation that ssDNA competes with dsDNA for binding to the RecA nucleoprotein filament implied that the secondary binding site of the filament is the same site that binds dsDNA during the homology search (Mazin and Kowalczykowski, 1996). In addition, the high affinity of the secondary site for ssDNA prompted us to suggest that this site has an additional post-pairing role which is to accommodate the ssDNA strand that is displaced from the duplex during DNA strand exchange. Therefore, we wanted to determine whether the displaced DNA strand was indeed bound to the secondary site of RecA protein filament after DNA strand exchange. The differential sensitivity of ssDNA bound to the primary versus the secondary site towards challenge ssDNA was used as the criterion for occupancy by the displaced strand.

To determine the location of the displaced DNA strand, DNA strand exchange was examined using a pre-synaptic filament formed with a 94mer oligonucleotide (#71) and paired with homologous 63mer dsDNA in which the strand identical in sequence to the 3′ end of the 94mer was labeled; this strand would be displaced during DNA strand exchange. The reaction products were analyzed on a polyacrylamide gel without (Figure 3A and B) or with deproteinization (Figure 3C). In the absence of

deproteinization, the amount of the displaced strand that remained associated with the nucleoprotein filament as a post-synaptic complex is measured, while in the absence of protein, the total amount of DNA displaced during DNA strand exchange is measured. In the presence of an ssDNA competitor with low affinity for the secondary site (htr#1) (Mazin and Kowalczykowski, 1996), almost all of the displaced labeled ssDNA remained in the complex with RecA protein after DNA strand exchange had occurred (Figure 3A). However, this complex showed complete sensitivity to challenge with ssDNA that has a high affinity for the secondary binding site (#41). As a result, this strand migrated as free ssDNA (Figure 3B). The observed pattern of sensitivities to ssDNA challenge indicates that the displaced strand binds to the secondary site in the RecA protein filament after DNA strand exchange.

SSB protein binds the displaced DNA strand that is associated with the post-synaptic complex

The high affinity of the secondary site for ssDNA is fully consistent with its proposed post-synaptic function in DNA strand exchange which is to bind the displaced strand. However, this high affinity can be an impediment to DNA strand exchange, since it renders the reaction sensitive to an excess of ssDNA that would compete with dsDNA for binding to this active site (Mazin and Kowalczykowski, 1996). Based on the dynamic nature of the complexes found between ssDNA and the RecA nucleoprotein secondary site, it was reasonable to expect that SSB protein could 'chase' ssDNA from the secondary site and thereby stimulate DNA strand exchange.

To test this hypothesis, RecA nucleoprotein filaments were formed in which labeled 48mer oligonucleotide was bound in either the primary or the secondary site, and these complexes were challenged with increasing concentrations of SSB protein. Figure 4 shows that ssDNA bound to the secondary site was sensitive to SSB protein. At 3 μM SSB protein, almost all of the ssDNA bound to the secondary site was displaced, and it migrated as a distinct band with characteristics typical of an SSB protein–ssDNA complex (Figure 4). The stoichiometry of ssDNA binding to SSB protein was ~4 nucleotides per protein monomer; this represents 2.5-fold more SSB protein than is required to bind the free 48mer oligonucleotide (data not shown), which probably reflects the need to compete with RecA protein. In contrast, ssDNA bound to the primary site of RecA protein could not be removed by SSB protein under the experimental conditions used.

This sensitivity of ssDNA bound to the secondary site of SSB protein provided another independent assay to determine the site to which the displaced DNA strand is bound. To examine whether the displaced ssDNA that was bound to the post-synaptic complex could be sequestered by SSB protein, DNA strand exchange was carried out between 94mer ssDNA and 63mer dsDNA described earlier. The post-strand exchange complex containing the displaced labeled DNA strand was challenged with increasing concentrations of SSB protein. The results show that this ssDNA species could be bound by SSB protein, a characteristic of ssDNA bound to the secondary site of the RecA nucleoprotein complex (Figure 5).

This result shows that SSB protein can remove ssDNA from the secondary site of both pre-synaptic and post-synaptic RecA nucleoprotein complexes. It is likely that this property of SSB protein is responsible for its stimulatory function in DNA strand exchange.

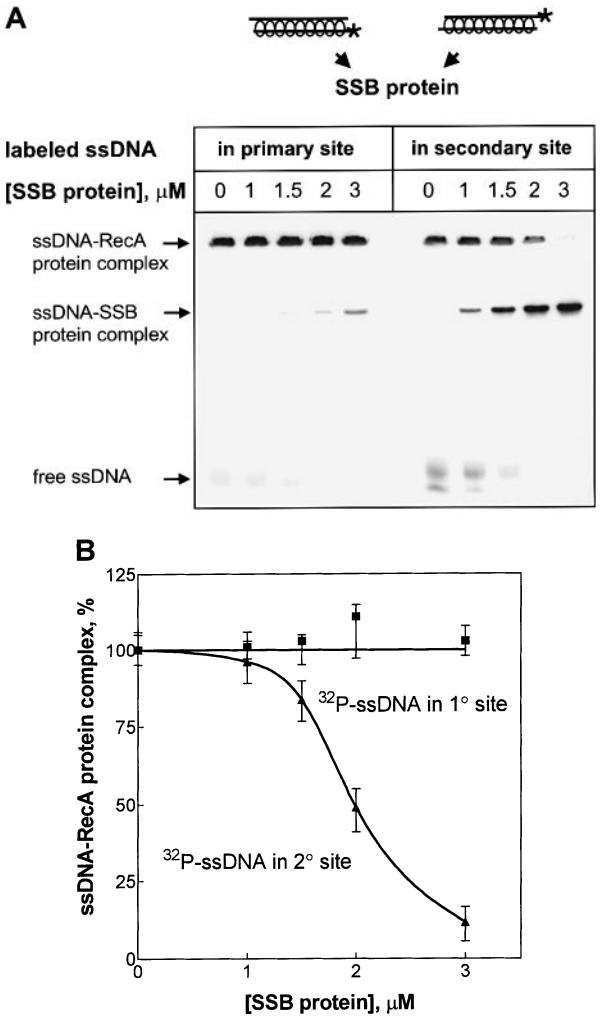

Fig. 4. The displacement of ssDNA from the primary and the secondary site of RecA by SSB protein. RecA protein (4 μM) was mixed with the 48mer oligonucleotide (#25) (12 μM) in standard buffer. After filament formation was completed, another aliquot (12 μM) of the same oligonucleotide was added to the filament. To distinguish between these two DNA molecules, either the first or the second DNA was ^{32}P labeled. These two RecA protein–ssDNA filaments were challenged with the concentrations of SSB protein indicated. Complexes were subjected to electrophoresis in a 10% polyacrylamide gel. (**A**) The autoradiograph of the gel; (**B**) a quantitation of these data.

Removal of ssDNA from the secondary site by SSB protein restores DNA strand exchange

The binding of ssDNA to the secondary site inhibits DNA strand exchange, since it prevents the binding of homologous dsDNA to this site (Mazin and Kowalczykowski, 1996). Here we tested whether the removal of ssDNA by SSB protein from the secondary site of the RecA nucleoprotein filament could restore DNA strand exchange.

DNA strand exchange was initiated between homologous double-stranded 48mer oligonucleotide and nucleoprotein filaments formed on a 63mer single-stranded oligonucleotide either in the presence or absence of a

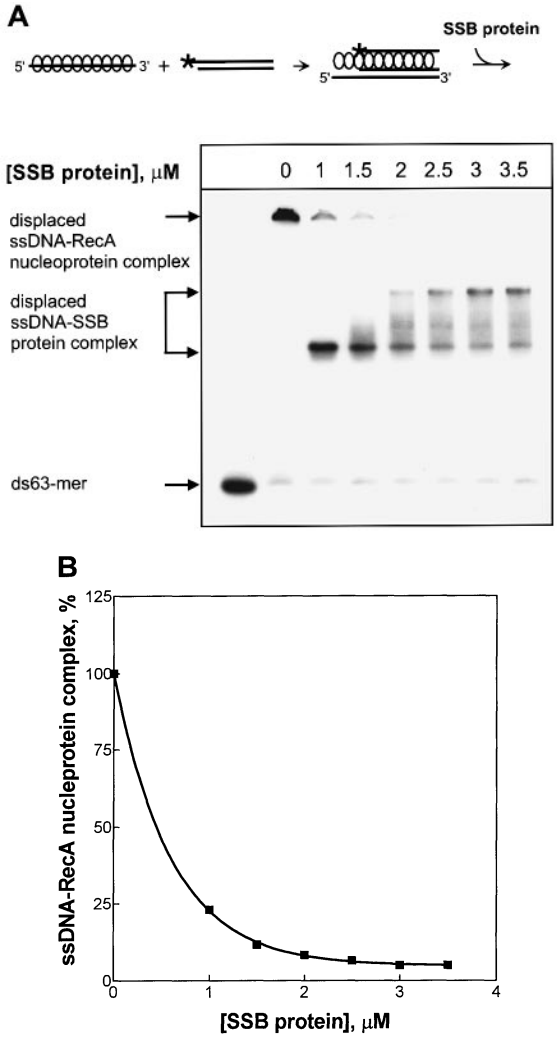

Fig. 5. SSB protein removes the displaced DNA strand from the secondary site of RecA protein. Nucleoprotein filaments were formed by incubation of RecA protein (4 μM) with the 94mer oligonucleotide (#71) (12 μM). DNA strand exchange was initiated by the addition of homologous 63mer dsDNA (#1–2) (16 μM) in which the strand identical to the 3′ region of the 94mer was labeled. After the indicated periods of time, aliquots from the reaction mixture were withdrawn and either loaded directly on a 10% polyacrylamide gel (lane marked '0'); or prior to the loading challenged with SSB protein at the concentrations indicated. (**A**) The autoradiograph of the gel; (**B**) quantitation of these data.

heterologous ssDNA inhibitor (#41) with high affinity for the secondary site. The ssDNA inhibitor was added to the reaction mixture after RecA nucleoprotein formation but prior to the addition of dsDNA. Figure 6 shows that a 3-fold excess of ssDNA nearly completely inhibited DNA strand exchange in the absence of SSB protein ('+ssDNA, 0 μM SSB' versus 'standard' reaction). This effect, however, was gradually suppressed when increasing amounts of SSB protein were added to the reaction. Addition of stoichiometric amounts of SSB protein with respect to the ssDNA inhibitor completely restored DNA strand exchange (Figure 6).

The secondary binding site of RecA protein can also be saturated with the identical strand of ssDNA during filament formation when the ssDNA is in excess over RecA protein (Figure 1); this excess ssDNA is inhibitory to DNA strand exchange (McEnree *et al.*, 1980). In this

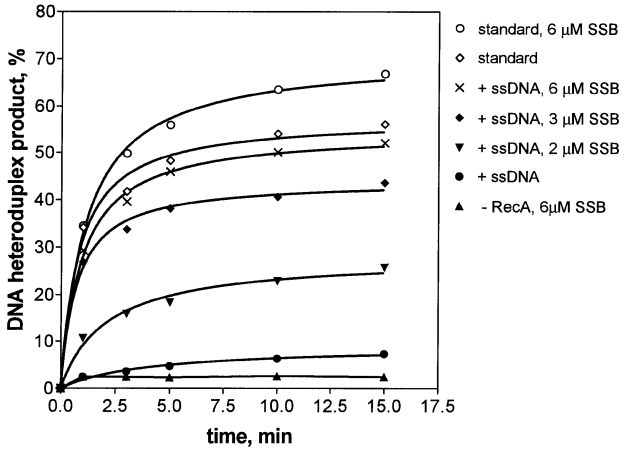

Fig. 6. SSB protein reverses the inhibitory effect of ssDNA on DNA strand exchange. Nucleoprotein filaments were formed by incubation of RecA protein (4 μM) with the 63mer oligonucleotide (#1) (12 μM) in standard buffer. After filaments were formed, oligonucleotide (#41) with high affinity to the secondary site was added (36 μM) to saturate the secondary site. SSB protein was added to the reaction mixtures at the concentrations indicated. DNA strand exchange was initiated by the addition of homologous 48mer dsDNA (#25–26) (18 μM) in which the strand (#26) complementary to the 3′ region of the 63mer was labeled. After the indicated periods of time, aliquots from the reaction mixture were removed, deproteinized and loaded on a 10% polyacrylamide gel. Gels were quantitated and data plotted on the graph.

case, we may also expect a significant stimulatory affect of SSB protein on DNA strand exchange. Indeed, an excess of homologous ssDNA (#1) (12 nucleotides per RecA protein monomer) significantly inhibited DNA strand exchange (Figure 7). However, SSB protein in a sufficient amount restored DNA strand exchange (Figure 7).

This result shows that SSB protein plays an important accessory role in DNA strand exchange by removing ssDNA from the secondary site. Saturation of the secondary site of the RecA nucleoprotein filaments with ssDNA inhibits DNA strand exchange regardless of whether it is achieved by the addition of ssDNA to the pre-formed RecA nucleoprotein filaments or during the normal course of DNA strand exchange. The addition of SSB protein in both cases alleviates the inhibition by ssDNA.

Discussion

Our results define the functional significance of the primary and the secondary DNA-binding sites within the RecA nucleoprotein filament, during the DNA strand exchange process. The primary site is responsible for the binding to ssDNA that is needed for both filament assembly and generation of a contiguous secondary site. The secondary site plays several important roles in this process. In the initial phase of DNA strand exchange, this site binds dsDNA as part of the homology search process. The secondary site also has a post-pairing function in DNA strand exchange, which is to bind the displaced DNA strand. This function is important for DNA strand exchange, since binding of the displaced strand stabilizes the nascent heteroduplex product of DNA strand exchange. Finally, this secondary site is the location at which SSB protein exerts its stimulatory effects, by abstracting ssDNA

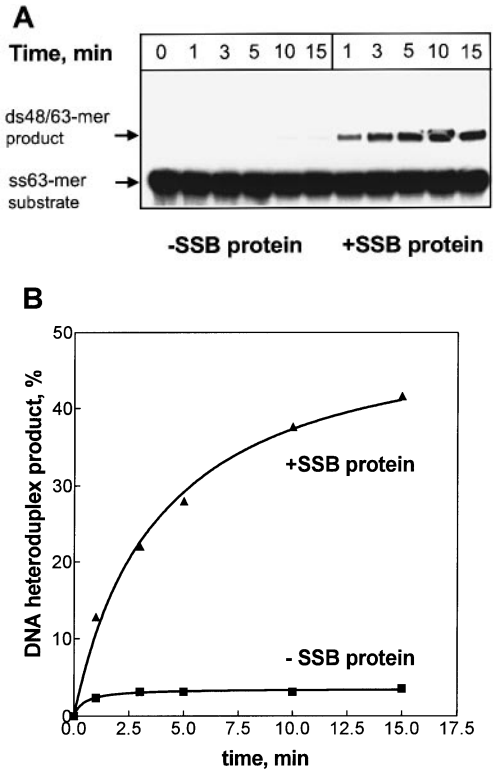

Fig. 7. SSB protein stimulates DNA strand exchange when an excess of ssDNA is present. Nucleoprotein filaments were formed by incubation of RecA protein (4 μM) with the ^{32}P-labeled 63mer oligonucleotide (#1) (48 μM) in standard buffer. The mixture was split in two and either SSB protein (6 μM) or an equivalent amount of SSB storage buffer was added to the reaction mixtures. DNA strand exchange was initiated by the addition of homologous 48mer dsDNA (#25–26) (18 μM). After the indicated periods of time, aliquots were withdrawn from the reaction mixture, deproteinized and loaded on a 10% polyacrylamide gel. (**A**) The radiograph of the gel; (**B**) quantitation of these data.

from the secondary site and, therefore, rendering DNA strand exchange insensitive to an excess of ssDNA.

The secondary site binds the displaced DNA strand

The DNA-binding experiments described here reveal the presence of two DNA-binding sites within the RecA nucleoprotein filament. The primary and secondary binding sites display distinctly different affinities for ssDNA: in the presence of ATP-γ-S, the primary site binds ssDNA much more strongly than the secondary site. The relatively lower DNA-binding affinity of the secondary site results in dynamic complexes that exchange bound DNA molecules rapidly. The dynamic nature of this DNA binding is fully consistent with a function for this site in the homology search process which requires rapid inspection of numerous dsDNA sequences. The two sites bind an equivalent amount of ssDNA, with each site accommodating ~2.5 nucleotides per RecA protein monomer. The result is consistent with the ssDNA binding stoichiometries obtained in the presence of ATP-γ-S that were determined using different methods (Bryant *et al*., 1985; Takahashi *et al*., 1989).

We show here that, after DNA strand exchange, the displaced DNA strand forms a complex with RecA nucleoprotein filament. This complex is indistinguishable from the complex formed by deliberately binding ssDNA to the secondary site. Both of these complexes were sensitive to challenge with competitor ssDNA or with SSB protein. These results are consistent with previously published data, obtained primarily using significantly longer DNA substrates and in the presence of hydrolyzable ATP. Electron microscopic observations with phage DNA show that, after DNA strands have exchanged, the displaced DNA strand is not released immediately from the protein complex but, rather, it remains temporarily associated with the RecA protein–DNA filament (Stasiak *et al*., 1984); however, the displaced strand is removed rapidly from the filament in the presence of SSB protein. In contrast, under the same conditions, SSB protein does not displace ssDNA from the pre-synaptic complex (Stasiak and Egelman, 1987). These two observations, which have been considered as contradictory, can be rationalized by assuming that RecA protein in the pre-synaptic and post-synaptic complexes contains ssDNA bound in two different binding sites, i.e. the primary and the secondary sites. As we demonstrate here, SSB protein can sequester the displaced DNA strand that is bound to the secondary site of the post-synaptic complex, while ssDNA bound to the primary site is not available for binding by SSB protein. The ability of the post-synaptic RecA nucleoprotein complex to bind the displaced DNA strand in the secondary site is also consistent with the observations that the RecA nucleoprotein filament with dsDNA bound to its primary site (which probably represents the product of DNA strand exchange) (Ullsperger and Cox, 1995), could still bind a stoichiometric amount of ssDNA (Takahashi *et al*., 1989; Muller *et al*., 1990). Together, these results argue for an important function of the secondary site at the post-synaptic step of DNA strand exchange, which is the binding of the displaced ssDNA strand.

Sequestration by SSB protein of the DNA strand displaced during DNA strand exchange

Previously, we demonstrated that ssDNA competes with dsDNA for binding to the secondary site and that it inhibits DNA strand exchange (Mazin and Kowalczykowski, 1996). The present results show that SSB protein can sequester ssDNA bound to the secondary site and thereby alleviate the inhibition due to ssDNA. The stimulatory role of SSB protein on DNA strand exchange *in vitro* has been thoroughly documented (McEntee *et al*., 1980; Shibata *et al*., 1980; Cox and Lehman, 1982; Kahn and Radding, 1984; Kowalczykowski and Krupp, 1987; Lavery and Kowalczykowski, 1992). Most significantly, SSB protein stimulates DNA strand exchange under sub-optimal conditions when ssDNA is present in excess over RecA protein (McEntee *et al*., 1980), i.e. under conditions when we observe saturation of the secondary site of the RecA nucleoprotein filament with ssDNA. While SSB protein certainly has several stimulatory functions in DNA strand exchange, including disruption of ssDNA secondary structures to promote RecA protein binding, our data demonstrate that the sequestration of ssDNA by SSB protein, preventing binding to the secondary site, can alone significantly stimulate DNA strand exchange. Again, these results, obtained with oligonucleotide substrates in the presence of ATP-γ-S, are consistent with the results obtained on long DNA substrates

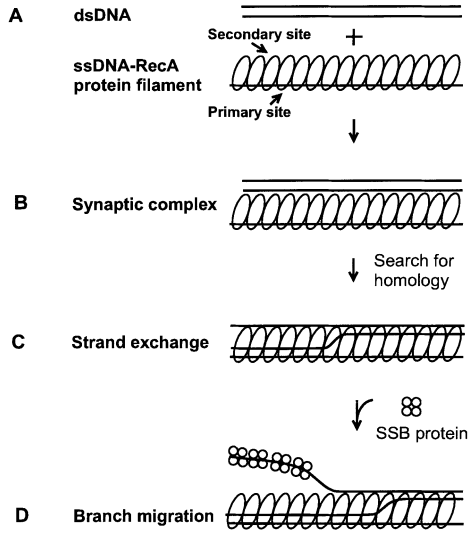

Fig. 8. Functions of the DNA-binding sites of RecA protein in DNA strand exchange. RecA protein binds ssDNA in the primary site and forms a RecA–ssDNA filament (**A**). As part of homology search process, this nucleoprotein filament binds dsDNA, forming a synaptic complex upon homologous alignment (**B**). In the synaptic complex, dsDNA is weakly bound to the secondary site of the RecA nucleoprotein filament. Local strand switching occurring in the synaptic complex generates the displaced DNA strand that binds to the secondary site with high affinity and stabilizes the nascent heteroduplex DNA (**C**). SSB protein abstracts the displaced DNA strand from the post-synaptic complex and renders the reaction essentially irreversible (**D**).

in the presence of ATP, validating the generality of these conclusions.

Previously, it was shown that with an excess of ssDNA, RecA protein forms aggregates which dissociate in the presence of SSB (Tsang *et al*., 1984). Our results suggest that the aggregation is caused by the saturation of the secondary site of the RecA nucleoprotein filament with ssDNA which, apparently in the case of long ssDNA, is capable of binding to the secondary sites of several different nucleoprotein filaments. SSB protein removes ssDNA from the secondary site and at the same time eliminates the aggregates.

Function of DNA-binding sites during DNA strand exchange

A model for homologous pairing and DNA strand exchange catalyzed by RecA protein, proposed by Howard-Flanders and co-workers (1984), points to two DNA-binding sites within the RecA nucleoprotein filament as essential: the primary site which binds ssDNA during filament formation and the secondary site which binds the dsDNA partner. Our study, whose aim was to examine the properties of the secondary binding site of the RecA protein, allows us to augment this model with some specific features. The salient feature of the secondary site is its dual affinity for ss and dsDNA. Our data demonstrate that the secondary site is involved in several important steps of DNA strand exchange (Figure 8). At first, this site weakly binds heterologous dsDNA as a part of the homology search process. When homology is found, pairing occurs and leads to the formation of both heteroduplex DNA and displaced ssDNA. Since the secondary site has a higher affinity for ssDNA than for dsDNA, it binds the displaced strand tightly, stabilizing the initially unstable heteroduplex. In accord with this model, we localized the displaced DNA strand in the secondary binding site of the post-exchange complex. There are a number of reports indicating that the heteroduplex product is found in the primary site of the filament after DNA strand exchange (Chow *et al*., 1986; Pugh and Cox, 1987; Rosselli and Stasiak, 1990; Ullsperger and Cox, 1995). The model described above allows the SSB protein to fulfill its stimulatory role in DNA strand exchange in at least two different ways. In the first, SSB protein can sequester the excess ssDNA which blocks DNA strand exchange by competing with dsDNA for the binding to the secondary site. In the second role, SSB protein can abstract the displaced ssDNA strand initially located in the secondary site of post-exchange complexes. Such abstraction can provide additional momentum for the extension of joint molecules and prevent the reversal of DNA strand exchange.

Materials and methods

Proteins and DNA

RecA protein was purified from strain JC12772 using a procedure based on precipitation by spermidine acetate (S.C.Kowalczykowski, unpublished observations). SSB protein was purified as described (LeBowitz, 1985). Oligodeoxyribonucleotides were prepared as described (Mazin and Kowalczykowski, 1996). The concentrations of the oligonucleotides were determined spectrophotometrically using the extinction coefficient ε_{260} = 9833/M/cm (1 OD_{260} = 33 μg/ml). DNA concentrations are expressed in moles of nucleotides. Oligonucleotide labeling and dsDNA preparations were as described (Sambrook *et al*., 1989). Oligonucleotides were stored at –20°C.

Binding of ssDNA to the RecA nucleoprotein filament

RecA nucleoprotein filaments were formed by incubation of ssDNA with RecA protein in a standard buffer containing 33 mM HEPES (pH 7.0), 1.2 mM magnesium acetate, 2 mM dithiothreitol, 100 μg bovine serum albumin and 1 mM ATP-γ-S at 37°C for 12 min, followed by an increase in the concentration of magnesium acetate to 15 mM (magnesium shift) and by incubation for 4 min. To bind ssDNA to the secondary site, the RecA protein–ssDNA filament was incubated with a new aliquot of oligonucleotide for 6 min. Alternatively, both sites of RecA nucleoprotein filament were saturated by incubation of RecA protein with an excess of ssDNA in the standard buffer. In the latter case, incubation was continued after the magnesium shift for 10 min. Where indicated, complexes were challenged with unlabeled ssDNA. Complexes were detected by a shift of mobility after electrophoresis in a 10% polyacrylamide gel (90 mM Tris-borate pH 8.3, and 0.5 mM EDTA) gels. Complex formation was quantitated using a Storm 840 PhosphorImager (Molecular Dynamics).

DNA strand exchange

The RecA nucleoprotein filament was formed by incubation of RecA protein with ssDNA in the standard buffer at 37°C for 12 min followed by an increase in the concentration of magnesium acetate to 11.2 mM, and by an additional incubation for 4 min. Where indicated, the secondary site of the filament was saturated by the addition of ssDNA competitor followed by a 6 min incubation. Where appropriate, SSB protein was added at the indicated concentration and incubation was continued for 15 min. DNA strand exchange was initiated by the addition of homologous dsDNA. Aliquots were withdrawn from the reaction mixture and were either mixed with a one-tenth volume of loading buffer (20% Ficoll, 0.1% bromophenol blue) and directly loaded on a 10% polyacrylamide gel, or were deproteinized prior to gel electrophoresis by the addition of EDTA to 50 mM, SDS to 1% and proteinase K to 500 μg/ml followed by incubation for 5 min at 37°C.

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