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The specificity of the secondary DNA binding site of RecA protein defines its role in DNA strand exchange

(genetic recombination/homologous pairing/DNA–protein interactions)

ALEXANDER V. MAZIN* AND STEPHEN C. KOWALCZYKOWSKI†

Division of Biological Sciences, Sections of Microbiology and of Molecular and Cellular Biology, University of California, Davis, CA 95616-8665

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ABSTRACT **The RecA protein–single-stranded DNA (ssDNA) filament can bind a second DNA molecule. Binding of ssDNA to this secondary site shows specificity, in that polypyrimidinic DNA binds to the RecA protein–ssDNA filament with higher affinity than polypurinic sequences. The affinity of ssDNA, which is identical in sequence to that bound in the primary site, is not always greater than that of nonhomologous DNA. Moreover, this specificity of DNA binding does not depend on the sequence of the DNA bound to the RecA protein primary site. We conclude that the specificity reflects an intrinsic property of the secondary site of RecA protein rather than an interaction between DNA molecules within nucleoprotein filament—i.e., self-recognition. The secondary DNA binding site displays a higher affinity for ssDNA than for double-stranded DNA, and the binding of ssDNA to the secondary site strongly inhibits DNA strand exchange. We suggest that the secondary binding site has a dual role in DNA strand exchange. During the homology search, it binds double-stranded DNA weakly; upon finding local homology, this site binds, with higher affinity, the ssDNA strand that is displaced during DNA strand exchange. These characteristics facilitate homologous pairing, promote stabilization of the newly formed heteroduplex DNA, and contribute to the directionality of DNA strand exchange.**

The RecA protein of *Escherichia coli* binds single-stranded DNA (ssDNA) in the presence of ATP to form a characteristic nucleoprotein filament. The structure of this filament is highly conserved evolutionarily: filaments formed by RecA protein analogs from prokaryotes and eukaryotes are remarkably similar (1). This RecA protein–ssDNA filament is essential for all biologically important reactions mediated by RecA protein (2–5). In particular, this presynaptic filament particpates in DNA strand exchange with homologous double-stranded DNA (dsDNA), a basic step of homologous recombination.

It had been suggested (6) that the presynaptic filament, with ssDNA bound to the primary site, has a secondary site; this site binds dsDNA nonspecifically during the search for DNA sequence homology. Indeed, a number of observations, using a variety of methods, indicate that the RecA protein–ssDNA filament can bind additional ssDNA or dsDNA, albeit weakly (7–11). These data support the existence of potential sites for the various DNA strands; herein we use the convention that the primary site binds the initial ssDNA, and the secondary site may accommodate either one (ssDNA) or two strands (dsDNA) of DNA.

Recently, Rao and Radding (12, 13) 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 secondary site of the nucleoprotein filament, so-called "self-recognition," as a part of a homology search process.

Despite rather extensive characterization, the precise nature and function of the secondary DNA binding site remains unknown. 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 this site is important for DNA strand exchange. Furthermore, the data presented herein show 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. The salient feature of the secondary site is that it binds both ss- and dsDNA, but it has a higher affinity for ssDNA. By taking advantage of these affinity differences, we demonstrate that the secondary binding site has a functional role in DNA strand exchange, as saturation of this site with high-affinity competitor ssDNA strongly inhibits DNA strand exchange.

The binding specificity of the secondary binding site suggests a dual role 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 that homologously aligned complexes are recognized due to stabilization of the strand-exchanged complex and that the direction of DNA strand exchange is biased toward DNA heteroduplex formation.

MATERIALS AND METHODS

Proteins and DNA. RecA protein was purified from strain JC12772 using a procedure based on precipitation by spermidine acetate (S.C.K., unpublished observations). Oligodeoxyribonucleotides were synthesized using a Milligene DNA synthesizer (Millipore) and were purified by electrophoresis in 8–10% polyacrylamide gels containing 8 M urea. Annealing and 5′-end labeling of oligonucleotides were as described (14).

Interaction of RecA Protein–ssDNA Filaments with ssDNA and dsDNA. Typically, RecA protein–ssDNA filaments were formed by incubation of 63-mer oligonucleotide 1 (ACAGCACCAGATTCAGCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAAAGGA) (12 μM nucleotides) with RecA protein (8 μM) in buffer containing 33 mM Hepes (pH 7.0), 1.2 mM magnesium acetate, 2 mM

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Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

*On leave from Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Science, Lavrentjeva, 10, Novosibirsk 630090, Russia.

†To whom reprint requests should be addressed at: Section of Microbiology, Hutchison Hall, University of California, Davis, CA 95616-8665. e-mail: sckowalczykowski@ucdavis.edu.

DTT, 1 mM adenosine 5′-[γ-thio]triphosphate, and BSA at 100 μg/ml for 12 min at 37°C; these conditions were used previously (13). Excess RecA protein was sequestered by addition of a heterologous 32-mer oligonucleotide (htr1, GTGAGTCGACAAGCCTGACTCAACATTATCCT) (120 μM), followed by an increase in the magnesium acetate concentration to 15 mM. Complexes containing ssDNA bound to the secondary site of the RecA protein–ssDNA filaments were formed by incubation with ^{32}P-labeled ssDNA for 6 min. When RecA protein filaments were formed at a stoichiometric ratio of 1 RecA protein monomer to 3 nucleotides of ssDNA (1:3 ratio), there was no excess free RecA protein (data not shown) and, consequently, addition of the heterologous oligonucleotide (htr1) was omitted. DNA strand exchange was initiated by addition of homologous dsDNA to RecA protein filaments prepared using the same conditions. Complexes of RecA protein and DNA, and DNA strand exchange products were detected by electrophoretic separation in 10% polyacrylamide gels (90 mM Tris-borate, pH 8.3/0.5 mM EDTA). In the presence of adenosine 5′-[γ-thio]triphosphate, the complexes were stable under the conditions of gel electrophoresis. Electrophoresis in either 10% polyacrylamide gels or 0.55% agarose gels (data not shown) gave quantitatively similar results; polyacrylamide gels had, however, the advantage of providing a sharp separation between the slowly migrating RecA protein–ssDNA ternary complexes and free DNA. All complexes were RecA-protein-dependent and did not survived deproteinization. Gels were quantitated using a Betascope 603 radioisotopic analyzer (Betagen, Waltham, MA).

RESULTS

Specificity of DNA Binding to the RecA Nucleoprotein Filament. As described in the Introduction, the RecA protein–ssDNA filament can bind a second molecule of ssDNA. Further, RecA nucleoprotein filaments are reported to preferentially bind ssDNA with a sequence identical to that bound in the primary site (12); certain modifications in the sequence of this "identical" oligonucleotide prevented binding, whereas others did not (13). To further understand the nature and the functional importance of this binding, we quantified the effect of base replacement and sequence heterogeneity on the binding efficiency of the second DNA molecule.

Complex formation between ssDNA oligonucleotides and RecA protein was monitored using a mobility-shift gel assay (12, 13). RecA protein–ssDNA filaments were assembled on a single-stranded 63-mer and their affinity for a variety of labeled oligonucleotides, either 32 or 33 nt long, was examined (Fig. 1*A*). A complex of RecA protein, primary ssDNA, and secondary ssDNA (referred to simply as "complex" hereafter) was detected for all oligomers except oligo(dA). 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 13 adenosine residues were replaced with thymidine residues (A → T), bound to the RecA protein–ssDNA filament with the highest yield (Fig. 1*A*). Oligonucleotides enriched with adenosines were among the least efficient: oligo(dA) and the modified oligonucleotide, in which all 9 cytidine residues were replaced by adenosine residues (C → A), showed the lowest level of binding. In part, our data are in agreement with the base substitution experiments of Rao and Radding (13): A → T replacements did not prevent binding to the RecA protein–ssDNA filament, whereas C → A replacements diminished binding.

The data also show that oligonucleotide 5, whose sequence is identical to part of the 63-mer bound to the primary site, was not the tightest-binding ligand (Fig. 1*A*, lane ident); it bound to the RecA nucleoprotein filament with an intermediate affinity that is comparable to that of a random 32-mer, synthesized using an equimolar mixture of each nucleotide at every position. (Fig. 1*A*, lane random). We also observed a

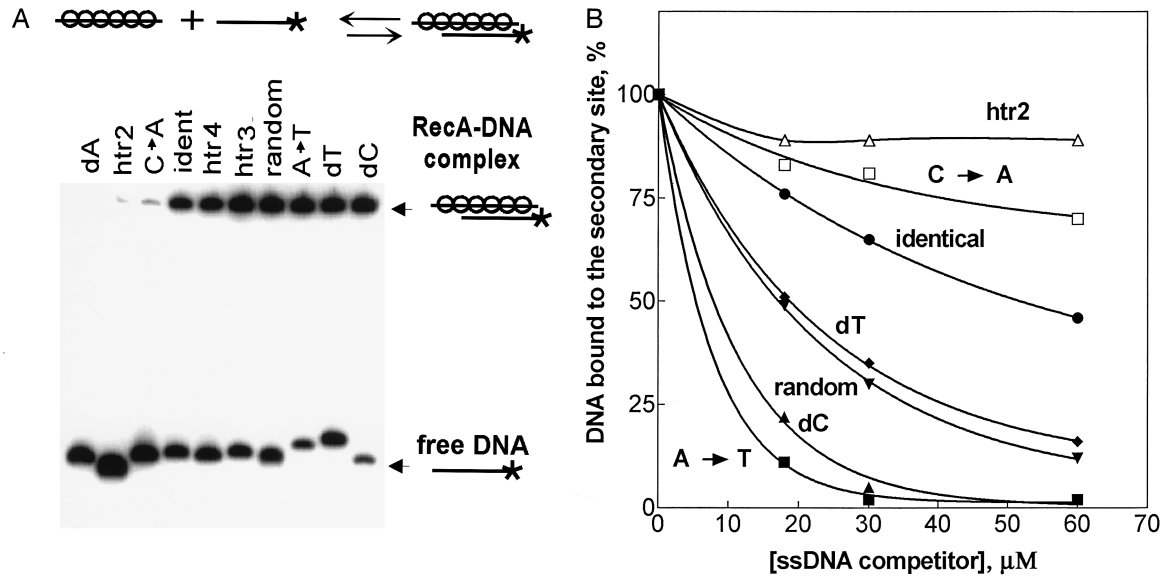

FIG. 1. Specificity of ssDNA binding to the RecA nucleoprotein filament. The RecA protein–ssDNA filament was formed using the 63-mer oligonucleotide 1. (*A*) This filament was incubated with equimolar (molecule) amounts (6 μM nucleotides) of ^{32}P-labeled different 32- or 33-mer oligonucleotides in the presence of heterologous ssDNA (htr1) (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 (oligonucleotide 5: CCATCCGCAAAAATGACCTCTTATCAAAAGGA) that is identical in sequence to the 3′ end of oligonucleotide 1. This was followed by a challenge for 6 min with the concentrations of unlabeled ssDNA 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 cytidine residues by adenosine residues and all adenosine residues by thymidine residues, respectively, and dA, dT, and dC are homooligomeric 32-mers. Heterologous oligonucleotides htr2, htr3, and htr4 have the following sequences: AATTCTTCGAAGCTAGCCCTCAGGCCTAGGCA, TTCACAAACGAATGGATCCTCATTAAAGCCAGA, and CATGGAGCAGGTCGCGGATTTCGACACAATTTA, respectively.

large variation in affinity of the filament for different oligonucleotides of mixed base composition, fortuitously chosen, that had no homology to the resident 63-mer oligonucleotide. Heterologous oligonucleotide 2 (htr2) displayed a low affinity, while other heterologous oligonucleotides (htr3 and htr4) bound to the RecA protein–ssDNA filament with an efficiency higher than that of the identical 32-mer (Fig. 1*A*). 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 (15).

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 filament assembled on a 63-mer oligonucleotide 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 min (data not shown), the 6-min 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. 1*A*: 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. By contrast, oligonucleotides enriched for adenosines, 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 (unpublished observations). This behavior is typical for complexes formed with adenosine 5′-[γ-thio]triphosphate (7).

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 (oligonucleotide 1; labeled I in Fig. 2), homologous to M13, was the same oligonucleotide that we used in the previous experiments (Fig. 1); the second one (labeled M) contained three 9-nt patches of heterologous DNA 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 (htr1). Each of these nucleoprotein filaments was then incubated with three different ^{32}P-labeled 32-mer oligonucleotides: one that is identical to 32 nt of the 63-mer oligonucleotide 1 (M13, no. 5), a second in which all of the adenosine residues were replaced with thymidine residues (A → T), and a third with cytidine residues replaced with adenosine residues (C → A).

The results show that each of the five 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. 2). 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. 1), 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. 2).

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.

ss- and dsDNA Compete for Binding to the Secondary Site. We further asked whether the secondary binding site is the same site that interacts with dsDNA during the DNA strand exchange reaction. Therefore, we tested whether dsDNA can

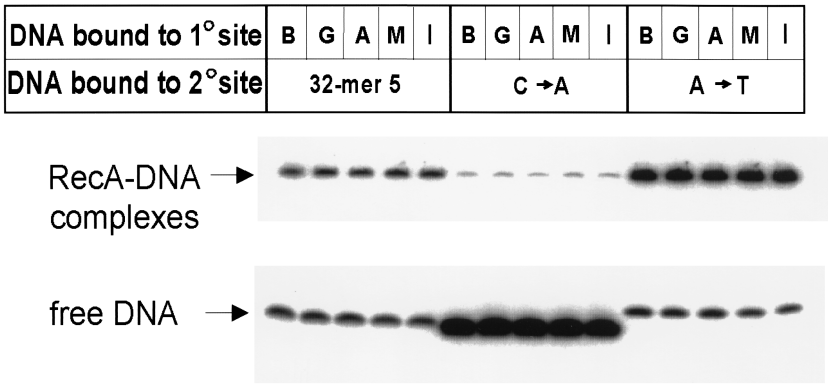

FIG. 2. The sequence of the ssDNA within RecA nucleoprotein filament does not determine the specificity of additional ssDNA binding. Five different RecA protein–ssDNA filaments were formed, using four 63-mer and one 32-mer oligonucleotides. Oligonucleotide G (ACAGCACCAGATTCAGCAATTAAGCTCTAAGGGCGGGCGGCGCGGCCGGGCGGCGGGCGCGCG), oligonucleotide A (ACAGCACCAGATTCAGCAATTAAGCTCTAAGAATAAATAATATAATTAAATAATAAATATATA), and oligonucleotide M (ACAGCACCAATGAAATCTATTAAGCTCCTCATCGTCCGCAAAAATATCGTCACCTCAAAAGGA) are derivatives of the 63-mer oligonucleotide 1 in which the 3′ region was replaced with guanine-rich sequences (lanes G), adenosine-rich sequences (lanes A), or mosaic patches of heterology (lanes M), respectively. Oligonucleotide 1 is designated I. These nucleoprotein filaments were incubated in the presence of heterologous ssDNA (htr1) (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 (lanes B), a 63-mer was omitted and RecA protein was mixed with heterologous 32-mer ssDNA (htr1) (120 μM). The underlined sequences represent substitutions in oligonucleotide 1.

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. 3). 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.

ssDNA 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 (oligonucleotide 1) and homologous dsDNA 48-mer (oligonucleotides 25 and 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 preincubated with the RecA nucleoprotein filament that was formed with the homologous 63-mer bound to the primary site. Fig. 4 shows that when the competitor ssDNA was present, only a 2-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 htr1) had no significant effect on DNA strand exchange. This inhibitory effect correlates with the affinity of the ssDNA bound to the secondary site: the 32-mer oligonucleotide with A → T substitutions has a larger inhibitory effect than hrt1, 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.

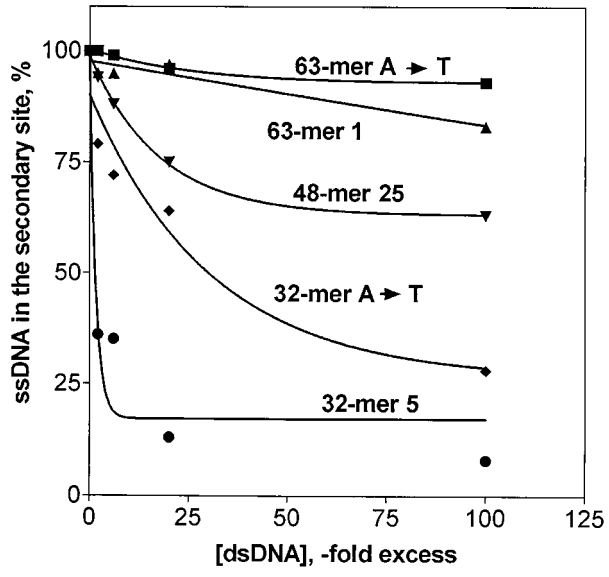

FIG. 3. Competition between ssDNA and dsDNA for secondary site binding. The RecA nucleoprotein filament was formed by incubation of 63-mer oligonucleotide 1 (12 μM) with RecA protein (4 μM) in the absence of heterologous (htr1) oligonucleotide. The filament was incubated for 6 min with an equimolar (molecule) amount of the ^{32}P-labeled ssDNA indicated. These complexes were then challenged for 15 min 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 (oligonucleotide 1).

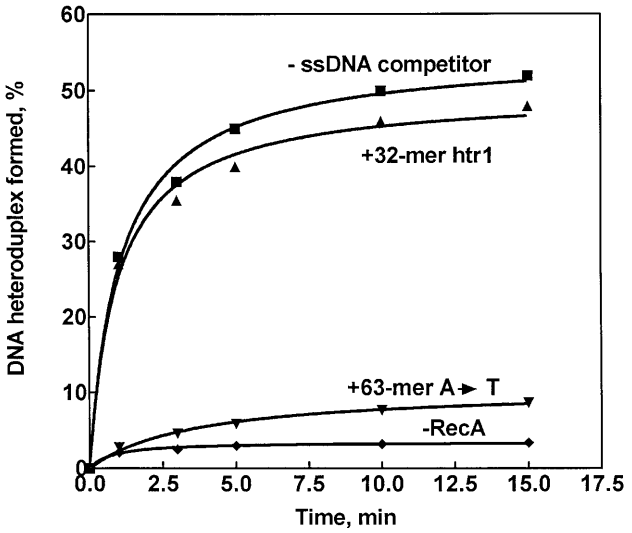

FIG. 4. Binding of ssDNA to the secondary site of the presynaptic filament inhibits DNA strange exchange. The RecA protein–ssDNA filament was formed by incubation of 63-mer oligonucleotide 1 (12 μM) with RecA protein (4 μM). DNA strand exchange was initiated by addition of homologous 48-mer dsDNA (oligonucleotides 25 and 26) in either the presence or absence a 2-fold molar excess of 63-mer oligonucleotide (A → T) (oligonucleotide 41: TCTGCTCCTGTT-TCTGCTTTTTTGCTCTTTGCCTTCCGCTTTTTTGTC-CTCTTTTCTTTTGGT) or in the presence of a 2-fold molar excess of 32-mer (htr1). The products were deproteinized with SDS/EDTA and analyzed by polyacrylamide gel electrophoresis.

DISCUSSION

The results presented herein 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 (13) that base replacements in the single-stranded oligonucleotides have significant effects on the binding affinity. Specifically, A → T replacements enhanced, while others, such as 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. The rules that govern the fine differences in the affinities 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 (12, 13). 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 (12, 13). However, as far as we can see from the experimental results of these authors, the replacement of all thymidine residues with cytidine 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 figures 1 and 4 in ref. 13]; this result is consistent with our data.

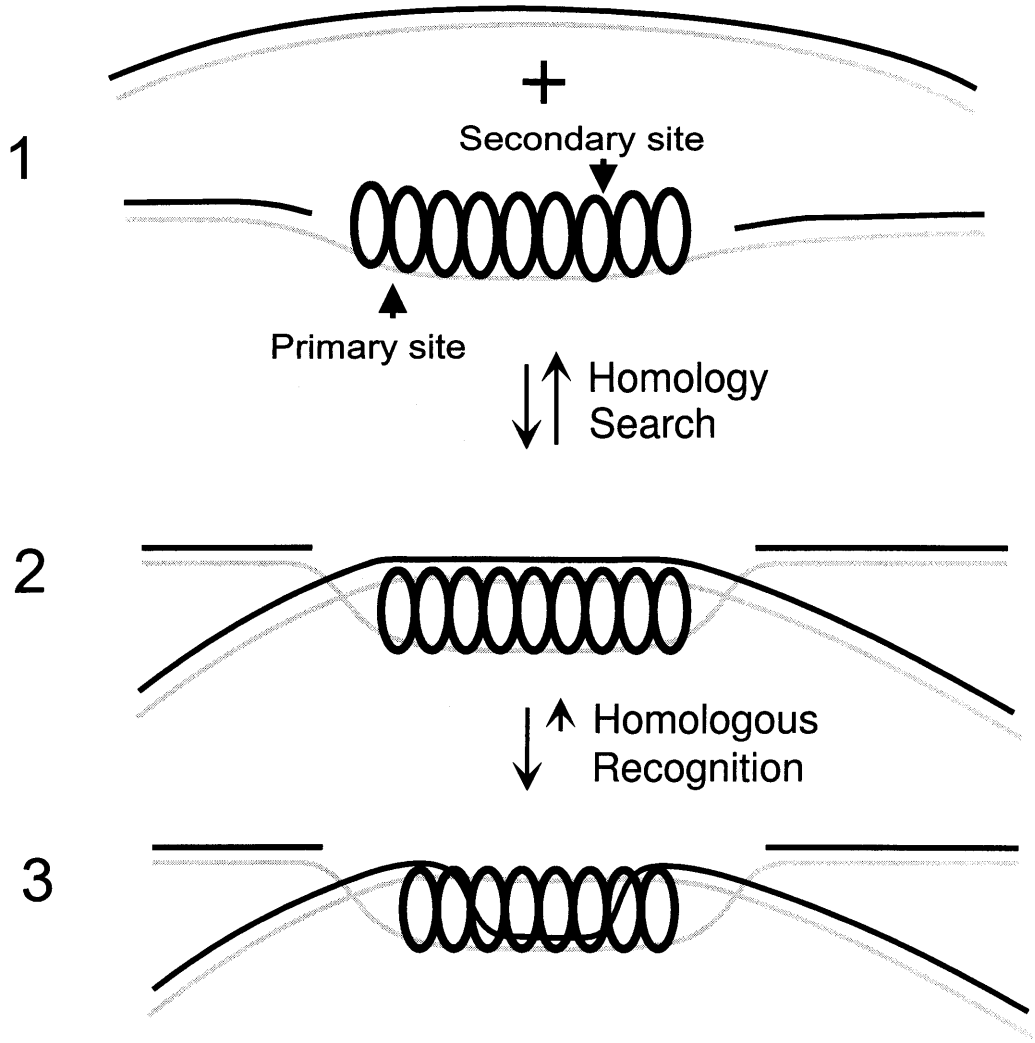

FIG. 5. Proposed function of the secondary DNA binding site of RecA protein in DNA strand exchange. In the first step, ssDNA binds to the primary DNA binding site of RecA protein; in the second step, dsDNA binds to the secondary DNA binding site as part of the homology search process; and in the third step, after homology recognition and DNA strand exchange, the displaced ssDNA strand is bound with high affinity and the resulting DNA heteroduplex is stabilized.

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.

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 (Fig. 5, steps 1 and 2). This interaction with nonhomologous DNA is both weak and necessarily transient, characteristics that 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 step after pairing in DNA strand exchange. Chemical modification experiments show that after DNA strand exchange, the identical strand of the homoduplex is displaced and is single-stranded in behavior (16). Furthermore, both electron microscopic observations (17) and experiments involving cross-linking (18) show that after DNA strands have exchanged, the displaced strand is not released immediately from the nucleoprotein complex but rather it remains temporally associated with the RecA protein–DNA filament. Given the properties of the secondary DNA binding site described herein, we suggest that the displaced DNA strand is transiently bound to this site within the nucleoprotein filament (Fig. 5, step 3). 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. This view does not exclude the existence of a transiently paired three-stranded intermediate but it does, instead, focus attention on the post-pairing complex as a means of stabilizing DNA strand exchange. 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 dsDNA. A prominent length dependence displayed by the secondary site in binding to ssDNA ensures 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* (19).

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