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The synergistic interaction between RecA protein and SSB protein during DNA strand exchange*

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ABSTRACT

RecA protein catalyzes DNA strand exchange, a basic step of homologous recombination. RecA protein possesses two DNA binding sites. During DNA strand exchange, the primary site binds to ssDNA, forming the helical RecA nucleoprotein filament. The weaker secondary site binds dsDNA during the homology search process. Surprisingly, the secondary DNA binding site displays a higher affinity for ssDNA than for dsDNA. Binding of ssDNA to this secondary site shows base compositional specificity, in that polypyrimidinic DNA binds to the RecA protein-ssDNA filament with higher affinity than polypurinic sequences. We found that this specificity reflects an intrinsic property of the secondary site of RecA protein rather than an interaction between DNA molecules within nucleoprotein fila-

ment, i.e., self-recognition. 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 SSB 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.

INTRODUCTION

Recombination between homologous DNA is catalyzed by the ubiquitous family of RecA-like proteins. The members of this family are conserved among Bacteria, Eucarya and Archaea (Dunderdale and West, 1994; Kowalczykowski and Eggleston, 1994; Radding, 1991; Roca and Cox, 1990). *In vitro*, RecA protein promotes DNA strand exchange, a central step of homologous recombination. RecA protein alone can carry out DNA strand exchange, but the single-stranded DNA binding protein (SSB) greatly stimulates the reaction (Cox and Lehman, 1982; Kowalczykowski and Krupp, 1987; Muniyappa et al., 1984).

DNA strand exchange is a multi-step process that initiates with the binding of RecA protein to single-stranded DNA (ssDNA), resulting in formation of a helical nucleoprotein filament. Conventionally, the RecA protein binding site responsible for this DNA binding is referred to as the "primary" site. RecA protein can assemble on either ssDNA or dsDNA to a form helical filament (McEntee et al., 1981; Pugh and Cox, 1988); however, ssDNA is the preferred and likely biologically important substrate for RecA protein (Kowalczykowski and Eggleston, 1994).

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 (Muller et al., 1990; Takahashi et al., 1989); the resultant complexes

hardly survive gel electrophoresis (Muller et al., 1990) and can be detected only by sensitive, thought indirect assays (Chow and Radding, 1985; Conley and West, 1990; Tsang et al., 1985). The RecA nucleoprotein filament can also bind non-homologous ssDNA (Menetski and Kowalczykowski, 1987; Muller et al., 1990; Takahashi et al., 1989; 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 (Mazin and Kowalczykowski, 1996; Muller et al., 1990; Rao and Radding, 1994; Rao and Radding, 1993). It had been suggested that the presynaptic filament, with ssDNA bound to the primary site, has a *secondary* site; this site binds dsDNA non-specifically during the search for DNA sequence homology (Howard-Flanders et al., 1984).

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).

Despite rather extensive characterization, the precise nature and function of the secondary DNA binding site remains unknown. Recently, Rao and Radding (Rao and Radding, 1994; Rao and Radding, 1993) showed that various ssDNA molecules interact with the RecA protein-ssDNA filament with different affinities. Unexpectedly, they found that an oligonucleotide identical in the sequence to ssDNA within nucleoprotein filament, displayed a higher affinity to the

filament than any heterologous oligonucleotide examined. They suggested that the observed binding specificity is determined by non-Watson-Crick bonds that are formed between ssDNA in the primary site and ssDNA bound to the nucleoprotein filament, so-called "self-recognition," as a part of a homology search process. Consequently, we sought to determine its role in DNA strand exchange and the basis for the apparently novel sequence-specificity attributed to this site. We have established that the specificity of ssDNA binding to a RecA protein-ssDNA filament is determined by an intrinsic property of the RecA protein secondary site rather than by self-recognition.

We demonstrate that the secondary site binds both ss- and dsDNA, but has a higher affinity for ssDNA. The high affinity of the secondary site for ssDNA has two important implications for the function of this site during DNA strand exchange. *First,* it suggests a dual role of the secondary site in DNA strand exchange: this site binds the incoming dsDNA weakly as part of the homology search process and then, upon finding local homology, it binds the resulting displaced ssDNA strand with a higher affinity. Binding of the displaced DNA strand ensures both that homologously aligned complexes are recognized due to stabilization of the strand-exchanged complex, and that the direction of DNA strand exchange is biased towards DNA heteroduplex formation. *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 the secondary site did indeed bind the displaced DNA strand following DNA strand exchange. Therefore, in addition to its role of dsDNA binding during the homology search, the secondary DNA site provides the 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.

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 (Table 1) were synthesized using a Milligene DNA synthesizer (Millipore) and were purified by electrophoresis in 8-10% polyacrylamide gels containing 8M urea. Annealing and 5'-end labeling of oligonucleotides were as described (Sambrook et al., 1989). The concentrations of the oligonucleotides were determined spectrophotometrically using the extinction coefficient ε_{260} = 9833 $M^{-1} \cdot cm^{-1}$ (1 OD_{260} = 33 mg/ml). DNA concentrations are expressed in moles of nucleotides. Oligonucleotides were stored at -20°C.

Table 1. List of the oligodeoxyribonucleotides used in this study

N, length	Sequence
#1, 63-mer	ACAGCACCAGATTCAGCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAAAGGA
#2, 63-mer	TCCTTTTGATAAGAGGTCATTTTTGCGGATGGCTTAGAGCTTAATTGCTGAATCTGGTGCTGT
#5 (ident), 32-mer	CCATCCGCAAAAATGACCTCTTATCAAAAGGA
#7, 32-mer	GGCGGGCGGCGCGGCCGGGCGGCGGGCGCGCG
#19, (C→A), 32-mer	AAATAAGAAAAAATGAAATATTATAAAAAGGA
#21 (A→T), 32-mer	CCTTCCGCTTTTTTGTCCTCTTTTCTTTTGGT
#25, 48-mer	GCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAAAGGA
#26, 48-mer	TCCTTTTGATAAGAGGTCATTTTTGCGGATGGCTTAGAGCTTAATTGC
#39BT, 32-mer	GCGTGTGTGGTGGTGTGCGCGTGTGTGGTGGT
#40BT, 32-mer	ACCACCACACACGCGCACACCACCACACACGC
#41, 63-mer	TCTGCTCCTGTTTCTGCTTTTTTGCTCTTTGCCTTCCGCTTTTTTGTCCTCTTTTCTTTTGGT
#71, 94-mer	CTTTAGCTGCATATTTACAACATGTTGACCTACAGCACCAGATTCAGCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAAAGGA
"G"*, 63-mer	ACAGCACCAGATTCAGCAATTAAGCTCTAAG<u>GGCGGGCGGCGCGGCCGGGCGGCGGGCGCGCG</u>

"A"[*], 63-mer	ACAGCACCAGATTCAGCAATTAAGCTCTAAG<u>AATAAATAATATAATTAAATAATAAATATATA</u>
"M"[*], 63-mer	ACAGCACC<u>AATGAAATCTATT</u>AAGCTC<u>CTCATCGTCCGCAAAAATATCGTCACC</u>TCAAAAGGA

Table 1. *continued*

N, length	**Sequence**
htr#1, 32-mer	GTGAGTCGACAAGCCTGACTCAACATTATCCT
htr#2, 32-mer	AATTCTTCGAAGCTAGCCCTCAGGCCTAGGCA
htr#3, 33-mer	TTCACAAACGAATGGATCCTCATTAAAGCCAGA
htr#4, 33-mer	CATGGAGCAGGTCGCGGATTTCGACACAATTTA

*Sequences of these oligonucleotides were derived from the sequence of the oligonucleotide #1, the underlined sequences indicate the regions that deviate from the oligonucleotide #1 sequence.

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 DTT, 100 µg/ml bovine serum albumin, and 1 mM ATPγS at 37°C for 12 minutes, followed by an increase in the concentration of magnesium acetate to 15 mM (magnesium shift) and by incubation for 4 minutes. To bind ssDNA to the secondary site, the RecA protein-ssDNA filament was incubated with a new aliquot of oligonucleotide for 6 minutes. 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 minutes. 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 minutes followed by an increase in the concentration of magnesium acetate to 11.2 mM, and by an additional incubation for 4 minutes. 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 either were mixed with a $^1/_{10}$ 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.

RESULTS

Stoichiometry of ssDNA binding to the RecA nucleoprotein filament

As described in the Introduction, the RecA nucleoprotein filament binds ssDNA molecules. If this binding occurs at a specific DNA binding site within the RecA nucleoprotein filament it should display a defined stoichiometry. Therefore, we sought to determine this stoichiometry and further characterize the function of the RecA protein site responsible for this binding.

When RecA protein, at the indicated concentrations, is incubated with an excess of a ^{32}P-labeled 32-mer oligonucleotide (#5), protein-ssDNA complexes are detected by virtue of their retarded mobility in a 10% polyacrylamide gel (Fig. 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 (Fig 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 5 nucleotides per RecA protein monomer.

When RecA-ssDNA complexes are formed, and then incubated with an excess of a challenge 32-mer oligonucleotide (#21; referred to as "A → T") which displays a very high affinity to the RecA nucleoprotein filament (see in the next sections Fig. 3 and table 2), half of the labeled ssDNA is displaced; importantly, 42-50% of labeled DNA remains bound at all concentrations of challenge ssDNA (Fig. 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 there exist two distinct species of protein-bound ssDNA 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 sites of
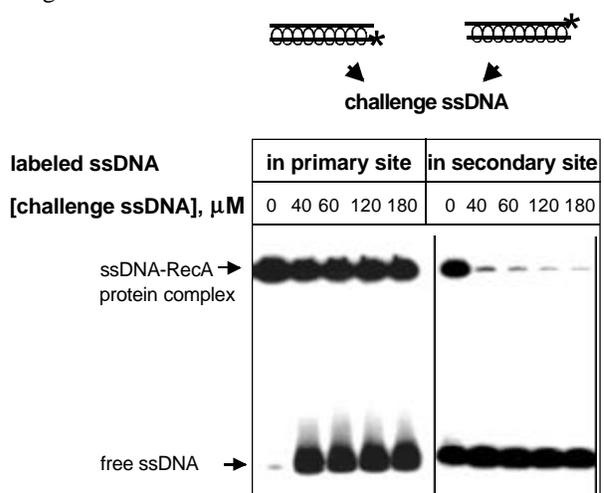
A

[ssDNA competitor], µM

0.75   1.5   3.0   4.0

[RecA protein], µM

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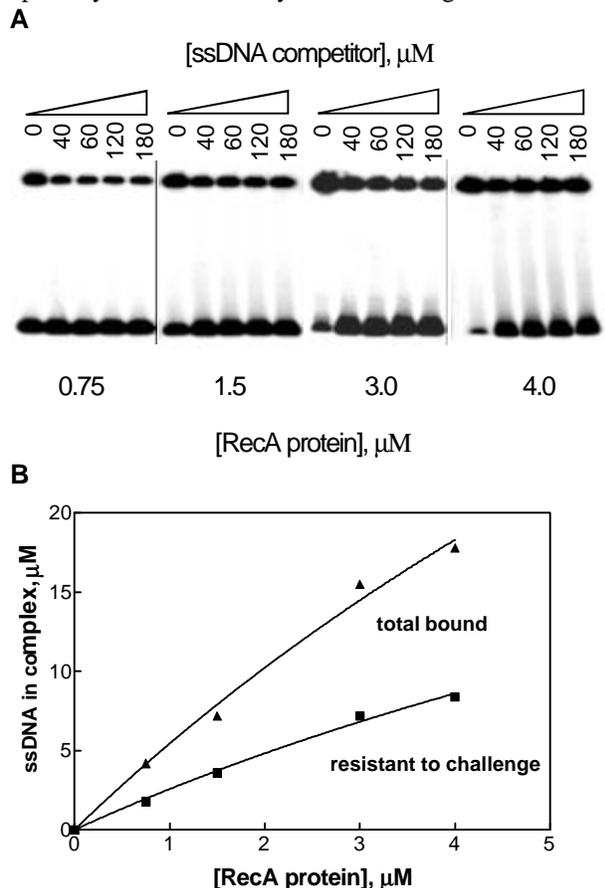
Fig. 1. Stoichiometry of RecA protein binding to ssDNA. RecA protein, at the indicated concentrations, was mixed with a ^{32}P-labeled 32-mer oligonucleotide (#5) (24 µM nucleotides) in the standard buffer. The resultant RecA protein-ssDNA filaments were either directly loaded on a 10% polyacrylamide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

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 approximately 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 Fig. 1 can be attributed to the presence of two unique saturable DNA binding sites, then the site which binds ssDNA with the higher 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 32-mer oligonucleotide (#5) at the stoichiometric ratio of 1 RecA protein monomer per 3 nucleotides of ssDNA. Based on the data in Fig.1, this concentration of ssDNA would be in slight excess (20%) of 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 32-mer (#21). As shown in the Fig. 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 32-mer 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.

challenge ssDNA

labeled ssDNA	in primary site					in secondary site				
[challenge ssDNA], µM	0	40	60	120	180	0	40	60	120	180

ssDNA-RecA protein complex →

free ssDNA →

Fig. 2. The existence of two non-equivalent DNA binding sites within the RecA nucleoprotein filament. RecA protein (4 µM) was mixed with the 32-mer 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 32-mer oligonucleotide (#21). Complexes were analyzed by electrophoresis in a 10% polyacrylamide gel.

In the second set of experiments, complexes were formed just as in the first series, except the order of addition of labeled and unlabeled oligonucleotide was inverted: 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 (Fig. 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, the primary and the secondary, in the RecA nucleoprotein filament which bind equal amounts of ssDNA, but show different affinities in this binding.

The secondary site shows base-compositional specificity of DNA binding

RecA nucleoprotein filaments are reported to preferentially bind ssDNA with a sequence identical to that bound in the primary site (Rao and Radding, 1993) certain modifications in the sequence of this "identical" oligonucleotide prevented binding, whereas others did not (Rao and Radding, 1994). To further understand the nature and the functional importance of this binding, we quantified the effect of base replacement and sequence heterogeneity on the efficiency of DNA binding to the secondary RecA protein site. RecA protein-ssDNA filaments were assembled on a single-stranded 63-mer and their affinity for a

The extent of complex formation showed a distinct hierarchy and suggested that polypyrimidinic oligonucleotides bound more efficiently. Oligo dC and the modified 32-mer oligonucleotide, in which all thirteen A residues were replaced with T (A→T), bound to the RecA protein-ssDNA filament with the highest yield (Fig. 3A). Oligonucleotides enriched with adenines were among the least efficient: oligo dA and the modified oligonucleotide, in which all nine C residues were replaced by A (C→A), showed the lowest level of binding. Similarly, GC-rich oligonucleotides (#7) show poor affinity for the secondary site (Table 2). In part, our data are in agreement with the base substitution experiments of Rao and Radding (Rao and Rad-

variety of labeled oligonucleotides, either 32- or 33-nucleotides in length, was examined (Figure 3A). A complex of RecA protein, primary ssDNA, and secondary ssDNA (referred to simply as "complex" hereafter) was detected for all oligomers except oligo dA.

ding, 1994): replacement of A residues with T residues did not prevent binding to the RecA protein-ssDNA filament, whereas replacement of C residues with A residues diminished binding. The data also show that oligonucleotide (#5), whose sequence is identical to
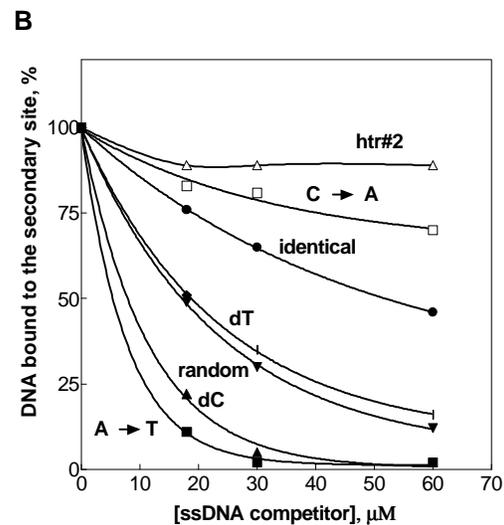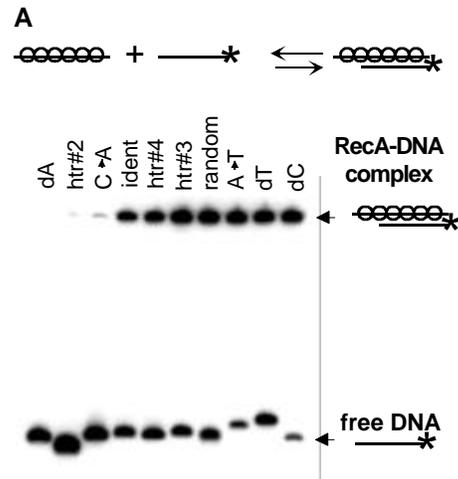
Fig. 3. Specificity of ssDNA binding to the RecA secondary site. The RecA protein-ssDNA filament was formed using a 63-mer oligonucleotide (#1) (Table 1) as described in the Materials and Methods. A) This filament was incubated with equimolar (molecule) amounts (6 µM nucleotides) of ^{32}P-labeled different 32 or 33-mer oligonucleotides in the presence of heterologous ssDNA (htr#1) (120 µM) and the resulting complex formation was analyzed by gel electrophoresis and subsequent autoradiography. B) This RecA nucleoprotein filament was incubated with equimolar (molecule) amounts (6 µM, nucleotides) of a ^{32}P-labeled 32-mer oligonucleotide (#5) which is identical in sequence to the 3'- end of oligonucleotide #1. This was followed by a challenge for 6 minutes with the concentrations of unlabeled ssDNA

6

indicated. The amount of labeled DNA remaining in the complex was determined by electrophoresis, and is plotted. The oligonucleotides designated "C → A" and "A→ T" were 32-mers derived from oligonucleotide #5 by replacement of all C residues by A residues and all A residues by T residues, respectively, and dA, dT, and dC are homo-oligomeric 32-mers.

part of the 63-mer bound to the primary site, was not the tightest-binding ligand (Fig. 3A, lane 4, labeled "ident"; Table 2, labeled "ident"); it bound to the secondary site with an intermediate affinity which is comparable to that of a random 32-mer, synthesized using an equimolar mixture of each nucleotide at every position. (Fig. 3A, lane 7, labeled "random"). We also observed a large variation in affinity of the filament for different oligonucleotides of mixed base composition, fortuitously chosen, which had no homology to the resident 63-mer oligonucleotide. Heterologous oligonucleotide #2 (htr#2) displayed a low affinity, while other heterologous oligonucleotides (htr#3 and htr#4) bound to the RecA protein-ssDNA filament with an efficiency higher than that of the identical 32-mer (Fig. 3A). This difference in the affinity is not explained simply by differences in the content of pyrimidines/purines but may, instead, reflect a distinct bias of RecA protein for oligonucleotides of a particular nucleotide composition (Tracy and Kowalczykowski, 1996).

Table 2. Sequence-specificity in ssDNA binding to the RecA secondary site.

Oligonucleotide	complex, %
#40BT	82.0
#39BT	71.0
A→T	71.0
ident	51.0
#7 (GC-rich)	7.0
C→A	5.0

We also tested the affinity for the secondary site of an oligonucleotide enriched with T and G residues. Previously, using SELEX protocol it was established that RecA protein shows preferential affinity for GTT-rich sequences (Tracy and Kowalczykowski, 1996). However, SELEX, a technique based on several sequential cycles of RecA protein-ssDNA filter binding and PCR amplification of bound DNA, does not allow to determine in which of the RecA protein site DNA binding occurs. Using gel-retardation assay, we found here that a GTT-rich oligonucleotide ("#39BT") binds to the secondary site very efficiently (Table 2). Its complement, CCA-rich sequence (#40BT), shows even higher affinity for the secondary site (Table 2).

There is a hierarchy of affinities for binding to the RecA protein-ssDNA filament

To further examine the relative DNA binding affinities of the RecA nucleoprotein filament, we performed competition experiments. Complexes formed between a RecA protein assembled on a 63-mer oligonucleotide filament and an identical ^{32}P-labeled 32-mer oligonucleotide were challenged with unlabeled single-stranded oligonucleotides of different sequences, but of the same length (32-mers). The residual complexes were analyzed by gel electrophoresis. Since the half-time for exchange is less than 1 minute (data not shown), the 6 minute incubation ensures that equilibrium is achieved. The ability of various oligonucleotides to replace the oligonucleotide that is bound to the secondary site directly correlates with their extent of complex formation seen in Fig. 3A: oligopyrimidinic oligonucleotides were the most efficient competitors and the oligonucleotide in which the identical sequence was modified with A→T replacements was also an efficient competitor (Fig. 3B). By contrast, oligonucleotides enriched for adenines, such as the 32-mer with C→A replacements in the original identical sequence, were poor competitors.

As expected, and characteristic of a difference between primary site binding and secondary site binding, the 32-mer oligonucleotide bound to the primary site was resistant to increasing concentrations of competitor (Mazin & Kowalczykowski, unpublished observations). This behavior is typical for complexes formed with ATPγS (Menetski and Kowalczykowski, 1987).

The sequence of the DNA molecule bound to the primary site of RecA protein does not determine the specificity of ssDNA binding to the secondary site
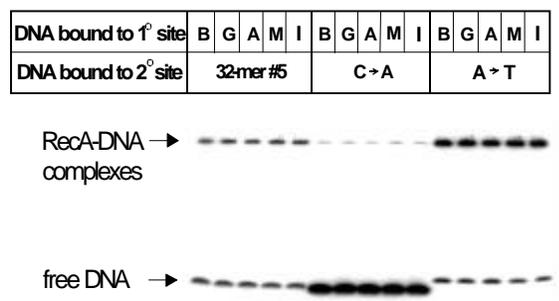
We found that the oligonucleotide whose sequence is identical to that bound in the primary site is a rather average ligand for binding to the RecA nucleoprotein filament. This finding led us to question whether self-recognition between the first and the second DNA strands is responsible for the observed specificity of ssDNA binding.

To address this question, we formed presynaptic filaments with four different 63-mer oligonucleotides bound to the primary site of RecA protein. The first one (#1; labeled "I" in Fig. 4), homologous to M13, was the same oligonucleotide that we used in the previous experiments (Figure 3); the second one (labeled "M") contained three patches of heterologous DNA, 9 nucleotides each, resulting in a mosaic of homology; and finally, in both the third (labeled "G") and the fourth (labeled "A"), the 3'-region of the 63-mer that

is identical in sequence to the 32-mer was replaced with G-rich or A-rich sequences, respectively. In the fifth experiment, the 63-mer was omitted (labeled "B" for blank), and RecA protein was mixed with heterologous 32-mer oligonucleotide (htr#1). Each of these nucleoprotein filaments was then incubated with three different ^{32}P-labeled 32-mer oligonucleotides: one that is identical to 32 nucleotides of the 63-mer oligonucleotide #1 ("M13, #5"); a second in which all of the A residues were substituted by T residues ("A→T"); and a third with C residues substituted by A residues ("C→A").

The results show that each of the five different presynaptic filaments displays an equal extent of complex formation for each ^{32}P-labeled 32-mer oligomer, regardless of the DNA sequences that occupy the primary DNA binding site (Fig. 4). Thus, binding to the secondary site of RecA nucleoprotein filament depends only on the composition of the added oligonucleotide and not on the sequence of the DNA bound to the primary site. In agreement with the results described above (Fig. 3), the modified 32-mer with A→T substitutions showed the highest level of binding; the identical 32-mer DNA was less efficient; and the modified 32-mer with C→A substitutions showed the lowest affinity for each of the different presynaptic filaments tested (Fig. 4).

We conclude that the sequence of the DNA bound to the first site of RecA protein does not determine the binding affinity of the second DNA molecule. Consequently, the observed specificity of DNA binding to the secondary site is an intrinsic property of RecA protein.

| DNA bound to 1° site	B	G	A	M	I	B	G	A	M	I	B	G	A	M	I
DNA bound to 2° site	32-mer #5					C→A					A→T				

RecA-DNA → complexes

free DNA →

Fig. 4. The sequence of the ssDNA bound to the primary site of RecA nucleoprotein filament does not determine the specificity of ssDNA binding to the secondary site. Five different RecA protein-ssDNA filaments were formed, using four 63-mer and one 32-mer oligonucleotides. Oligonucleotide "G", oligonucleotide "A" and oligonucleotide "M" are derivatives of the 63-mer oligonucleotide #1 in which the 3' region was replaced with guanine-rich sequences (G), adenine-rich sequences (A), or mosaic patches of heterology (M), respectively. Oligonucleotide #1 is designated as "I". These nucleoprotein filaments were incubated in the presence of heterologous ssDNA (htr#1) (120 μM) with each of three different ^{32}P-labeled 32-mer oligonucleotides: 32-mer #5 (see Fig. 1) which is identical to 3'-

part of oligonucleotide #1; modified A→T (see Fig. 1) and modified C→A (see Fig. 1) and analyzed by electrophoresis. In the blank experiment (B), a 63-mer was omitted and RecA protein was mixed with heterologous 32-mer ssDNA (htr#1) (120 μM). The underlined sequences represent substitutions in the oligonucleotide #1.

Single- and double-stranded DNA compete for binding to the secondary site

We further asked whether the secondary binding site is the same site which interacts with dsDNA during the DNA strand exchange reaction. Therefore, we tested whether dsDNA can bind to the secondary binding site. Since we could not detect complex formation with dsDNA directly by gel retardation due to weak binding, the ability of dsDNA to compete with ssDNA bound to the secondary site was measured (Fig. 5).

The results show that the ssDNA oligonucleotides, particularly the shorter ones, can be displaced from the secondary site by competing heterologous dsDNA. The fact that both ssDNA and dsDNA compete for binding to the RecA protein-ssDNA filament suggests that they bind to the same, or at least, an overlapping site. This result is consistent with the suggestion that the secondary binding site functions in DNA strand exchange.
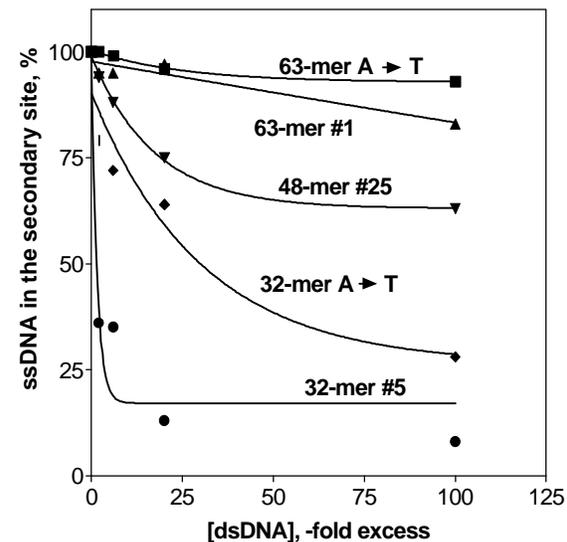
Fig. 5. Competition between ssDNA and dsDNA for secondary site binding. The RecA nucleoprotein filament was formed by incubation of 63-mer oligonucleotide (#1) (12 μM) with RecA protein (4 μM) in the absence of heterologous (htr#1) oligonucleotide. The filament was incubated for 6 minutes with an equimolar (molecule) amount of the ^{32}P-labeled ssDNA indicated. These complexes were then challenged for 15 minutes with various concentrations (nucleotides) of a *Hae*III digest of pBR322 DNA and analyzed by electrophoresis. The 48-mer oligonucleotide #25 was identical in the sequence to the 3'-end of 63-mer (#1).

Single-stranded DNA bound to the secondary site inhibits DNA strand exchange

Since ssDNA binds to the secondary site with higher affinity than dsDNA, its binding to this site should be

inhibitory for DNA strand exchange. To test this notion, DNA strand exchange between a ssDNA 63-mer (#1) and homologous dsDNA 48-mer (#25-26) was measured in either the presence or the absence of a ssDNA 63-mer containing the A → T substitutions that confer a high affinity for the secondary DNA binding site. When present, the competing ssDNA 63-mer was pre-incubated with the RecA nucleoprotein filament that was formed with the homologous 63-mer bound to the primary site. Figure 6 shows that when the competitor ssDNA was present, only a two-fold excess of such ssDNA was sufficient to almost completely block the reaction. In contrast, ssDNA with a very low affinity for the secondary site, (like htr#1), had no significant effect on DNA strand exchange. This inhibitory effect correlates with the affinity of the ssDNA bound to the secondary site: a 32-mer oligonucleotide with A → T substitutions has a smaller inhibitory effect, which could be reverted by an excess of homologous dsDNA (data not shown).

These data show that the secondary DNA binding site has a functional role in DNA strand exchange: it is involved in the binding of dsDNA, despite displaying a preferential affinity for ssDNA.
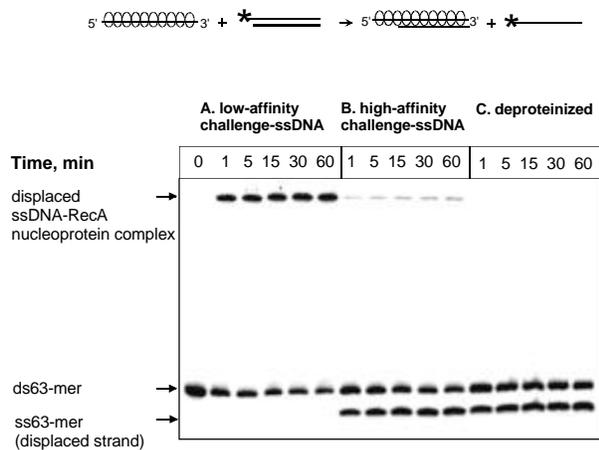
The DNA strand that is displaced during DNA strand exchange binds to the secondary site

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. 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 *vs.* the secondary site towards challenge ssDNA was used as the criterion for occupancy by the displaced strand.
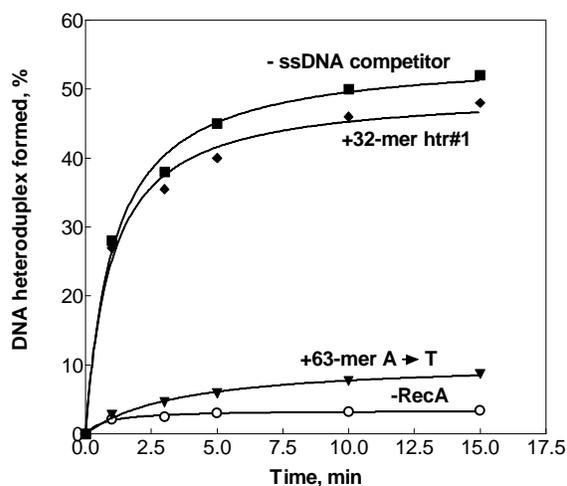
Fig. 6. Binding of ssDNA to the secondary site of the presynaptic filament inhibits DNA strange exchange. The RecA protein-ssDNA filament was formed by incubation of 63-mer oligonucleotide (#1) (12 μM) with RecA protein (4 μM). DNA strand exchange was initiated by addition of homologous 48-mer dsDNA (#25-26) in either the presence or absence a 2-fold molar excess of 63-mer oligonucleotide (A→T) (#41) or in the presence of a 2-fold molar excess of 32-mer (htr#1). The products were deproteinized with SDS-EDTA and analyzed by polyacrylamide gel electrophoresis.

To determine the location of the displaced DNA strand, DNA strand exchange was examined using a presynaptic filament formed with a 94-mer oligonucleotide (#71) and paired with homologous 63-mer dsDNA in which the strand identical in sequence to 3'-end of the 94-mer was labeled; this strand would be displaced during DNA strand exchange. The reaction products were analyzed on a polyacrylamide gel without (Fig. 7A and 7B) or with deproteinization (Fig. 7C). 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 a affinity for the secondary site (htr#1), almost all of the displaced labeled ssDNA remained in the complex with RecA protein after DNA strand exchange had occurred (Fig. 7A). 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 (Fig. 7B). 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.

Fig. 7. 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 94-mer oligonucleotide (#71) (12 µM). DNA strand exchange was initiated by addition of homologous 63-mer dsDNA (#1-2) (16 µM) in which the strand identical to the 3'-region of the 94-mer 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

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. Based on the dynamic nature of the complexes found between ssDNA and the RecA nucleoprotein secondary site, it was reasonable to expect that single-stranded DNA binding (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 48-mer oligonucleotide was bound either in the primary or the secondary site, and these complexes were challenged with increasing concentrations of SSB protein. Figure 8 shows that ssDNA bound to the secondary site was sensitive to SSB protein. At 3 µM SSB protein, almost all of ssDNA bound to the secondary site was displaced, and it migrated as a distinct band with characteristics typical for an SSB protein-ssDNA complex (Fig. 8). The stoichiometry of ssDNA binding to SSB protein was about 4 nucleotides per 1 protein mono-

mer; this represents 2.5-fold more SSB protein than required to bind the free 48-mer 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 under the experimental conditions used.

This sensitivity of ssDNA bound to the secondary site to 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 94-mer ssDNA and 63-mer 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 (Fig. 9).

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.


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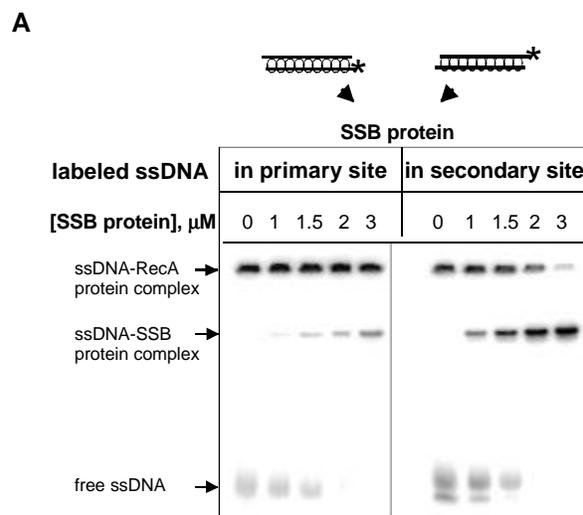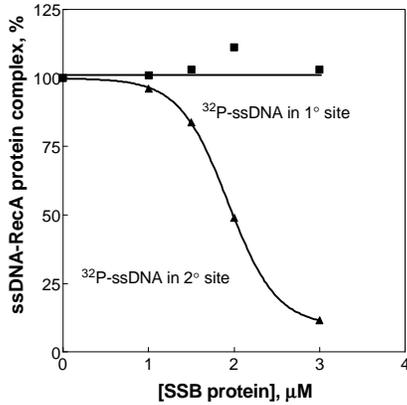
Fig. 8. The displacement of ssDNA from the primary and the secondary site of RecA by SSB protein. RecA protein (4 μM) was mixed with the 48-mer oligonucleotide (#25) (12 μM) in standard buffer. After filament formation was completed, another aliquot (12 μM) of the same oligonucleotide was added to the filament. To distinguish between these two DNA molecules, either the first (A) or the second (B) 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. Panel A shows the autoradiograph of the gel; panel B shows a quantitation of these data.

Removal of ssDNA from the secondary site by SSB 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. Here we tested whether the removal of ssDNA by SSB from the secondary site of the RecA nucleoprotein filament could restore DNA strand exchange.

DNA strand exchange was initiated between homologous double-stranded 48-mer oligonucleotide, and nucleoprotein filaments formed on a 63-mer single-stranded oligonucleotide either in the presence, or absence, of a 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. Fig. 10 shows that 3-fold excess ssDNA nearly completely inhibited DNA strand exchange in the absence of SSB ("+ssDNA, 0 μM SSB" *vs* "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 with respect to the ssDNA-inhibitor completely restored DNA strand exchange (Fig. 10).
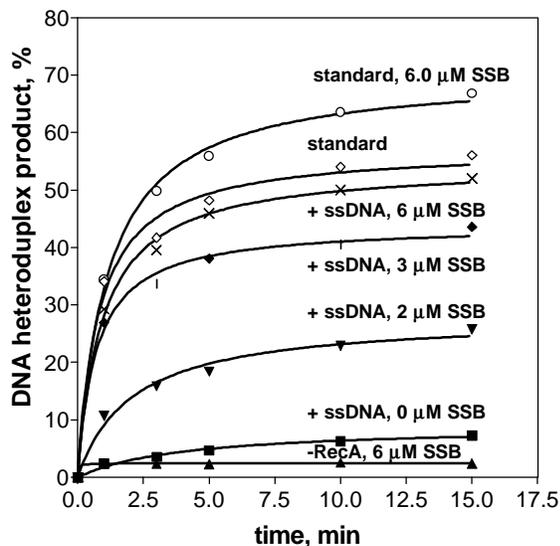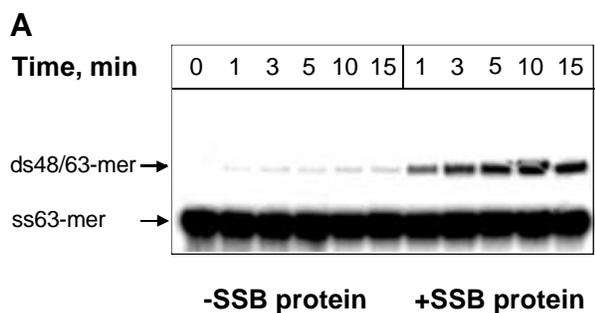
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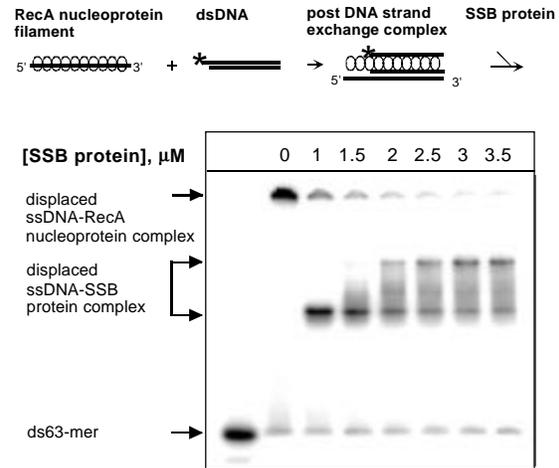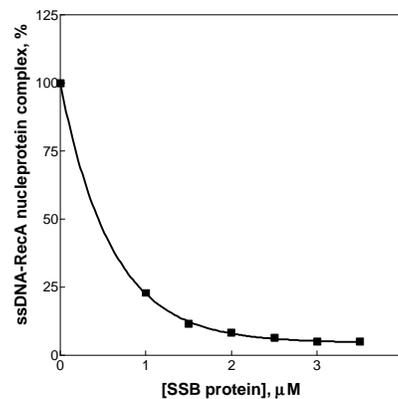
Fig. 9. SSB protein removes the displaced DNA strand from the secondary site of RecA protein. Nucleoprotein filaments were formed by incubation of RecA protein (4 μM) with the 94-mer oligonucleotide (#71) (12 μM). DNA strand exchange was initiated by the addition of homologous 63-mer dsDNA (#1-2) (16 μM) in which the strand identical to the 3'-region of the 94-mer was labeled. After the indicated periods of time, aliquots from the reaction mixture were withdrawn and either directly loaded on a 10% polyacrylamide gel (lane marked "0"); or prior to the loading challenged with SSB protein at the concentrations indicated. Panel A shows the radiograph of the gel; panel B shows quantitation of these data.

Fig. 10. 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 63-mer 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 48-mer dsDNA (#25-26) (18 µM) in which the strand (#26) complementary to the 3'-region of the 63-mer was labeled. After 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.

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 (Fig. 3); this excess ssDNA is inhibitory to DNA strand exchange (McEntee et al., 1980). In this case, we may also expect a significant stimulatory affect of SSB on DNA strand exchange. Indeed, an excess of homologous ssDNA (#1) (12 nucleotides per 1 RecA protein monomer) significantly inhibited DNA strand exchange (Fig. 11). However, SSB protein in a sufficient amount restored DNA strand exchange (Fig. 11).

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 preformed 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.
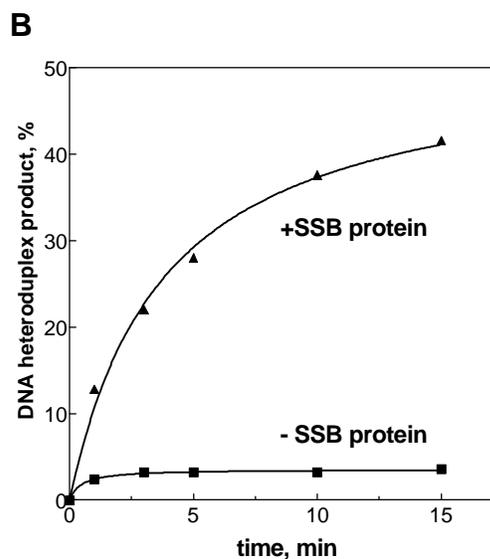
Fig. 11. SSB protein stimulates DNA strand exchange when an excess of ssDNA is present. Nucleoprotein filaments were formed by incubation of RecA protein (4 µM) with the ^{32}P-labeled 63-mer oligonucleotide (#1) (48 µM) in standard buffer. The mixture was split in two and either SSB protein (6 µM) or equivalent amount of SSB storage buffer was added to the reaction mixtures. DNA strand exchange was initiated by the addition of homologous 48-mer 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. Panel A shows the radiograph of the gel. Panel B shows quantitation of these data; percent (%) of DNA heteroduplex product was expressed relative to the initial concentration of dsDNA substrate.

DISCUSSION

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

Stoichiometry and sequence specificity of ssDNA binding to the secondary site

The DNA binding experiments described here reveal the presence of two DNA binding sites within 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. This 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 about 2.5 nucleotides per RecA protein monomer. The result is consistent with the ssDNA binding stoichiometries obtained in the presence of ATPγS that were determined using different methods (Bryant et al., 1985; Takahashi et al., 1989).

The results presented here show that binding of ssDNA to the RecA protein presynaptic filament displays a pronounced hierarchy: oligopyrimidinic DNA binds to the RecA nucleoprotein filament best, while oligoadenylic DNA binds poorly. We confirmed the observations of Rao and Radding (Rao and Radding, 1994) that base replacements in the single-stranded oligonucleotides have significant effects on the binding affinity. Specifically, A→T replacements enhanced, while others, like C→A replacements, prevented binding to the filament. However, we did not find a requirement for sequence identity between the ssDNA within the nucleoprotein filament and the second ssDNA molecule. Furthermore, the random 32-mer oligonucleotides bound to the RecA protein filament as well as the identical oligonucleotide. We also tested a number of different heterologous oligonucleotides. Some of them bound to the RecA protein filament with an affinity lower than that of the identical oligonucleotide, whereas others bound with a similar or higher affinity. We found also that an oligonucleotide enriched with TGG-trinucleotide (#39BT) binds to the secondary site with high efficiency. Previously, this oligonucleotide was selected using SELEX protocol as the most potent ligand for RecA protein (Tracy and Kowalczykowski, 1996). The complement of the #39BT, #40BT showed the highest overall affinity for the secondary site in all series of our experiments. The SELEX protocol did not allow to distinguish in which of the RecA protein sites the binding actually occurs. Since the SELEX did not detect sequences enriched with CCA-trinucleotide we believe that it shows predominantly DNA binding to the primary site. The fact, that the selected TGG-rich oligonucleotide was also among the strongest ligands for the secondary site may indicate that the base compositional preferences of the primary and the secondary DNA binding sites are overlapped. The rules which govern these preferences remain to be established, but appear to be an intrinsic property of RecA protein.

An important question was whether the ssDNA bound to the primary site of RecA protein affects the observed specificity of binding for the second DNA molecule. Previously, it was suggested that non-Watson-Crick bonds between the first and the second DNA molecules within the RecA nucleoprotein filament are essential for the self-recognition of ssDNA (Rao and Radding, 1994; Rao and Radding, 1993). Our results show that the DNA sequence within the primary site of the RecA protein filament has no effect on binding of ssDNA to the secondary site. This apparently contradicts the conclusion of Rao and Radding. However, as far as we can see from the experimental results of these authors, the replacement of all T residues by C residues in the homologous part of their 83-mer oligonucleotide within RecA nucleoprotein filament had no visible effect on the specificity of ssDNA binding [compare Fig. 1 and Fig. 4 in (Rao and Radding, 1994)]; this result is consistent with our data. We conclude that an intrinsic property of RecA protein, rather than non-Watson-Crick bonding between two DNA molecules, determines specificity of ssDNA binding to the secondary site.

The secondary site binds the displaced DNA strand

What is the function of the site responsible for binding of the second DNA molecule? It almost certainly plays a role during the pairing phase of DNA strand exchange, by binding dsDNA during the search process. This interaction with non-homologous DNA is both weak and necessarily transient, characteristics which ensure a rapid homology search. But given these attributes, how then is homologous alignment recognized? We suggest that the secondary binding site plays an additional role in homologous recognition, but that this function is at the post-pairing step of DNA strand exchange. Given the properties of the secondary DNA binding site described here, we suggest that the displaced DNA strand is transiently bound to this site within the nucleoprotein filament. This displaced strand is produced only upon homologous alignment and exchange of DNA strands, and thereby serves as a *de facto* signal of homologous recognition. Since the affinity of the secondary site is significantly greater for ssDNA than for dsDNA, this affinity bias provides a means for stabilizing productively paired homologs. Thus, the binding of the displaced strand to the secondary site may serve the important function of stabilizing the nascent short DNA heteroduplexes formed between a resident ssDNA and the complementary strand of an incoming double-stranded DNA.

We, indeed, demonstrate 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 using different DNA substrates and different methods. Chemical modification experiments show that after DNA strand exchange, the identical strand of the homoduplex is displaced and is single-stranded in behavior (Adzuma, 1992). Furthermore, experiments involving cross-linking reagent (Podyminogin et al., 1995; Zhou and Adzuma, 1997) show that, the displaced strand remains associated with the RecA protein-DNA filament after DNA strands have exchanged. Similarly, electron microscopic observations with phage DNA in the presence of hydrolyzable ATP 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 rapidly removed 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 rationalize 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. 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 likely represents the product of DNA strand exchange) (Ullsperger and Cox, 1995), could still bind a stoichiometric amount of ssDNA (Muller et al., 1990; Takahashi et al., 1989). 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. A prominent length dependence displayed by the secondary site in binding to ssDNA insures that random short homologies present in the genome would not impair the search for homology. This binding efficiency decreases rapidly for oligonucleotides shorter than 63 bases, thus providing a rationale for the minimal homology length required for productive RecA protein-dependent recombination *in vivo* (Watt *et al,* 1985).

Sequestration by SSB protein of the DNA strand displaced during DNA strand exchange
We demonstrate here that ssDNA competes with dsDNA for binding to the secondary site and that it inhibits DNA strand exchange. Our results also 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 (Cox and Lehman, 1982; Kahn and Radding, 1984; Kowalczykowski and Krupp, 1987; Lavery and Kowalczykowski, 1992; McEntee et al., 1980; Shibata et al., 1980). 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 ATPγS are consistent with the results obtained on long DNA substrates 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., 1985). 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 (Howard-Flanders et al., 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, which was aimed towards examining 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 (Fig. 12). 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). Recently, several other scenarios of DNA strand switching have been discussed in literature. For instance, the heteroduplex DNA product could be transferred into the secondary site leaving the primary site vacant for the occupation by the displaced DNA strand. Thus, our data speak against this and several other conceivable patterns of DNA strand switching. Our model 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 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.

Fig. 12. Functions for 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 post-synaptic complex and renders the reaction essentially irreversible (D).
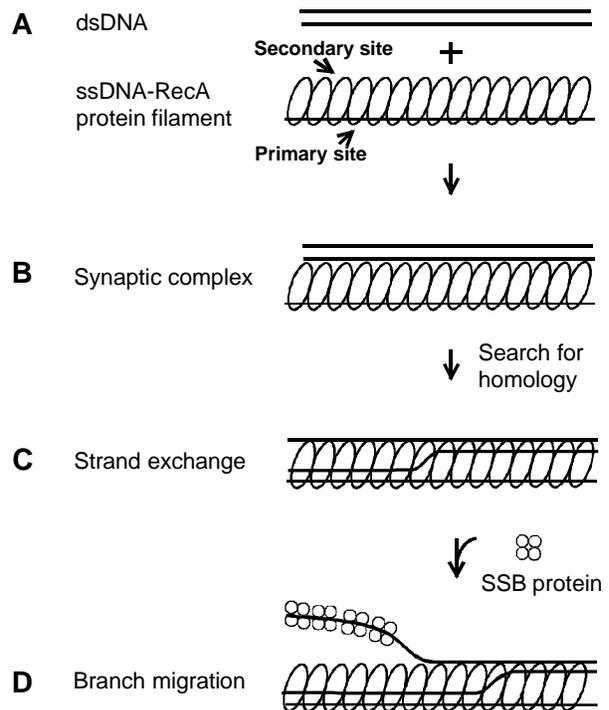
ACKNOWLEDGMENTS

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