

Binding, annealing and strand exchange between oligonucleotides in different sites of the RecA protein filament

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Abstract The efficiency of single-stranded (ss) oligonucleotides binding at the secondary site of the RecA protein filament is demonstrated to depend on the strandedness of DNA bound at the primary site. When the primary site is occupied by a ss-oligonucleotide, the binding of another ss-oligonucleotide at the secondary site is characterized by higher affinity and a lower rate of dissociation than is the case when the primary site is occupied by a double-stranded oligonucleotide. In contrast to a DNA strand exchange reaction suppressed by a heterologous oligonucleotide bound at the secondary site of the RecA filament, the occupation of the secondary site by a heterologous oligonucleotide does not prevent renaturation between the oligonucleotides bound at the primary site and complementary oligonucleotides from solution demonstrating that the binding of a DNA strand in the secondary site is not a necessary intermediate step in RecA-promoted DNA renaturation.

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Key words: RecA protein; Fluorescent dye-labeled oligonucleotide; RecA–oligonucleotide complex; DNA strand exchange

1. Introduction

A suggestion that the RecA filament might have several sites for binding of DNA strands was put forward by Howard-Flanders et al. [1] and specified in more detail by Kubista et al. [2]. According to this idea, the RecA-promoted DNA strand exchange reaction proceeds as a series of consecutive interactions of DNA strands with different active sites within the RecA filament and between each other. The first step of the strand exchange reaction is the formation of a RecA–single-stranded (ss-) DNA complex. The RecA filament site that binds this DNA is referred to as the primary binding site or site I of the RecA filament. The RecA–ss-DNA complex can further bind ss- or, less efficiently, double-stranded (ds-) DNA at the secondary site (site II) of the filament. The functions of this site in the strand exchange reaction remain to be determined.

Earlier, we demonstrated that fluorescently labeled oligonucleotides do form complexes with RecA protein and allow one

to follow strand exchange reaction with very short oligonucleotides [3,4]. We believe that this system is generally more efficient and simpler in interpretation than complexes of RecA protein with long natural DNAs or polynucleotides. In the present work, we characterized interaction of fluorescent dye-labeled ss-oligonucleotides with the secondary DNA binding site of the RecA filament.

2. Materials and methods

RecA protein purification and synthesis of dye-tagged oligonucleotides was done as described earlier [3]. The oligonucleotides used are listed in Table 1. Presynaptic complexes were formed by incubation of stoichiometric amounts of RecA protein (6 μ M) and dye-labeled oligonucleotides (18 μ M of bases) at 37°C for 30 min in the presence of 0.5 mM adenosine-5'-O-(3-thiotriphosphate) and 2 mM MgAc₂. The mixture was then cooled to 7°C, and the MgAc₂ concentration was raised to 10 mM, followed by the addition of other oligonucleotides. After appropriate incubation, the mixtures were loaded onto 2% agarose gels equilibrated with a buffer containing 20 mM TEA Ac, pH 7.5, and 2 mM MgAc₂ in a cold room and electrophoresed for 30 min at 10 V/cm. To estimate the content of dye-labeled oligonucleotides in the RecA protein complexes, the bands corresponding to the complexes and free oligonucleotides were excised from the gel, eluted and their contents were analyzed by high resolution polyacrylamide gel electrophoresis (PAGE) on 12% denaturing gels. Electrophoretic profiles were detected with an ABI 370A DNA sequencer and treated with the use of an available program for analysis of chromatographic data (a product of Ampersand, Moscow, Russia).

Alternatively, the complexes were disrupted by the addition of sodium dodecyl sulfate (SDS). Oligonucleotides were fractionated by non-denaturing PAGE, and the bands corresponding to ds- and ss-oligonucleotides were eluted and analyzed as above.

3. Results

3.1. Binding of ss-oligonucleotides at site II of the RecA protein filament

To follow oligonucleotides binding to different sites of RecA filaments, we added fluorescent dye-labeled oligonucleotides of different lengths (18-, 19-, 20-mers, see Table 1) and incubated with the RecA protein in different order. So, to saturate site II of the RecA filament with dye-labeled ss-oligonucleotides, an increasing amount of 18*-mers (–strand) (for designations, see Table 1) was added to the presynaptic complex of RecA protein with 20*-mers (–strand). The mixtures were incubated for 5 min and analyzed by agarose gel electrophoresis. As shown in Fig. 1, the saturation of site II with 18*-mers results in the presence of free oligonucleotide in lanes where the ratio of 18*- to 20*-mers is more than one (lanes 5 and 6, Fig. 1a).

The molar ratio of the oligonucleotides in the complex was estimated by excising the bands corresponding to RecA–oligonucleotide complexes and analyzing as described in Section

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; ds-, ss-, double- and single-stranded unmodified oligonucleotides, respectively; ds*-, ss*-, double- and single-stranded dye-labeled oligonucleotides, respectively; SDS, sodium dodecyl sulfate

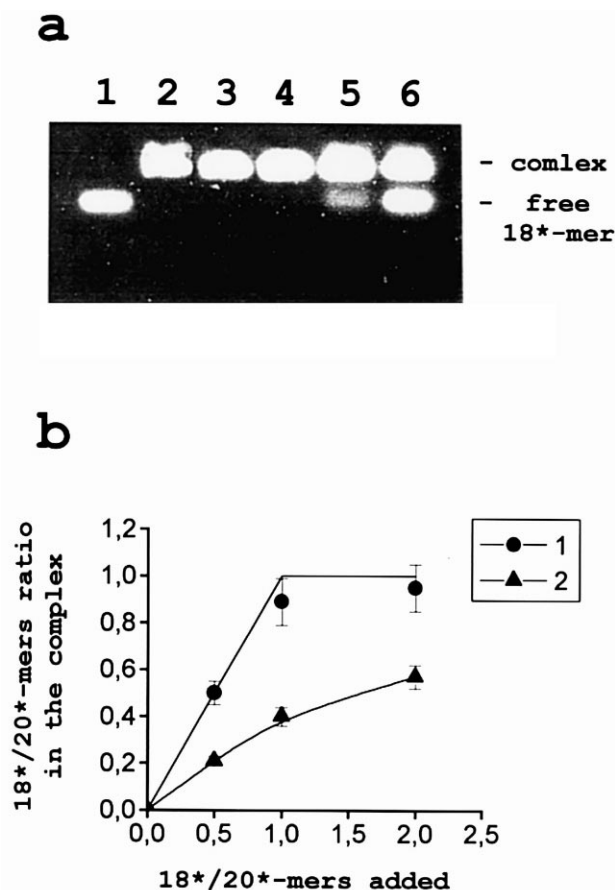


Fig. 1. Saturation of site II of the RecA filament with ss-oligonucleotides. Different amounts of the 18*-mers (–strand) were added to the presynaptic complex of RecA protein with 20*-mers (–strand). (a) Agarose electrophoresis of free oligonucleotides (lane 1) and the presynaptic complexes obtained after addition of 0 (lane 2), 0.5 (lane 3), 1.0 (lane 4), 2.0 (lane 5) and 3.0 (lane 6) molar equivalents of the 18*-mers. (b) Quantitation of the dye-labeled oligonucleotides in the complexes with RecA protein containing ss- (1) and ds-oligonucleotides (2) bound at site I.

2. Fig. 1b shows that the saturation of the presynaptic complex with added 18*-mers is achieved at a near equimolar amount of 18*-mers to 20*-mers, in accord with the assumption that 18*-mer oligonucleotides bind to site II of the nucleoprotein filament.

This assumption is additionally confirmed by an experiment illustrated by Fig. 2. In this experiment, an excess of unlabeled complementary oligonucleotides was added after the formation of a presynaptic complex with dye-labeled 20*-mers and the saturation of site II with 18*-mers. The annealing with the complementary oligonucleotides resulted in the dissociation of 18*-mers from the complex (Fig. 2b, scan 3) whereas 20*-mers remained bound (scan 2), demonstrating that 18*- and 20*-mers were bound differently. This difference in the behavior of the annealing products corresponds to the previously described properties of DNA binding centers of the RecA filaments. As we demonstrated earlier, the annealing of a complementary oligonucleotide from solution to a dye-labeled oligonucleotide bound at site I resulted in the formation of a very stable complex of RecA with ds-oligonucleotide [3]. In contrast, annealing between an oligonucleotide from solution and that bound in site II entails dissociation of the resulting

ds-oligonucleotide from the complex due to the lower affinity of site II to ds-DNA than to ss-DNA [2].

To estimate the dissociation rate of the oligonucleotides bound at site II, we formed the RecA protein complex with 20*-mers (–strand) as above and saturated site II of this complex with a two-fold excess of 18*-mers (–strand). 19*-mers (–strand) also in a two-fold excess over the complex were then added. The complexes taken at various time intervals were separated from free oligonucleotides by agarose electrophoresis and analyzed in a DNA sequencer as described above. Fig. 3 shows that the dissociation of 18*-mers from site II under these conditions is rather slow. Since the reaction is not quenched and the extent of exchange at the initial part of the kinetic curve is, to some extent, overestimated due to ignoring the time required for electrophoretic separation, it may be concluded that the time of half exchange under these conditions is no less than 5–10 min. There was no difference in the exchange rate when the order of oligos addition was reversed (19*-mer for the initial binding at site II followed by 18*-mer for exchange) (not shown).

To characterize the interaction of ss-oligonucleotides with site II of the RecA filament containing ds-DNA bound at site I, we annealed a preformed presynaptic complex containing a 20*-mer (–strand) with an equal amount of complementary, unlabeled oligonucleotide. The affinity of site II in this complex for ss-oligonucleotides was then studied as described above. As demonstrated in Figs. 1b and 3, in this case, the binding was substantially less efficient as compared to the case when site I was occupied by a ss-oligonucleotide. The difference was evident both in the experiments on site II saturation with ss-oligonucleotides (Fig. 1b) and on the exchange between oligonucleotides bound at site II and free in solution (Fig. 3). In the last case, the exchange proceeded practically instantly when site I was occupied by ds-oligonucleotides whereas a rather slow process was observed when site I was occupied by ss-oligonucleotides.

3.2. Strand annealing activity of oligonucleotides bound to RecA protein

To follow the renaturation between oligonucleotides bound to the RecA filament and oligonucleotides free in solution, we consecutively added dye-labeled and non-labeled oligonucleotides to preformed presynaptic complexes with complementary 18*-mers (+strand) at 1 min time intervals (Fig. 4). The

Table 1
Oligonucleotides used for the formation of complexes with RecA protein

Designation (polarity)	Sequence
<i>Dye-labeled oligonucleotides:</i>	
18*-mer (+strand)	Flu-TGTAAAACGACGGCCAGT-3'
19*-mer (+strand)	Flu-ATGTAAAACGACGGCCAGT-3'
20*-mer (+strand)	Flu-GATGTAAAACGACGGCCAGT-3'
18*-mer (–strand)	Flu-ACTGGCCGTCGTTTACA-3'
19*-mer (–strand)	Flu-GACTGGCCGTCGTTTACA-3'
20*-mer (–strand)	Flu-AGACTGGCCGTCGTTTACA-3'
20*-mer (hetero)	tetramethylrhodamine-GGGATGTTACAACACCATGC-3'
<i>Unlabeled oligonucleotides:</i>	
16-mer (–strand)	CTGGCCGTCGTTTACA-3'
19-mer (–strand)	GACTGGCCGTCGTTTACA-3'
19-mer (+strand)	ATGTAAAACGACGGCCAGT-3'

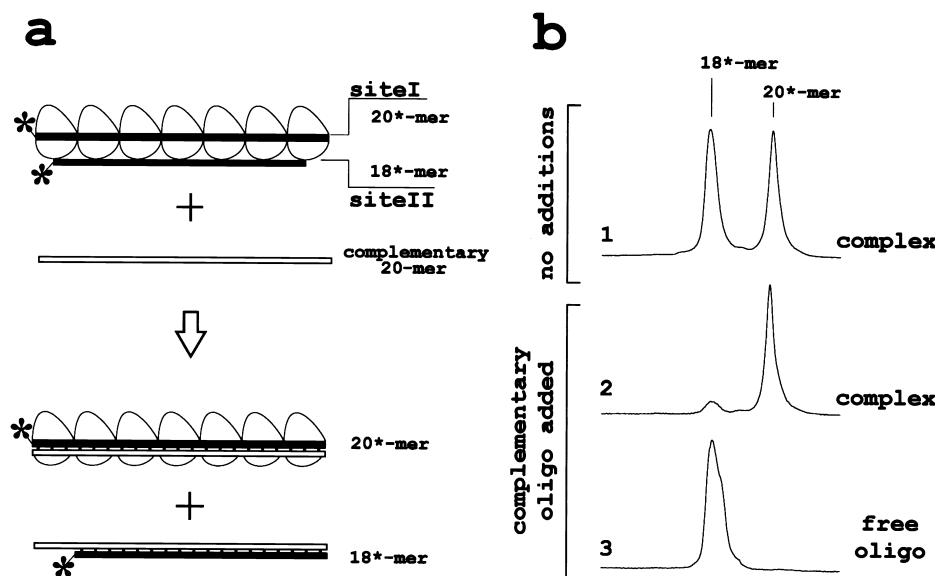


Fig. 2. Displacement of 18*-mers from site II caused by the addition of complementary oligonucleotides. (a) A scheme of the experimental approach. After formation of a presynaptic complex with 20*-mers (–strand) and its saturation with 18*-mers (–strand), an excess of complementary unlabeled oligonucleotides was added. (b) Content of the complex and free oligonucleotide fractions separated by agarose electrophoresis.

complexes were then deproteinized with SDS, and the ds- and ss-reaction products were separated by non-denaturing PAGE. The ds-product bands were eluted and analyzed in a DNA sequencer.

As shown in Fig. 4a, addition of unlabeled complementary oligonucleotides (–strand) completely inhibited the annealing between the dye-labeled 18*-mers (+strand) bound at site I and complementary 19*-mers (–strand) added later as confirmed by the absence of the 19*-mers in the ds-fraction (scan 3, Fig. 4a). Addition of the unlabeled oligonucleotides after that of the 19*-mers (–strand) did not affect the annealing between the dye-labeled oligonucleotides (scan 2, Fig. 4a). This result was confirmed by the successive addition of two

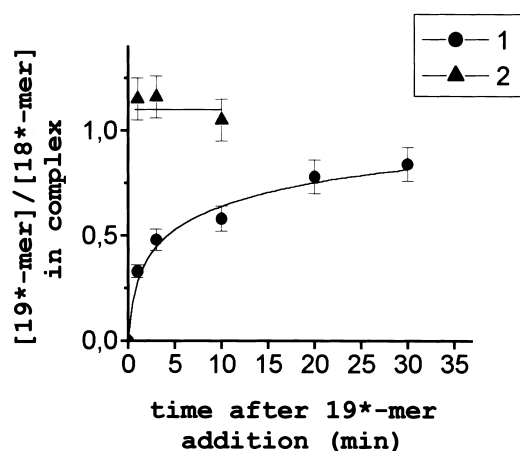


Fig. 3. Exchange of ss-oligonucleotides at site II of the RecA filament. Site II of a presynaptic complex preformed with 20*-mers (–strand) was saturated with 18*-mers (–strand), then an equal amount of 19*-mers (–strand) was added. The kinetic curves for the complexes with ss- (1) and ds-oligonucleotides (2) bound at site I are presented.

dye-labeled oligonucleotides of different lengths (19*-mer and 20*-mer (–strands)) (scans 4 and 5). In both cases, the fraction of ds-oligonucleotides along with 18*-mer (+strand) formerly bound at site I contained the complementary dye-labeled oligonucleotide that had been added first (compare scans 4 and 5, Fig. 4a). Therefore, the strand annealing in these conditions is a very fast process being completed in less than 1 min.

Since strong binding of ss-oligonucleotides at site II of the RecA filament takes place in these conditions, we wished to elucidate the relationship between strand annealing and ss-DNA binding at site II. Site II of a complex of RecA with dye-labeled 18*-mer (+strand) was saturated with an excess of heterologous oligonucleotide prior to the addition of a complementary oligonucleotide (19*-mer (–strand)). If the binding of the complementary oligonucleotide to site II is a necessary intermediate step in the process of the annealing, the occupation of the site II with heterologous oligonucleotide would result in a delay in the annealing corresponding to the time needed for equilibration between the oligonucleotide bound at site II and free in solution (5–10 min as demonstrated in Fig. 3). Fig. 4b shows that in fact the instant annealing is not prevented by the saturation of site II with an excess of heterologous oligonucleotides demonstrating a capability of the oligonucleotide from solution to rapidly interact with complementary oligonucleotide bound at site I.

In this experiment to prevent spontaneous annealing between dye-labeled oligonucleotides that could proceed after deproteinization, an excess of unlabeled oligonucleotides of appropriate polarity (19-mer (–strand)) was added before the addition of SDS. The efficiency of this termination procedure was demonstrated above (Fig. 4a, scan 3).

3.3. RecA protein-promoted strand exchange reaction

To follow the RecA-promoted strand exchange, the presynaptic complex was formed with 20*-mer (+strand). The

product of annealing of 19*-mers (+strand) with unlabeled complementary 16-mers (–strand) was used as the second, ds-substrate of the reaction. The presynaptic complex and the ds-substrate were incubated for the desired time at 7°C. The reaction was terminated by the addition of SDS, and the spectrum of the ds-products was analyzed as described above.

As shown in Fig. 5, the presynaptic complex is active in the strand exchange reaction under the conditions used. The reaction rate depends on the presynaptic complex/ds-substrate molar ratio (curves 1 and 2). In contrast to the case of ss-oligonucleotides annealing, the strand exchange reaction is strongly susceptible to the inhibition by heterologous ss-oli-

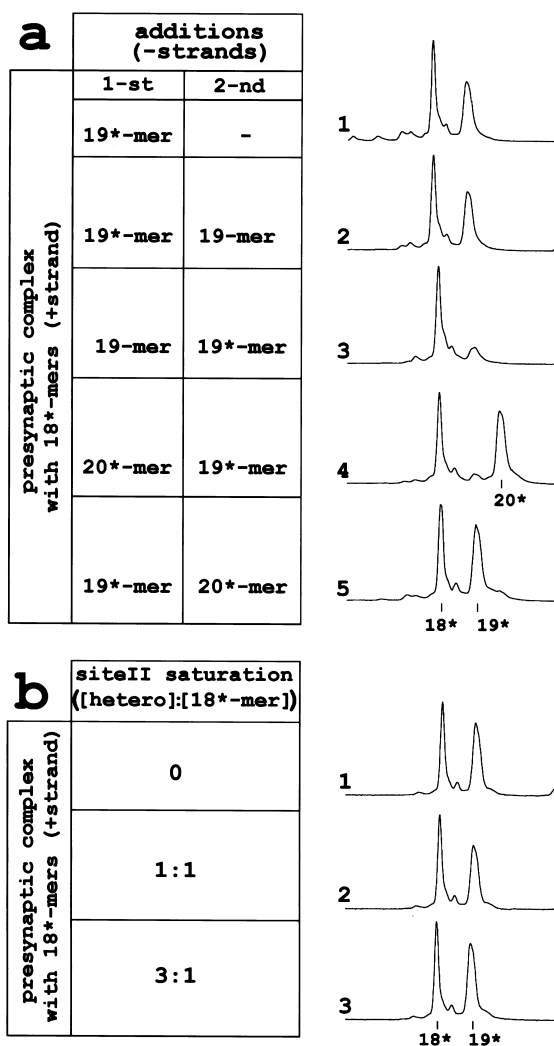


Fig. 4. Strand annealing between oligonucleotides bound at site I and free in solution. To a presynaptic complex with 18*-mers (+strand), an equal amount of complementary dye-labeled or an excess of unlabeled oligonucleotides was added in different order. (a) Influence of the order of additions on the content of ds-fraction. Order of additions is indicated by corresponding scans. (b) Occupation of site II with heterologous oligonucleotides does not retard strand annealing. Site II of the presynaptic complex with 18*-mer (+strand) was saturated with a different amount of heterologous oligonucleotide and then 19*-mer (–strand) was added. After 1 min incubation, the annealing was terminated with addition of an excess of unlabeled 19-mer (–strand) and the ds-oligonucleotides fraction was analyzed in a DNA sequencer. Molar ratio of heterologous oligonucleotide to 18*-mer is indicated by corresponding scans.

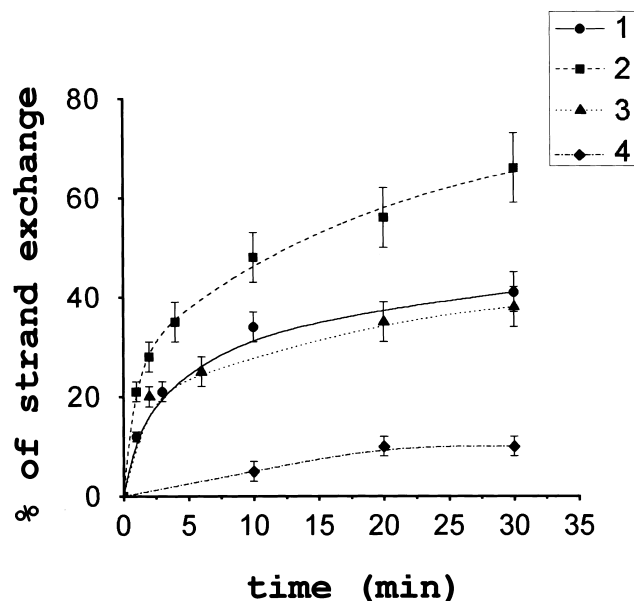


Fig. 5. RecA promoted strand exchange reaction. A ds-reaction substrate was added to a presynaptic complex with 20*-mers (+strand). Time course of the strand exchange reactions at equimolar amount (1, 4) and a two-fold molar excess of the presynaptic complex over the ds-substrate (2) is shown. The cases when site II of the presynaptic complex was saturated with heterologous oligonucleotides (4) and when equimolar amounts of the presynaptic complexes formed with 20*-mers (+strand) and heterologous oligonucleotides were mixed before addition of the ds-substrate (3) are also presented.

gonucleotides added before the ds-substrate (curve 4). Addition of an equimolar amount of presynaptic complexes formed with heterologous oligonucleotides had no significant effect on the reaction (curve 3).

4. Discussion

Earlier, it was demonstrated that the binding of homopolynucleotides at site II of the RecA protein filaments prevented their annealing with complementary polynucleotides bound at site I of these filaments [5]. The annealing occurred only after saturation of site II with ss-DNA demonstrating the capability of ss-DNA bound at site I of the RecA filament to interact immediately with complementary ss-DNA in solution. In our conditions, ss-oligonucleotides bound at site I of the RecA filament were also able to anneal with complementary oligonucleotides in solution without binding to site II. At the same time, this annealing was much faster than the binding of the oligonucleotides to site II, suggesting that this binding could not compete with the annealing. We believe that this disparity with the previous observation might be explained by the difference in length and/or nucleotide content of the DNA substrates used since both of these features have been demonstrated to affect RecA protein–DNA binding [6,7].

Modern models of structure of the presynaptic complex place the primary site of DNA binding deep in the interior of the RecA filament (for review, see [2,8]). The absence of influence of the secondary site occupation with heterologous DNA on the efficiency of the strand annealing demonstrated in the present study means that a DNA strand bound in this site does not prevent ss-DNA in solution from finding its way

inside the filament and annealing to complementary DNA bound in the primary site.

In contrast to the strand annealing and in accordance with the previously reported data [6], RecA-promoted strand exchange in our conditions is very susceptible to the inhibition by heterologous ss-oligonucleotides bound at site II that confirms an important role of ds-DNA interaction with site II in strand exchange reaction.

Binding of ss-DNA displaced from the ds-substrate of the strand exchange reaction was proposed to be one of the important functions of the secondary site of DNA binding in the RecA filament [9]. The relatively low efficiency of ss-DNA binding to site II of the RecA protein complex with ds-DNA demonstrated here does not support this proposal. We suppose that this intrinsic property of the RecA filament facilitates fast dissociation of the displaced DNA strand from the complex formed in the course of strand exchange reaction thus impeding the reverse reaction.

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