Affinity Cleavage of Double-Stranded DNA Directed by a RecA Nucleoprotein Filament Containing an Oligonucleotide-EDTA•Fe(II)

Thesis by

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Abstract

Escherichia coli RecA protein promotes strand exchange between two homologous DNAs by polymerizing on single-stranded DNA to form nucleoprotein filaments which then bind to a homologous sequence of duplex DNA. Joint formation between the RecA protein and three strands of DNA is followed by strand exchange in which the original single strand is inserted into the duplex while the homologous duplex strand is displaced out. The structures of the strand exchange intermediates formed by the RecA protein have not been elucidated. We review studies aimed at determining such structures and we report the use of affinity cleaving techniques to probe the structure of the joint molecule formed between a RecA-oligonucleotide filament and duplex DNA by incorporating a thymidine-EDTA•Fe (T*) into the oligonucleotide of the filament. In our study, we find that the nucleoprotein filament binds antiparallel to the complementary strand within the homologous sequence of DNA and that it is associated more strongly with the complementary strand than with the homologous strand of the duplex.

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Chapter 1: A Review of RecA-Mediated Strand Exchange

Introduction

The RecA protein of *E. Coli* (38 kD) plays a central role in homologous genetic recombination and repair and is involved in the cellular S.O.S. response to gene damage.[1-5] *In vitro*, RecA protein mediates a set of DNA strand exchange reactions which have been used to model homologous recombination *in vivo*. In the model studies, RecA-catalyzed strand exchange occurs between two DNA molecules of homologous sequence when one of the pieces of DNA is completely or partially single-stranded. In the presence of ATP, RecA polymerizes on single-stranded DNA forming a right handed helical nucleoprotein filament in which the DNA is significantly unwound relative to B-form DNA. Such filaments contain 18.6 bases per turn with an axial spacing between bases of 5.1 Å.[6] The filament binds sequence-specifically to a site on duplex DNA which is complementary to the sequence of the single strand bound in the filament. In this synapsis step, a joint molecule is formed which contains the three strands of DNA and numerous RecA monomers. The final step is the release of the strand exchange products; a displaced single strand and a heteroduplex. The process is polar occurring in the 5' to 3' direction with respect to the strand being displaced from the duplex. In the presence of the non-hydrolyzable ATP analog, γS-ATP, the reaction does not proceed past synapsis and the three strands of DNA remain bound in the joint molecule.[7-9]

The precise structure and mechanism of action of the joint molecule formed in RecA-mediated strand exchange have not been elucidated. It is generally accepted that the three DNA strands are held stably within the RecA filament during the synapsis step of the reaction.[10-17] Two basic mechanisms of strand exchange have been postulated which require different joint molecule

structures.[18,19] The first mechanism involves duplex strand separation before pairing. More specifically, local opening of a region of duplex is followed by Watson-Crick base-pairing of the single strand with its complementary strand of the denatured duplex. For the second mechanism, which involves pairing before strand separation, a fully base-paired duplex makes additional base-specific interactions with the homologous single strand within the RecA-associated complex. A three-stranded structure is formed which then undergoes strand exchange.

The first mechanism is supported by the strand exchange model proposed by Norden and coworkers based on findings from linear dichroism spectroscopy and small angle neutron scattering studies of RecA-DNA complexes formed in the presence of γS-ATP.[13-17] The RecA protein is proposed to have three DNA binding sites which each bind a single strand of DNA (figure 1.1). Double-stranded DNA (dsDNA) binds to two of these sites simultaneously. The sites are designated I, II, and III based on the order in which pieces of single-stranded DNA (ssDNA) bind to the protein. The sites differ in their DNA binding affinities and in the relative polarity of the bound strands. A DNA strand bound in site III is susceptible to nuclease cleavage whereas strands bound in sites I or II are fully protected.[15] Site III is thus proposed to be a more open binding site with weaker DNA binding affinity than sites I or II. RecA-catalyzed renaturation of complementary strands of DNA is most successful when two equivalents of one strand are bound to the protein before addition of its complement.[16] Renaturation is inefficient when the two complementary strands are added in a ratio of 1:1. A possible explanation for these findings is that base-pairing is not possible between strands in sites I and II because the strands are parallel to each other. The polarity of the strand bound in site III is opposite to the other two strands allowing site III-site I base-pairing and site III-site II base-pairing.

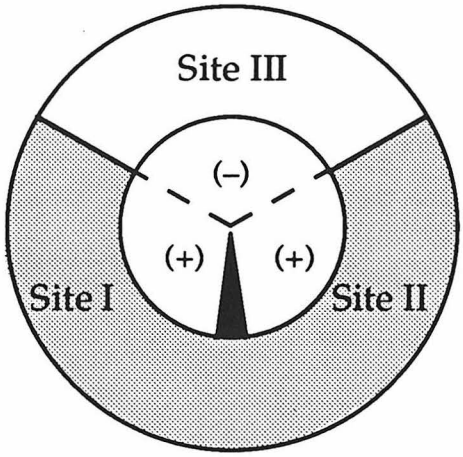

Figure 1.1. A model of a cross-section of a RecA filament illustrating its three DNA binding sites. The sites are numbered I, II, and III in the order that they are engaged in binding ssDNA. Site III is tentatively placed at the groove of the filament (unshaded sector) as suggested from nuclease accessibility studies.[15] The dark wedge symbolizes the inability of strands bound in site I and site II to base-pair. Strands in site I and II are bound parallel (+), and the strand in site III is bound with opposite polarity (–). (Adapted from Norden et al.[17])

Linear dichroism spectra of complexes formed between RecA, ssDNA, and dsDNA indicate that the bases in a complex in which ssDNA is added to the protein first (RecA-ssDNA-dsDNA) are all aligned perpendicular to the helical axis.[15] The bases of a complex in which ssDNA is added last (RecA-dsDNA-ssDNA), however, are not all coplanar. The authors suggest that the two complexes represent two strand exchange intermediates. The RecA-ssDNA-dsDNA complex, in which the ssDNA is located in site I and the dsDNA in sites II and III, represents an intermediate in which the coplanar bases are poised for strand exchange. The RecA-dsDNA-ssDNA complex, in which the dsDNA is located in sites I and III and the ssDNA in site II, represents a post strand exchange intermediate.

The strand exchange mechanism proposed by Norden and coworkers[17] is illustrated in figure 1.2. The RecA protein first binds ssDNA in site I (figure 1.2, **A**) followed by the binding of dsDNA to sites II and III (figure 1.2, **B**). The initial

contacts between the ssDNA and dsDNA are in nonhomologous regions and the dsDNA remains paired. When complementary regions meet in the search for homology, the strand in site III spontaneously flips over and pairs with the strand in site I, and the bases in the remaining strand of the duplex tilt away from their perpendicular geometry (figure 1.2, C).

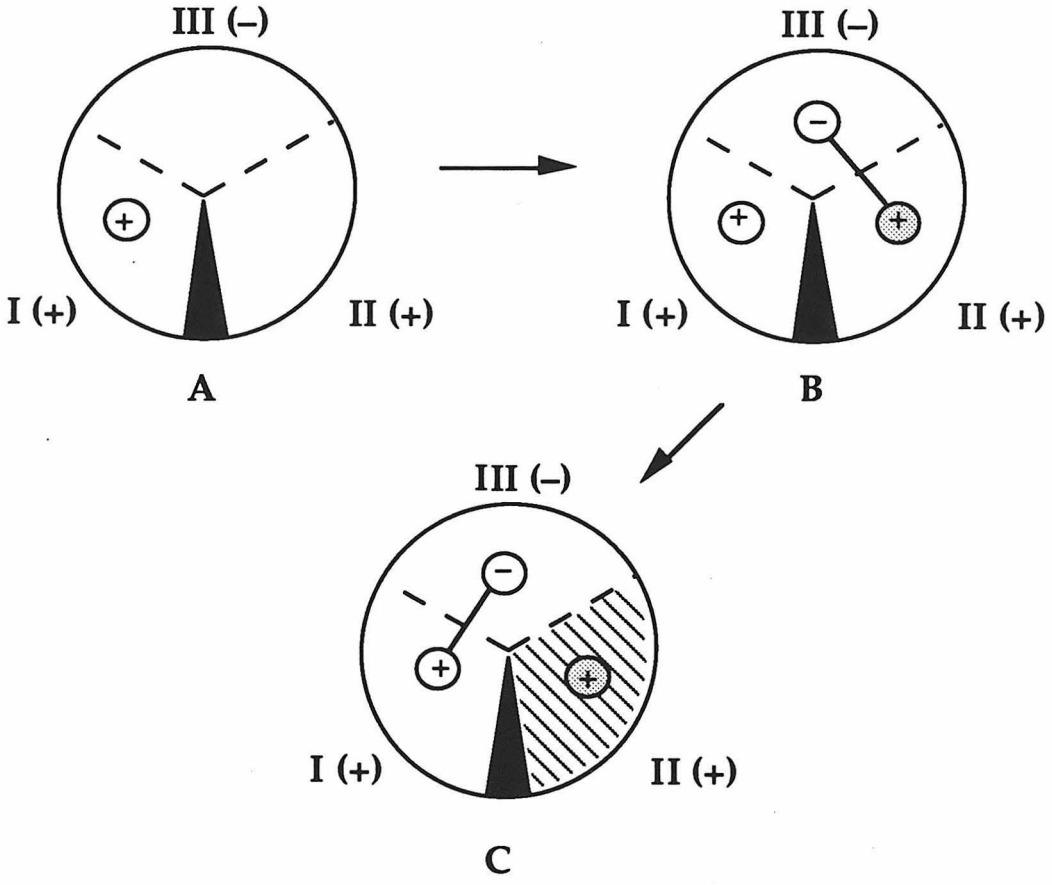

Figure 1.2. A possible mechanism of RecA-mediated strand exchange. The RecA protein is represented by a circle which is partitioned into three sections corresponding to DNA binding sites I, II, and III. ssDNA is represented by a small circle and dsDNA by a dumbbell. The relative orientation of the strands is indicated by a "+" or a "–" in the center of the circle. (**A**) ssDNA binds to site I forming a nucleoprotein filament. (**B**) dsDNA binds to sites II and III of the protein. Initial contacts are in nonhomologous regions and the duplex remains paired. All bases are coplanar. (**C**) When complementary regions of the ssDNA and dsDNA meet, strand exchange occurs. The strand in site III becomes paired with the strand in site I and the bases of the outgoing strand in site II tilt out of the plane of the other bases as indicated by shading of site II in **C**. (Adapted from Norden et al.[17])

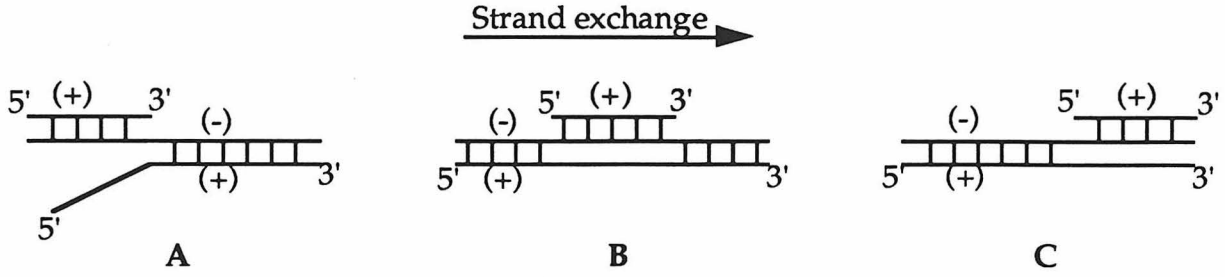

Figure 1.3. Schematic representations of joint molecules restricted to different sites in linear duplex DNA. (**A**) Proximal joint. (**B**) Medial joint. (**C**) Distal joint. (Adapted from Radding[3]).

Deproteinized Joint Molecules

For a mechanism in which pairing occurs before strand separation, a novel three-stranded DNA structure involving specific interactions defined by sequence homology between double-stranded and single-stranded DNA is required. Evidence for[12,20-27] and against[28,29] such a structure has been reported. Radding and coworkers[20-23] and Camerini-Otero and coworkers[24-26] have described the RecA-mediated formation of triple-stranded joint molecules which, after deproteinization, are thermostable and show chemical reactivity consistent with a novel triplex structure. For recombination structures at one terminus of a linear duplex (figure 1.3, **A** and **C**), complexes formed at the 5' end of the displaced duplex strand are known as proximal joints (i.e., the origin of strand exchange is proximal to the duplex terminus), while those formed at the 3' end of the displaced strand are known as distal joints (i. e., the origin of strand exchange is distal to the duplex terminus).[3] Because of their location on the duplex and the directionality of strand exchange, proximal joints may be more quickly resolved into heteroduplexes and displaced strands than distal joints. Thus, distal joints may be more triple-stranded in character than proximal joints. Both distal and proximal joints are thermostable after deproteinization whereas joints formed

internally on a linear duplex (medial joints, figure 1.3, **B**) are not.[20] Deproteinized distal joints were found to be more thermostable than duplex DNA, consistent with the possibility that this type of joint has a novel triple-stranded structure. Structural studies of deproteinized distal joints by Radding and coworkers[22,23] and Camerini-Otero and coworkers[26] have led to different models of the complex. The deproteinized structures are proposed by both groups to model recombination intermediates.

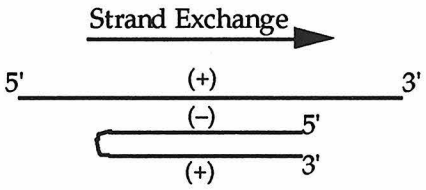

Figure 1.4. Schematic representation of a joint molecule formed between ssDNA and a complementary hairpin duplex. (+) and (–) indicate complementary sequences. The hairpin turn located near the 5'-end of the ssDNA mimics a distal joint in that separation of the original duplex is inhibited at the origin of strand exchange. (Adapted from Chiu et al.[23]).

Radding and coworkers have reported extensive chemical and enzymatic modification studies of deproteinized joint molecules formed using RecA, ATP, ssDNA, and hairpin duplexes containing regions complementary to the ssDNA.[22,23] Such structures model distal joints when the hairpin turn of the duplex is placed at the 5' end of the ssDNA (figure 1.4). Results from these studies are summarized in table 1.1. The two strands of the deproteinized joint which would be destined to form heteroduplex DNA (table 1.1, incoming strand and complementary strand) in strand exchange show chemical modification and enzymatic digestion patterns indistinguishable from those of control duplex DNA. The strand which would be displaced from the complex in strand exchange (table 1.1, outgoing strand) shows reactivity different from both ssDNA and dsDNA.

The authors propose that the deproteinized joint has a novel triplex structure in which the incoming strand and its complement are Watson-Crick base-paired with the outgoing strand interacting with the other two strands through novel interactions. Proposed base triplets are shown in figure 1.5 (triplets **1-3, 4A**).

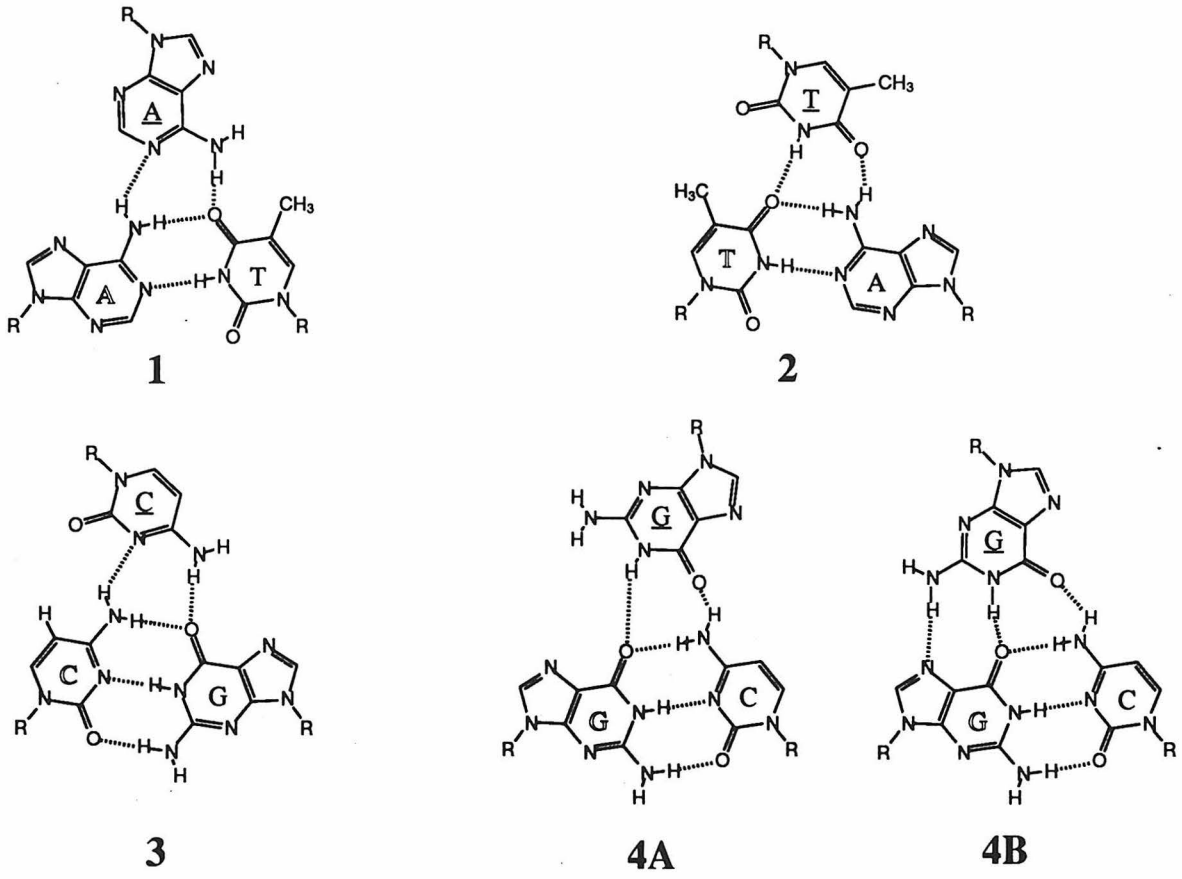

Figure 1.5. Proposed joint molecule base triplets. In the triplets proposed by Radding and coworkers[23] (**1-3, 4A**), the incoming strand (outlined letters) is Watson Crick base-paired with the complementary strand (plain text letters) while the outgoing strand (underlined letters) is located in the major groove of the heteroduplex. In the triplets proposed by Camerini-Otero and coworkers[26] (**1-3, 4B**), the incoming strand (underlined letters) is located in the major groove of the original Watson-Crick duplex (outgoing strand, outlined letters; and complementary strand, plain text letters).

Reagent	Incoming Strand	Complementary Strand	Outgoing Strand	Reference
Cu Phen	ND	hypersensitive	hypersensitive	22
	ND	ND	no reaction	26
DEPC	ND	no cleavage	A	23
DMS	G	G	G	23
	ND	G	no reaction	26
	G	G	G, C	28
$KMnO_4$	ND	no reaction	A, T	23
	T, non uniform	T, non uniform	T, uniform	28
DNase I	ND	like duplex	novel reactivity	22, 23
	ND	ND	like duplex	26
P1 Nuclease	ND	like duplex	novel reactivity	23

Table 1.1. Summary of enzymatic digestion and chemical modification studies of joint molecules. Chemical abbreviations are as follows; copper phenanthroline (Cu Phen), diethylpyrocarbonate (DEPC), dimethylsulphate (DMS), potassium permanganate ($KMnO_4$). ND indicates that no data was available. G, C, A, T indicate reactivity of guanine, cytosine, adenine, or thymine bases, respectively. References include Radding and coworkers,[22,23] Camerini-Otero and coworkers,[26] and Adzuma.[28]

Camerini-Otero and coworkers have studied deproteinized distal joints formed using RecA, ATP, M13mp18 (circular ssDNA), and a 128-bp duplex DNA which contains 57 base pairs at its terminus which are homologous to a region of M13mp18 (figure 1.6).[26] Results from this study are summarized in table 1.1. In contrast to the Radding studies,[22,23] the outgoing strand in this system is protected from attack by copper phenanthroline (Cu Phen) and by dimethylsulphate (DMS). The complementary strand, however, is not protected from DMS attack (in agreement with the Radding studies). From these and previous results, the authors present a structural model of the deproteinized complex in which the

original duplex maintains Watson-Crick base-pairing while making novel interactions with the incoming strand. Proposed base triplets are shown in figure 1.5 (triplets **1-3, 4B**). Protection of N7 of guanine (G) from DMS in the displaced strand is accounted for by interaction with N2 of G in the incoming strand (figure 1.5, triplet **4B**). The main difference between the two proposed structures of deproteinized joints is the location of Watson-Crick base-pairing. In the Camerini-Otero structure, the original duplex remains intact whereas a Watson-Crick heteroduplex has formed in the Radding structure. The discrepancy may be due to the two groups studying intermediates at different stages of the strand exchange process.

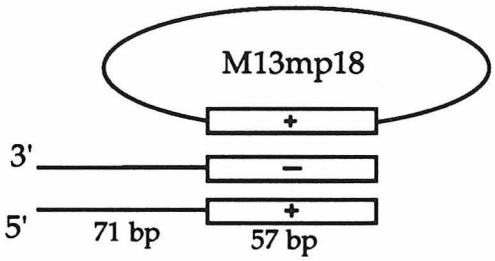

Figure 1.6. Schematic representation of the distal joints studied by Camerini-Otero and coworkers.[26] Boxes indicate regions of homology between duplex and single-stranded DNA, thin lines indicate heterologous regions. "+" and "–" indicate complementary sequences.

RecA-Coated Joint Molecules

Joint molecules formed in the presence of γS-ATP have been studied by Adzuma using restriction enzyme protection and chemical modification studies.[28] The joints studied were formed on internal sites of target duplex molecules and all modification experiments were carried out while RecA was bound to the complex. The results are summarized in table 1.1. N7 of guanine (G) residues of all three DNA strands and N3 of cytosine (C) residues of the outgoing strand of the complex reacted with DMS. All three strands reacted with potas-

sium permanganate ($KMnO_4$) but the incoming and complementary strands showed less uniform reactivity than did the outgoing strand. In contrast to the structures of deproteinized distal joints,[22,23,26] the outgoing strand of the RecA-coated joint molecule is single-stranded in character while the other two strands behave more like a canonical duplex. The lack of novel triple-stranded interactions is consistent with a mechanism in which strand separation occurs before pairing. However, it may be that the complexes studied by Radding and coworkers[20-23] and by Camerini-Otero and coworkers[24-26] represent transient novel three-stranded intermediates which precede the structure studied by Adzuma.[28]

The strand exchange model proposed by Adzuma[28] (figure 1.7) is similar to that proposed by Norden and coworkers.[17] The two models differ mainly in the location of the products following strand exchange. Both models contain three DNA binding sites (I, II, and III in figures 1.1, 1.2: A, B, and C in figure 1.7). Sites A, B, and C are labeled in order of DNA binding as well as according to DNA binding affinity with site A the strongest and site C the weakest DNA binding site. In the first step, ssDNA binds to site A (figure 1.7, **1**) followed by association of dsDNA with site B (figure 1.7, **2**). If the duplex is not homologous to the ssDNA, the duplex dissociates from site B. If a region of homology is found, local strand exchange occurs in which the ssDNA is transferred from site A to site B and the outgoing duplex strand is transferred to site C (figure 1.7, **3**). The final step, which requires ATP, is the release of the heteroduplex and the outgoing strand (figure 1.7, **4**).

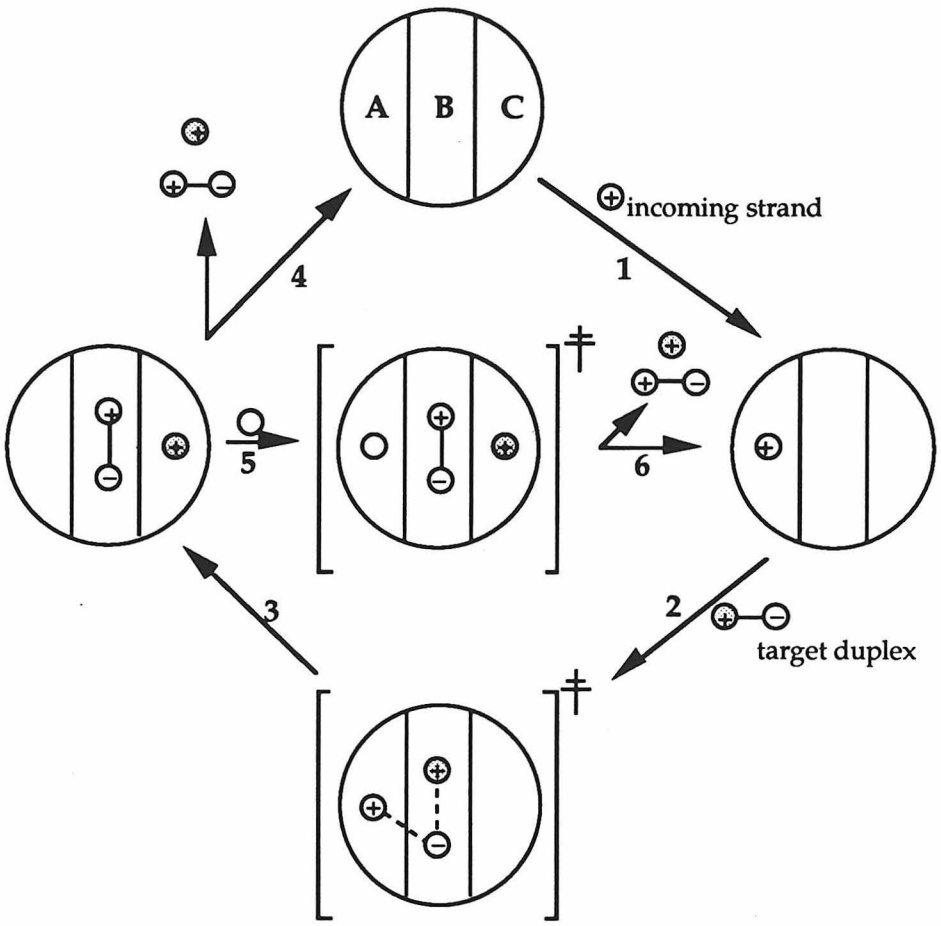

Figure 1.7. RecA-catalyzed strand exchange model proposed by Adzuma.[28] The RecA protein is represented by a circle partitioned into three DNA binding sites labeled A, B, and C. The sites are labeled in sequential order of DNA binding and also in order of DNA binding affinity. ssDNA is represented by a small circle and dsDNA by a dumbbell. "+" and "–" indicate the relative orientation of the strands. Brackets surrounding some of the complexes are meant to indicate the potentially transient nature of these complexes. **(1)** A ssDNA binds to site A forming a nucleoprotein filament. **(2)** Binding of duplex to site B. In case of non-homology, the duplex simply dissociates from site B. **(3)** If a homologous region is found, local strand exchange occurs. The heteroduplex occupies site B and the outgoing strand is transferred to site C. **(4)** Product release. **(5 & 6)** A putative pathway of how an additional nonhomologous ssDNA might induce rapid dissociation of RecA from the complex.

The results of restriction enzyme protection assays indicate that the joint molecules formed in the Adzuma study are not stable when challenged with an excess of nonhomologous ssDNA.[28] Without the ssDNA challenge, the complex-

es are very stable. The joint molecule instability is attributed to a rapid transfer of RecA molecules from the complex to the challenging ssDNA. Redistribution of RecA due to rapid equilibrium does not easily explain the results as the RecA molecules in the complexes seem to be relatively immobile without the ssDNA challenge. In Adzuma's model, site A is unoccupied in the stable joint molecule. The challenging ssDNA is proposed to bind to site A promoting the dissociation of RecA from the resident DNA (figure 1.7, **5-6**). The model proposed by Norden and coworkers[17] would require dissociation of the resident strands from the protein prior to binding of the challenging ssDNA.

Summary

Figure 1.8 summarizes the proposed models discussed above of RecA-mediated strand exchange. Nucleoprotein filament formation between RecA and ssDNA (figure 1.8, **1**) is followed by dsDNA binding to form a joint molecule (figure 1.8, **2**). Next is the most mysterious step of the process in which homology is recognized and strand exchange takes place (figure 1.8, **3**). The intriguing possibility of a novel stable triplex is supported by the studies of deproteinized distal joint molecules (figure 1.8, **A** and **B**).[20-26] It is not certain, however, that such structures represent true recombination intermediates as structural changes may be caused by the protein removal process. Joint molecules formed in the presence of γS-ATP[28] do not show triplex properties but this may be because these complexes represent intermediates which occur after the proposed novel triplex intermediate. The proposed strand exchange models of Norden and coworkers[17] and of Adzuma[28] dictate slightly different structures of the final complex in the γS-ATP reaction in which product release does not occur. The structure proposed by Norden and coworkers (figure 1.8, **C**) contains no empty DNA binding sites whereas that proposed by Adzuma (figure 1.8, **D**)

has an open binding site available for ssDNA binding. The final step of the strand exchange process, which requires ATP, is the release of the displaced strand, the heteroduplex, and free RecA (figure 1.8, **4**).

Further studies are needed to fully elucidate the mechanism of RecA-mediated strand exchange. In particular, more structural studies of stable joint molecules are needed to determine whether or not a novel triplex intermediate plays a role in strand exchange and, if so, to determine the structure of such a triplex.

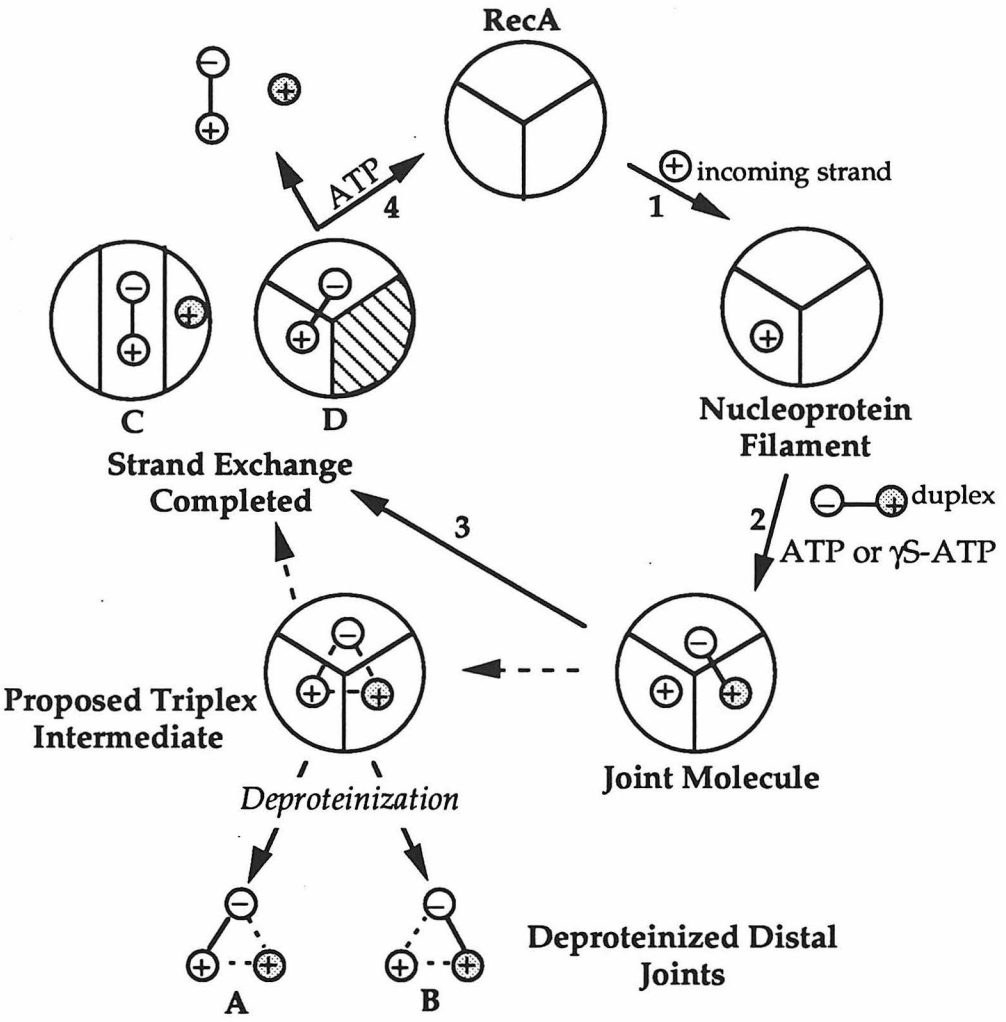

Figure 1.8. Summary of studies of RecA-catalyzed strand exchange. The RecA protein is represented by a circle partitioned into three DNA binding sites. ssDNA is represented by a small circle and dsDNA by a dumbbell. The relative orientation of the strands is indicated by "+ or "–". **(1)** Nucleoprotein filament formation between ssDNA and RecA. **(2)** Duplex binding results in joint molecule formation. This step requires a nucleotide cofactor (ATP or γS-ATP). **(3)** A search for homology ensues which results in strand exchange. A novel triplex has been proposed as an intermediate in the homology search process. Schematic structures of the deproteinized distal joints studied by Radding and coworkers[23] **(A)** and Camerini-Otero and coworkers[26] **(B)** are shown. Solid lines indicate Watson-Crick base-pairing and dashed lines indicate novel triplex interactions. Adzuma[28] and Norden and coworkers[17] have proposed slightly different structures of the joint molecule after strand exchange and before product release. In the structure proposed by Adzuma, the molecule has an open DNA binding site **(C)** whereas all DNA binding sites are occupied in the structure proposed by Norden and coworkers **(D)**. **(4)** The release of products requires ATP.

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Chapter 2: A Manuscript in Preparation for *PNAS*

Affinity Cleavage of Double-Stranded DNA Directed by a RecA Nucleoprotein Filament Containing an Oligonucleotide-EDTA•Fe(II)

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Abstract

Escherichia coli RecA protein promotes strand exchange between two homologous DNAs by polymerizing on single-stranded DNA to form nucleoprotein filaments which then bind to a homologous sequence of duplex DNA. Joint formation between the RecA protein and three strands of DNA is followed by strand exchange in which the original single strand is inserted into the duplex while the homologous duplex strand is displaced out. The structures of the strand exchange intermediates formed by the RecA protein have not been elucidated. We have used affinity cleaving techniques to probe the structure of the joint molecule formed between a RecA-oligonucleotide filament and duplex DNA by incorporating a thymidine-EDTA•Fe (T*) into the oligonucleotide of the filament. We find that the nucleoprotein filament binds antiparallel to the complementary strand within the homologous sequence of DNA and that it is associated more strongly with the complementary strand than with the homologous strand of the duplex.

Introduction

The RecA protein of *E. Coli* (38 kD) plays a central role in DNA recombination and repair. *In vitro*, RecA protein directs strand exchange between two DNA molecules of homologous sequence when one of the pieces of DNA is completely or partially single-stranded.[1-5] In the presence of ATP, RecA polymerizes on single-stranded DNA forming a right handed helical nucleoprotein filament in which the DNA is significantly unwound relative to B-form DNA. The filament then binds sequence-specifically to a site on duplex DNA which is complementary to the sequence of the single strand bound in the filament. In this synapsis step, a joint molecule is formed which contains the three strands of DNA and numerous RecA monomers. The final step is the release of the strand exchange products; a displaced single strand and a heteroduplex. In the presence of the non-hydrolyzable ATP analog, γ–S-ATP, the reaction does not proceed past synapsis and the three strands of DNA remain bound in the joint molecule.[4,6,7]

The precise structure and mechanism of action of the joint molecule formed in RecA-mediated strand exchange have not been elucidated. It is generally accepted that the three DNA strands are held stably within the RecA filament during the synapsis step of the reaction.[8-13] Two basic mechanisms of strand exchange have been postulated which require different joint molecule structures.[14,15] The first mechanism involves duplex strand separation before pairing. More specifically, local opening of a region of duplex is followed by Watson-Crick base-pairing of the single strand with its complementary strand of the denatured duplex. For the second mechanism, which involves pairing before strand separation, a fully base-paired duplex makes additional base-specific interactions with the homologous single strand within the RecA-associated

complex. A three-stranded structure is formed which then undergoes strand exchange.

The second mechanism requires the formation of a novel three-stranded DNA structure involving specific interactions defined by sequence homology between double-stranded and single-stranded DNA. Evidence for[10,16-22] and against[23,24] such a structure has been reported. Radding and coworkers[15-18] and Camerini-Otero and coworkers[20,21] have described the RecA-mediated formation of triple-stranded joint molecules which, after deproteinization, are thermostable and show chemical reactivity consistent with a novel triplex structure. The deproteinized joints are proposed to be structurally similar to an intermediate in the recognition of sequence homology in RecA-mediated strand exchange. However, the reactivities to dimethylsulphate and potassium permanganate of RecA-coated joint molecules formed in the presence of γ–S-ATP suggest that the strand to be displaced from the starting duplex is single-stranded in character while the other two strands behave more like a canonical duplex.[23] It is possible that the two observed structures represent recombination intermediates at different stages of synapsis. The novel triplex structure would presumably occur prior to the structure containing a Watson-Crick duplex and a displaced single strand. Alternatively, the deproteinized structures may not represent true recombination intermediates.

In order to characterize further the putative intermediate in RecA-mediated recombination, we have probed the structure of the joint molecule formed between a RecA nucleoprotein filament and duplex DNA by incorporating T*, a modified thymidine derivative wherein the DNA cleaving agent EDTA•Fe(II) is covalently attached at C5 of the thymine heterocycle, into the oligonucleotide used to form the nucleoprotein filament. By making use of

established EDTA•Fe(II) affinity cleavage techniques,[25,26] we hoped to obtain information about the structure of the joint molecule.

Materials and Methods

General

RecA protein was obtained from Pharmacia and was stored at -20 °C. Sonicated, deproteinized calf thymus DNA (Pharmacia) was dissolved in H_2O to a final concentration of 2.0 mM in base pairs and was stored at 4 °C. Glycogen was obtained from Boehringer-Mannheim as a 20 mg/ml aqueous solution. γ-S-ATP was purchased from Sigma and was stored at -20 °C. Nucleoside triphosphates labeled with ^{32}P were obtained from Amersham or ICN and were used as supplied. Cerenkov radioactivity was measured with a Beckman LS 2801 scintillation counter. Restriction endonucleases were purchased from Boehringer Mannheim or New England Biolabs and were used according to the supplier's recommended protocol in the activity buffer provided. Klenow fragment and T4 polynucleotide kinase were obtained from Boehringer Mannheim. Phosphoramidites were purchased from ABI or Cruachem.

Construction of pUCJWII47

This plasmid was prepared by standard methods.[27] Briefly, the plasmid was prepared by annealing two synthetic oligonucleotides, 5′-AATTCAGTTCTCCTCGACGAATTCTTTTTCTTTCTTCTTTTCTTCGAGTCGA GTCGAG-3′ and 5′-GATCCTCGACTCGACTCGAAGAAAAGAAGAAAGAAA-AAGAATTCGTCGAGGAGAACTG-3′, followed by ligation of the resulting duplex with pUC19 DNA previously digested with EcoRI and BamHI; this

ligation mixture was used to transform *E. Coli* XL1-Blue competent cells (Stratagene). Plasmid DNA from ampicillin resistant white colonies was isolated, and the presence of the desired insert was confirmed by restriction analysis and Sanger sequencing. Preparative isolation of plasmid DNA was performed using a Qiagen plasmid kit.

Synthesis and Purification of Oligodeoxyribonucleotides

Oligodeoxyribonucleotides were synthesized using standard automated solid-support chemistry on an Applied Biosystems Model 380B DNA synthesizer and *O*-cyanoethyl-*N,N*-diisopropyl phosphoramidites. Crude oligonucleotide products containing the 5′-terminal dimethoxytrityl protecting group were purified by reverse phase FPLC using a ProRPC 16/10 (C_2-C_8) column (Pharmacia LKB) and a gradient of 0-40% CH_3CN in 0.1 M triethylammonium acetate, pH 7.0, detritylated in 80% aqueous acetic acid, and chromatographed a second time.

5′-Dimethoxytrityl-2′-deoxyuridine-5-[2-(*N*′-(trifluoroacetyl)amino)-ethyl]propionamide-3′-(*O*-cyanoethyl)-*N,N*-diisopropyl phosphoramidite was prepared as described[28] and incorporated at the 5′ end of oligonucleotide **1**. Deprotection was carried out in concentrated NH_4OH at 55 °C for 24 h. Following trityl-on and trityl-off FPLC purification, the oligonucleotide containing a free amine attached to C5 of the 5′-thymidine was post-synthetically modified with EDTA monoanhydride as described[28] to give the T* oligonucleotide **1**. The modified oligonucleotide was chromatographed a third time by FPLC using a gradient of 0-40 % CH_3CN in 0.1 M NH_4OAc.

Thymidine-EDTA was prepared as described[25] and incorporated at the 3′ end of oligonucleotide **2** via the 5′-*O*-DMT-thymidine-EDTA-triethlyester 3′-succinyl controlled pore glass.[29] Deprotection was carried out in 0.1 N NaOH at 55 °C for 24 h.

Purified oligonucleotides were desalted on Pharmacia NAP-5 Sephadex columns. The concentrations of single-stranded oligonucleotides were determined by UV absorbance at 260 nm using extinction coefficients calculated by addition of the monomer nucleoside values (values for T were used in place of T* in the calculation). Oligonucleotide solutions were lyophilized to dryness for storage at -20 °C.

DNA Manipulations

The 300 bp HindIII/NdeI restriction fragment of the plasmid pUCJWII47 was isolated and labeled at the 5'- or 3'-end by standard procedures.[27] Adenine-specific sequencing reactions were carried out as previously described.[30]

Affinity Cleavage Reactions

A dried pellet of oligonucleotide-EDTA was dissolved in a solution of aqueous $Fe(NH_4)_2(SO_4)_2 \bullet 6H_2O$ to produce a solution that was 40 μM in oligonucleotide and Fe(II). This solution was allowed to equilibrate for 15 min at room temperature. A stock solution was prepared containing 60 μl 10x buffer (250 mM Tris-acetate, 40 mM Mg-acetate, 1 mM EGTA, 5 mM spermidine, 8 mM β-mercaptoethanol, pH 7.5), 30 μl calf thymus DNA (2 mM in bp), and 20 μl acetylated BSA (3 mg/ml). To a given reaction was added 5.5 μl stock solution, 3 μl of either the oligonucleotide-EDTA•Fe(II) at the appropriate concentration or H_2O (for control reactions), 3 μl of either oligonucleotide **3** at the appropriate concentration (for reactions in Figure 5 only) or H_2O, 9.5 μl of RecA solution (7.4 mg RecA/ml RecA storage buffer) plus RecA storage buffer (20 mM Tris-HCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, pH 7.5) to give the desired nucleotide to monomer ratio (excess RecA storage buffer was added to ensure that each reaction was run under the same conditions), and enough H_2O to give a final

reaction volume of 30 µl after DTT addition. Following a 1 min incubation at 37 °C, nucleoprotein filament formation was initiated by addition of 1 µl 30 mM γ–S-ATP. After 10 min, approximately 20,000 cpm 3'- or 5'-labeled duplex was added and joint molecule formation was allowed to proceed for 30 min at 37 °C. The cleavage reactions were initiated by the addition of 1.2 µl 100 mM DTT. The final reaction conditions were 25 mM Tris-acetate, 6 mM Tris-HCl, 4 mM $Mg(OAc)_2$, 1 mM EGTA, 32 µM EDTA, 0.5 mM spermidine, 0.8 mM β-mercaptoethanol, 15% glycerol, 4.3 mM DTT, pH 7.5. After 8 h, the cleavage reactions were stopped by precipitation of the DNA by the addition of glycogen, NaOAc (pH 5.2), and $MgCl_2$ to final concentrations of 140 µg/ml, 0.3 M, and 10 mM, respectively, and 100 µl ethanol. The DNA was isolated by centrifugation and removal of the supernatant. The precipitate was dissolved in 20 µl H_2O, frozen, and lyophilized to dryness. The DNA in each tube was resuspended in 5 µl of formamide-TBE loading buffer containing 0.1% SDS and transferred to a new tube. The DNA solutions were assayed for Cerenkov radioactivity by scintillation counting and diluted to 2000 cpm/µl with more formamide-TBE loading buffer containing 0.1% SDS. The DNA was denatured at 90 °C for 5 min, and loaded onto an 8% denaturing polyacrylamide gel. The DNA cleavage products were electrophoresed in 1x TBE buffer at 50 V/cm. The gel was dried on a slab dryer and then exposed to a storage phosphor screen. The gel was visualized with a Molecular Dynamics 400S PhosphorImager. The gel was then exposed to X-ray film (Amersham Hyperfine-MP) to obtain an autoradiogram.

Results and Discussion

Affinity cleavage directed by a RecA•oligonucleotide-EDTA filament has been used to characterize the structure of the joint molecule formed in RecA-mediated homologous recombination. T* was incorporated at either the 5′- or 3′-end of an oligonucleotide (**1** and **2**, respectively, in Figure 2) which was homologous to a 31-bp target site in the plasmid pUCJWII47 (Figure 2). RecA was incubated with either oligonucleotide **1** or **2** in the presence of γ–S-ATP to form a nucleoprotein filament after which 5′- or 3′-^{32}P-labeled duplex substrate was added to form the joint molecule (Figure 1). This joint molecule is trapped because there is no hydrolyzable ATP present to complete the reaction. DTT was then added to initiate the cleavage reactions. The cleavage products were separated using denaturing polyacrylamide gel electrophoresis and analyzed by autoradiography.

The results using labeled complementary strand (3′-^{32}P-labeled restriction fragment) and labeled homologous strand (5′-^{32}P-labeled restriction fragment) are shown in Figure 3. No cleavage is observed on either strand in control experiments in which RecA (lanes 3 and 15) or both RecA and the T* oligonucleotide are absent from the reaction (lanes 2 and 14). Surprisingly, when RecA is present in the absence of T* oligonucleotide, weak cleavage is observed on the complementary strand several residues outside of the target site (lane 4). This cleavage may result from RecA polymerizing on the duplex, rendering certain sequences susceptible to nicking. When both RecA and T* oligonucleotide are present, cleavage is observed near the ends of the designated target sequence (lanes 5-11, 17, and 18). The efficiency of cleavage is dependent both on the ratio of nucleotides to RecA monomers and on the concentration of nucleoprotein filament. Using labeled complementary strand as substrate, the nucleotide-to-monomer

ratio is varied from 4:1 to 2:1 in lanes 5-7 while the concentration of **1** is held at 4 μM. Maximum cleavage of the complementary strand is observed at 4 μM **1** with a nucleotide-to-monomer ratio of 2:1 (lane 6). In lanes 8-10, the nucleotide-to-monomer ratio is held at the optimum value of 2:1 while the concentration of the RecA-**1** filament is varied from 1 μM to 4μM. The most efficient cleavage is observed at a RecA-**1** filament concentration of 4 μM (lane 10). Lane 11 shows cleavage observed with 4 μM RecA-**2** filament at a nucleotide to monomer ratio of 2:1.

The efficiency of cleavage produced at each nucleotide of the radiolabeled target DNA by T* oligonucleotide at 4 μM with 2 nucleotides per RecA monomer was measured from the photostimulable storage phosphor autoradiogram in Figure 3 (lanes 10, 11, 17, and 18). The results of this analysis are presented as histograms where the lengths of the arrows represent the relative amounts of cleavage found at each nucleotide (Figure 4). In reactions with labeled complementary strand, two cleavage sites are seen with each filament. The site of major cleavage in both cases is at the expected location of the T* and extends over seven contiguous nucleotides. The maxima occur within the joint molecule adjacent to the base to which the T* is targeted. Based on the role played by the RecA protein in homologous recombination, the predicted binding orientation of a RecA nucleoprotein filament on a homologous segment of duplex DNA is parallel to the homologous strand of the duplex. The locations of the major cleavage patterns observed with the RecA-T* oligonucleotide filaments in this study are consistent with such an orientation.

Both filaments also produce weaker cleavage which extends over five contiguous nucleotides and is centered at the end of the target site furthest from the expected location of the T* (Figure 4). This minor cleavage may result from a second nucleoprotein filament binding to the filament at the target site (Figure 5).

Because RecA polymerizes in a head-to-tail fashion,[31] nucleoprotein filaments may also polymerize head-to-tail through protein-protein contacts between the RecA monomers bound at the filament ends. The 3'-end of the filament positioned at the target site would be associated with the 5'-end of a second filament such that the 5'-T* of **1** in the second filament would be pulled in close enough to the duplex to effect cleavage at the minor cleavage site observed with the RecA-**1** filament. Similarly, the 3'-T* of a filament containing **2**, if associated with the 5'-end of the filament located at the target site, would be positioned correctly to produce the weaker cleavage observed with the RecA-**2** filament. Because the associated nucleoprotein filaments adjacent to the filament bound at the target site do not make sequence-specific contacts with the duplex DNA, the sequence of the oligonucleotide in the associated filament is not required to be identical to that of the bound filament.

In order to test this model (Figure 5), we examined the effect on cleavage of the complementary strand by the RecA-**1** and RecA-**2** filaments when a 30mer oligonucleotide of random sequence (Figure 2, **3**) was added to the reaction solution. The results of this experiment are shown in Figure 6. As in Figure 3, no cleavage is observed in the absence of RecA (lanes 2, 3) and weak cleavage is observed several residues outside of the target site when RecA is present in the absence of T* oligonucleotide (lane 4). In lanes 5-10, the concentration of either **1** (lanes 5-7) or **2** (lanes 8-10) is varied from 4 μM to 1 μM as the concentration of **3** is adjusted to maintain 4 μM total oligonucleotide concentration. The nucleotide to monomer ratio in lanes 5-10 is held at the optimum value of 2:1. The cleavage intensity at each of the seven and five nucleotide positions at the sites of major and minor cleavage, respectively, were summed. When the ratio of **1**:**3** was 4:0 (lane 5), 2:2 (lane 6), and 1:3 (lane 7), the ratio of the total cleavage measured at the predicted T* location to that measured at the opposite end of the target site

was 3:1, 6;1, and 9:1, respectively. Similarly, when the ratio of **2:3** was 4:0 (lane 8), 2:2 (lane 9), and 1:3 (lane 10), the ratios were 2: 1, 3:1, and 5:1, respectively. Thus, for both the RecA-**1** and RecA-**2** filaments, cleavage remains strong at the predicted T* location as the concentration of the RecA-**3** filament is increased while cleavage at the other end of the target site is reduced dramatically.

The RecA-**3** filament should not bind specifically to the labeled duplex but is capable of binding nonspecifically by polymerizing with other filaments. In the absence of RecA-**3** filament, the population of filaments located proximal to the target site consists exclusively of filaments containing T* oligonucleotides. As the concentration of RecA-**3** filament is increased, the minor cleavage patterns disappear because the percentage of RecA-T* oligonucleotide filaments bound proximally to the target site decreases. The population of filaments bound specifically at the target site, however, consists completely of RecA-T* oligonucleotide filaments, and thus the increasing RecA-**3** filament concentration has a much smaller effect on the cleavage patterns located at the predicted T* binding sites.

Cleavage of the homologous strand with the RecA-**1** filament produces a very weak cleavage pattern which extends over four nucleotides and is centered within the joint molecule 3 bases away from the expected location of the T*. No cleavage of the homologous strand is seen with the RecA-**2** filament. The disparity in the amount of cleavage observed on the homologous strand as compared to the complementary strand suggests that the T* of the oligonucleotide bound in the nucleoprotein filament is located closer in space to the complementary strand than to the homologous strand. The data is consistent with a joint molecule structure in which the single strand and the complementary strand interact through Watson-Crick base pairing while the homologous strand is displaced from the new duplex. A structure involving novel interactions between all three strands is inconsistent with the lack of significant cleavage on the homologous

strand.[26,32] The joint molecule characterized here is thus more like the recombination intermediate studied by Adzuma[23] than those studied by Camerini-Otero and coworkers[19,20] and Radding and coworkers.[16-19]

Conclusion

We have used affinity cleaving techniques to probe the structure of the joint molecule formed between a RecA-oligonucleotide filament and duplex DNA using an oligonucleotide with T* attached at either the 5'- or 3'-end. We observed cleavage of the complementary duplex strand at the site predicted by antiparallel binding of the filament to its complementary sequence. We also observed weaker cleavage of the complementary strand at the opposite end of the predic-ted binding site which disappears when a RecA filament containing a nonT*- oligonucleotide of random sequence is added to the reaction. Thus, we believe that this weaker cleavage is due to the T* of a second filament which binds through protein-protein interactions to the filament positioned at the target site. Almost no cleavage was observed on the homologous strand suggesting that the single strand of the joint molecule interacts mainly with the complementary strand perhaps through Watson-Crick base pairing.

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Figure Captions

Figure 1. Sequence-specific cleavage of duplex DNA directed by a RecA nucleoprotein filament formed with an oligonucleotide containing thymidine-EDTA•Fe(II) (T*). In the first step, RecA monomers polymerize on the T* oligonucleotide (* denotes the location of T*) to form a nucleoprotein filament. Synapsis follows in which the nucleoprotein filament binds to its complementary sequence on a ^{32}P-labeled duplex substrate forming a joint molecule. The cleavage reaction is initiated by the addition of DTT.

Figure 2. The duplex target site present on the 300-bp HindIII/NdeI restriction fragment from plasmid pUCJWII47. The expected binding site of the RecA nucleoprotein filament is boxed. The sequences of oligonucleotides **1**, **2**, and **3** are shown, where T* indicates the position of thymidine-EDTA•Fe(II).

Figure 3. Autoradiogram of an 8% denaturing polyacrylamide gel used to separate affinity cleavage products. The cleavage reactions were carried out as described in the methods section. Briefly, RecA was incubated with **1** or **2** for 10 min at 37 °C in the presence of γ–S-ATP to form a nucleoprotein filament. The 300 bp HindIII/NdeI restriction fragment of pUCJWII47 ^{32}P-labeled at either the 3'- (complementary strand labeled) or 5'-end (homologous strand labeled) was then added to allow joint molecule formation. After 30 minutes, the cleavage reactions were initiated by addition of DTT and allowed to proceed for 8 h at 37 °C. (Lane 1) Untreated 3'-labeled duplex. (Lanes 2-4) Intact 3'-labeled duplex obtained from control reactions in the absence of RecA and T* oligonucleotide (Lane 2), in the presence of RecA (62 μM) with no T* oligonucleotide (Lane 3), and in the presence of **1** with no RecA (Lane 4). (Lanes 5-11) Affinity cleavage

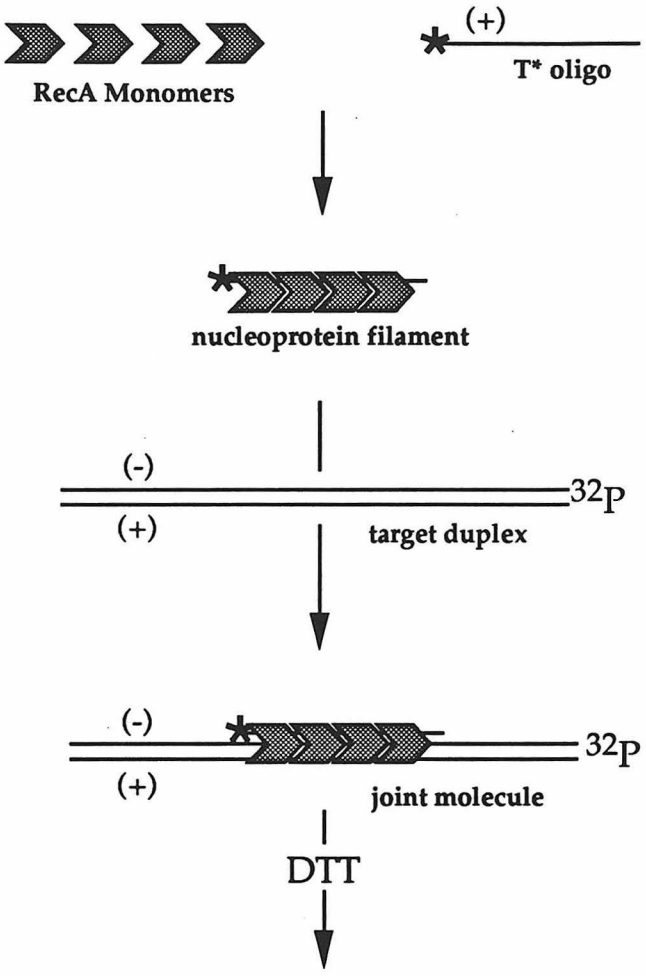
products of 3'-labeled duplex produced by RecA-**1** (Lanes 5-10) or RecA-**2** (Lane 11) filaments with oligonucleotide concentrations and nucleotide-to-monomer ratios as indicated above the autoradiogram. (Lane 12) Products of an adenine-specific sequencing reaction of 3'-labeled DNA. (Lane 13) Untreated 5'-labeled duplex. (Lanes 14-16) Intact 5'-labeled duplex obtained from control reactions in the absence of RecA and T* oligonucleotide (Lane 14), in the presence of RecA (62 μM) with no T* oligonucleotide (Lane 15), and in the presence of **1** with no RecA (Lane 16). Affinity cleavage products of 5'-labeled duplex produced by RecA-**1** (Lanes 17) or RecA-**2** (Lane 18) filaments. (Lane 19) Products of an adenine-specific sequencing reaction of 5'-labeled duplex.

Figure 4. Histograms showing cleavage patterns observed with **(A)** RecA-**1** and **(B)** RecA-**2** filaments. The target site is boxed and the lengths of the arrows represent relative cleavage efficiencies obtained with a Molecular Dynamics 400S Phosphorimager.

Figure 5. A model of head-to-tail binding of filaments proposed as an explanation for the minor cleavage sites observed in the presence of both RecA-**1** and RecA-**2** filaments. The filaments in the figure contain **1**. The center filament is located at the target site while the two outer filaments are associated with the joint molecule through protein-protein interactions between RecA monomers. (+) indicates the sequence of **1** as well as that of the homologous duplex strand and (-) indicates the sequence of the complementary duplex strand.

Figure 6. Autoradiogram of an 8 % denaturing polyacrylamide gel used to separate affinity cleavage products. The cleavage reactions were carried out as described in the methods section and in the Figure 3 caption. **3** was added at the

same time as **1** or **2**. (Lane 1) Untreated 3′-labeled duplex. (Lanes 2-4) Intact 3′-labeled duplex obtained from control reactions in the absence of RecA and T* oligonucleotide (Lane 2), in the presence of RecA (62 μM) with no T* oligonucleotide (Lane 3), and in the presence of **1** with no RecA (Lane 4). (Lanes 5-10) Affinity cleavage products of 3′-labeled duplex produced by RecA-**1** (Lanes 5-7) or RecA-**2** (Lanes 8-10) filaments with **1**, **2**, and **3** concentrations as indicated above the figure. The nucleotide to monomer ratio in lanes 5-10 was 2:1. (Lane 11) Products of an adenine-specific sequencing reaction of 3′-labeled duplex.


RecA Monomers
(+)
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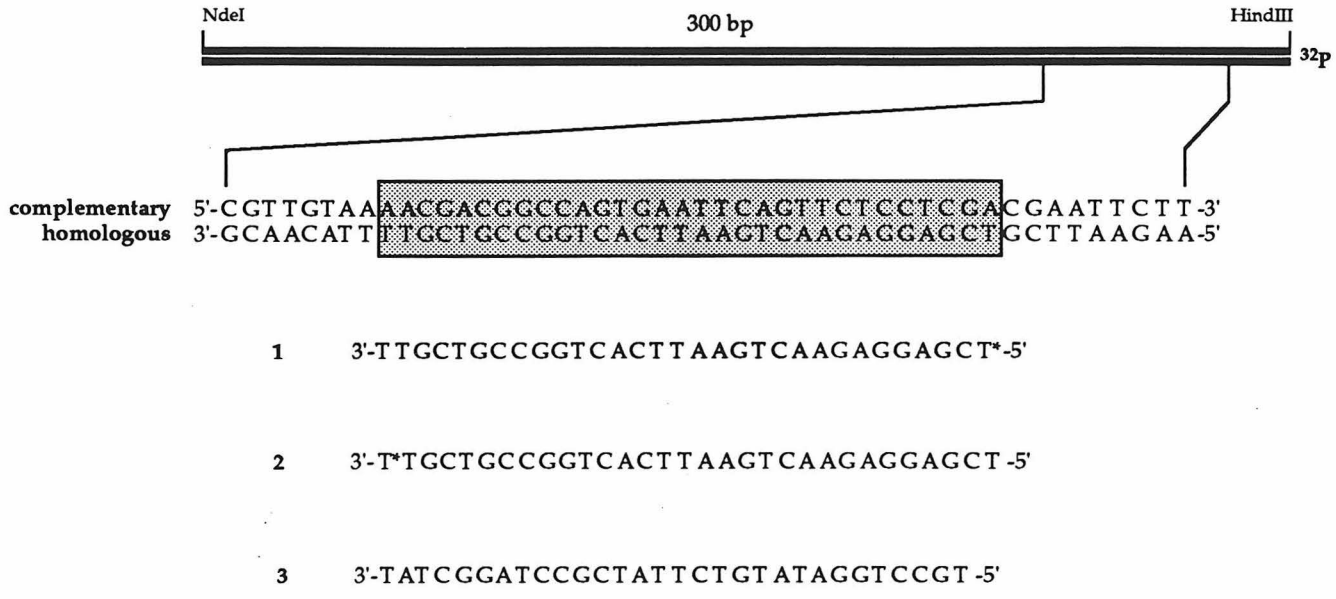
nucleoprotein filament
(-)
(+)
32P
target duplex
(-)
(+)
32P
joint molecule
DTT
Affinity Cleavage Products

Figure 1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300 bp
HindIII
32p
complementary 5'-CGTTGTAAAACGACGGCCAGTGAATTCAGTTCTCCTCGACGAATTCTT-3'
homologous 3'-GCAACATTTTGCTGCCGGTCACTTAAGTCAAGAGGAGCTGCTTAAGAA-5'
1 3'-TTGCTGCCGGTCACTTAAGTCAAGAGGAGCT*-5'
2 3'-T*TGCTGCCGGTCACTTAAGTCAAGAGGAGCT-5'
3 3'-TATCGGATCCGCTATTCTGTATAGGTCCGT-5'

Figure 2

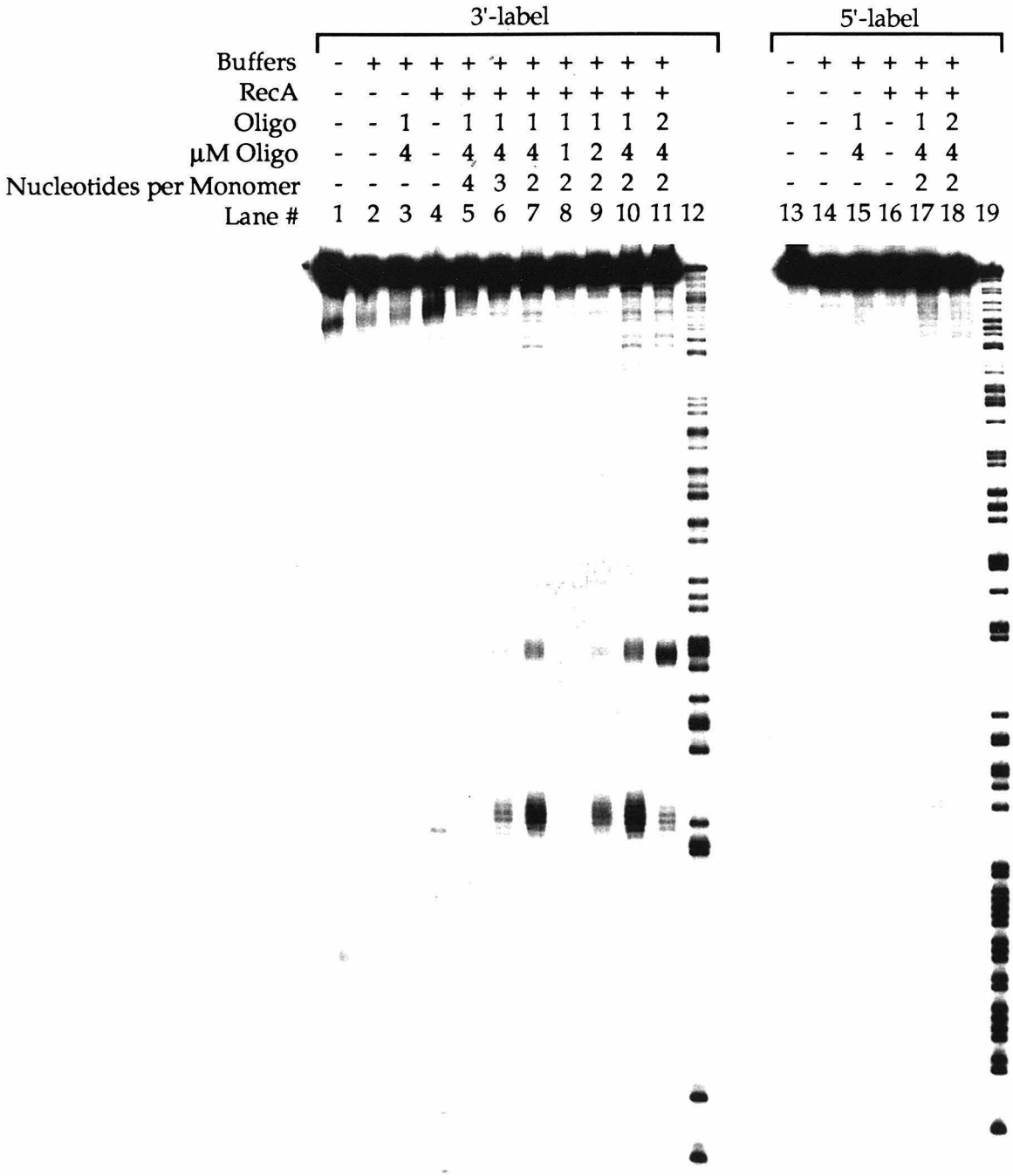
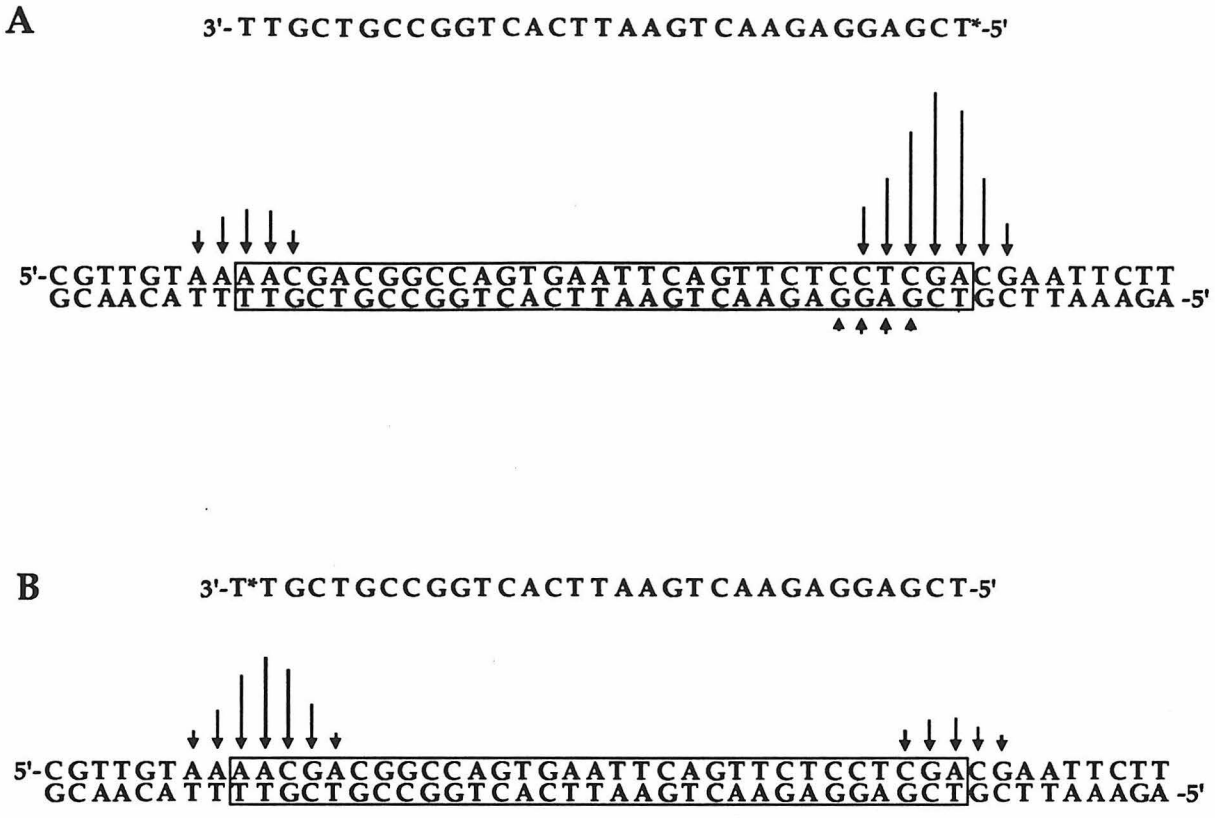

3'-label
5'-label
Buffers - + + + + + + + + + + - + + + + +
RecA - - - + + + + + + + + - - - + + +
Oligo - - 1 - 1 1 1 1 1 1 2 - - 1 - 1 2
µM Oligo - - 4 - 4 4 4 1 2 4 4 - - 4 - 4 4
Nucleotides per Monomer - - - - 4 3 2 2 2 2 2 - - - - 2 2
Lane # 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure 3


A
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5'-CGTTGTAAAACGACGGCCAGTGAATTCAGTTCTCCTCGACGAATTCTT
GCAACATTTTGCTGCCGGTCACTTAAGTCAAGAGGAGCTGCTTAAAGA-5'
B
3'-T*TGCTGCCGGTCACTTAAGTCAAGAGGAGCT-5'
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Figure 4

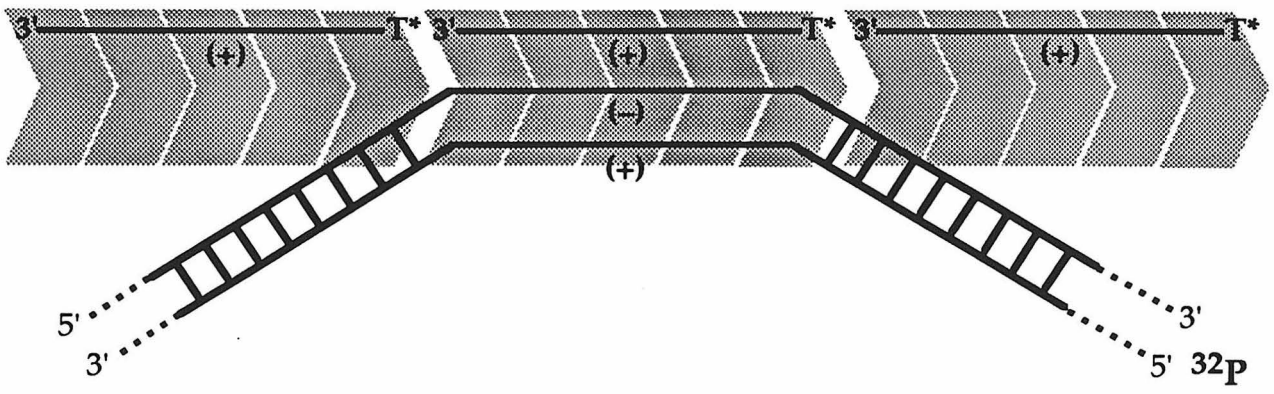

Figure 5

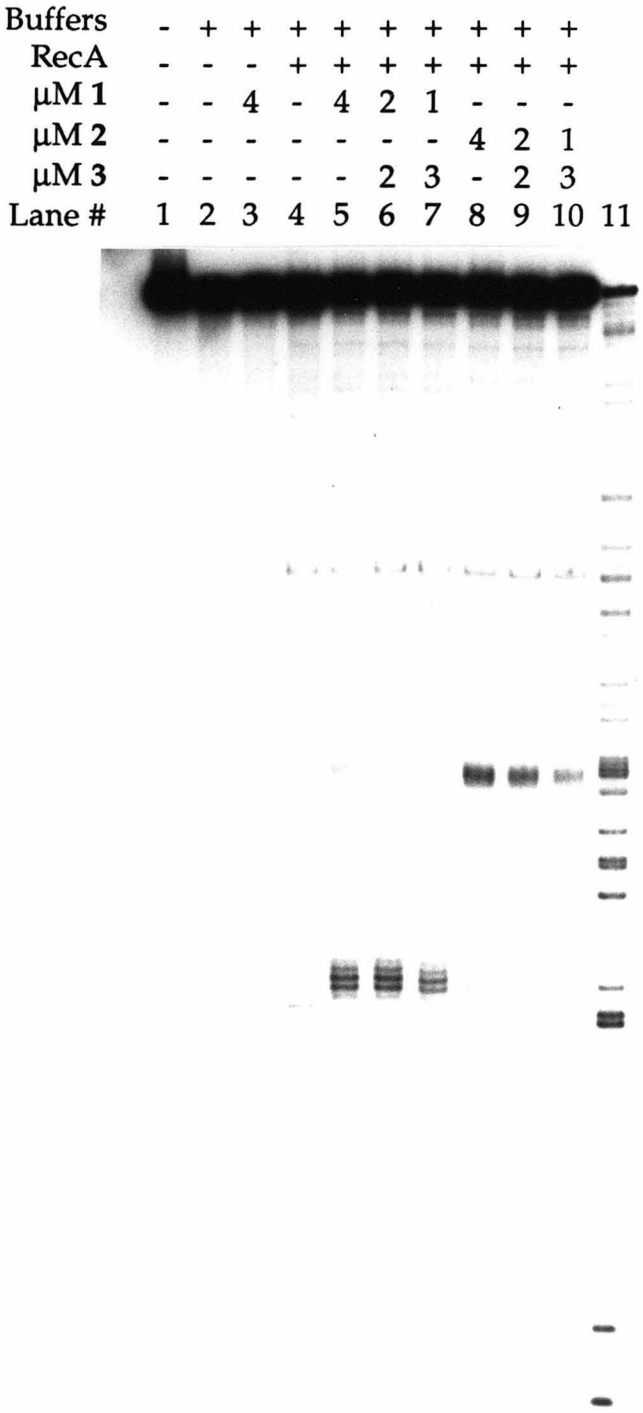

Figure 6

Appendix: Future Experiments

Affinity cleavage studies could be used to further investigate ternary complexes formed between RecA, ssDNA, and dsDNA. Norden and coworkers observe that when ssDNA is added to RecA followed by dsDNA (RecA-ssDNA-dsDNA), the bases of all three strands are perpendicular to the helix axis.[15] However, coplanarity of all bases is not observed when dsDNA is added to the protein before ssDNA (RecA-dsDNA-ssDNA). These complexes may represent different strand exchange intermediates. Affinity cleavage studies of such complexes could affirm that they are different and could provide information regarding how they differ.

Affinity cleavage could also be used to investigate the relative polarity of RecA-bound DNA strands. The strand exchange model proposed by Norden and coworkers[17] (figure 1.2) dictates that dsDNA must bind to the RecA-oligonucleotide filament with the outgoing duplex strand located in site II. The model is supported by renaturation studies which indicate that when three strands are added sequentially to RecA, the first two bind with the same polarity, which is opposite to the polarity of the third strand.[16] Direct evidence for such an arrangement could be provided by affinity cleavage studies of RecA-ssDNA complexes.

The experimental design for studying the differences between the RecA-ssDNA-dsDNA complex and the RecA-dsDNA-ssDNA complex is illustrated in figures A.1 and A.2. The DNA substrates are a 31-base oligonucleotide containing T* (figure A.1, **W***) and a 41-bp ^{32}P-labeled duplex complementary to **W*** with five extra bases on either end (figure A.1, **W-C**)). In the first experiment (figure A.2, **A**), **W*** is added to the protein followed by addition of an equivalent

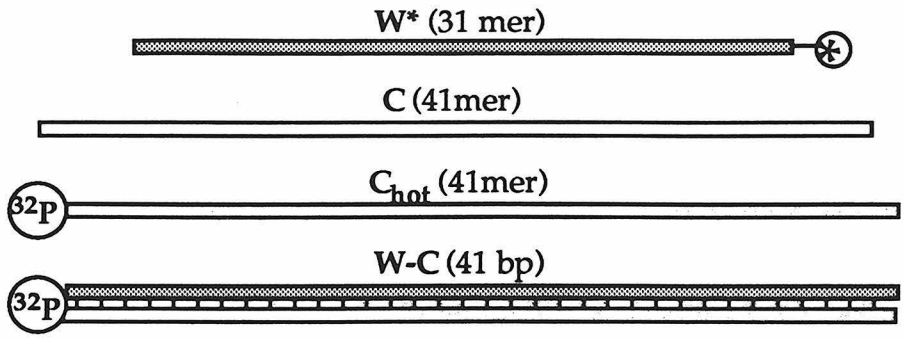

Figure A.1. Substrates for the proposed experiments. DNA strands are represented by rectangles. Shaded and unshaded rectangles represent oligonucleotides with complementary sequences. The location of T* is indicated by a circled "*". **W*** is a 31-base oligonucleotide containing T*. **C** is a 41-base oligonucleotide complementary to W* with five extra bases on either end. C_{hot} is a ^{32}P-labeled oligonucleotide identical to **C** in sequence. **W-C** is a 41-bp ^{32}P-labeled duplex formed between **C** and its complement.

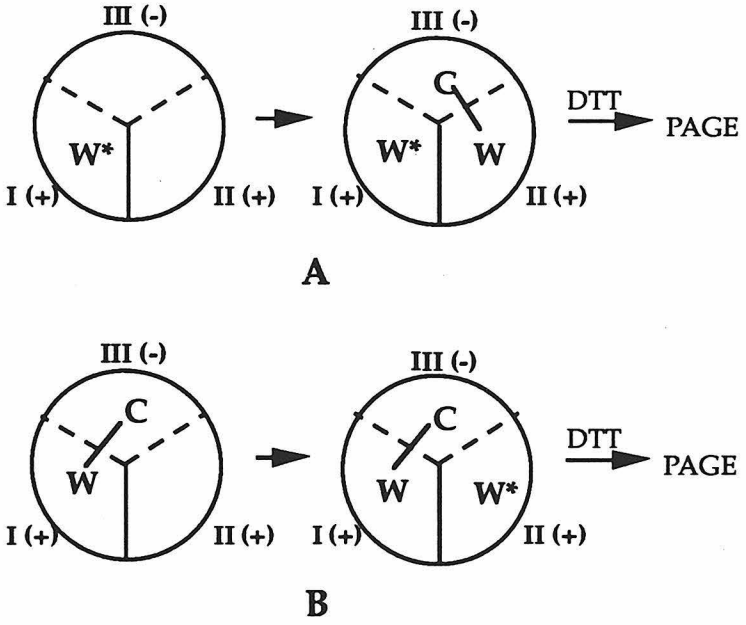

Figure A.2. Schematic illustrations of experiments designed to investigate the ternary complexes formed between RecA, dsDNA, and ssDNA. The RecA protein is represented by a circle partitioned into three DNA binding sites (I, II, and III). The predicted relative orientation of DNA bound to each of the sites[17] is indicated by (+) or (–). **A**, The RecA-ssDNA-dsDNA complex is formed by incubating RecA with 31mer **W*** followed by addition of the complementary duplex **W-C**. **B**, The RecA-dsDNA-ssDNA complex is formed by reversing the order of **W*** and **W-C** addition. In both **A** and **B**, the affinity cleavage reaction is initiated by addition of DTT and the products are resolved by denaturing PAGE.

concentration of **W-C** to form the RecA-ssDNA-dsDNA complex. In the second experiment (figure A.2, **B**), the order of addition is reversed to form the RecA-dsDNA-ssDNA complex. Comparison of the cleavage patterns from the two experiments may help to detect differences between the two complexes.

The experimental design for studying the relative polarity of DNA strands bound to RecA is shown in figures A.1 and A.3 and table A.1. The oligonucleotide substrates are shown in figure A.1. They are **W***, a T*-containing 31mer; **C**, a 41mer complementary to **W*** with five extra bases on either end; and $\mathbf{C_{hot}}$, a ^{32}P-labeled 41mer identical in sequence to **C**. Equivalent concentrations of the three oligonucleotides are added in varying order to RecA in the presence of γS-ATP. Following complex formation, DTT is added to initiate cleavage and the products are analyzed by denaturing PAGE. There are six possible ways in which the order of oligonucleotide addition can be varied (table A.1, experiments 1-6). Experiment 1 is shown schematically in figure A.3. In a given experiment, sequence specific cleavage of the labeled strand ($\mathbf{C_{hot}}$) indicates that it was bound with opposite polarity to the T* oligonucleotide (**W***). The results from all six experiments should help elucidate the relative polarity of the strands bound to the RecA protein.

Experiment	Order of Substrate Addition
1	W*, C, C_{hot}
2	W*, C_{hot}, C
3	C, W*, C_{hot}
4	C, C_{hot}, W*
5	C_{hot}, W*, C
6	C_{hot}, C, W*

Table A.1. Order of addition of the oligonucleotide substrates in the six experiments designed to investigate the relative polarity of DNA strands bound to the RecA protein.

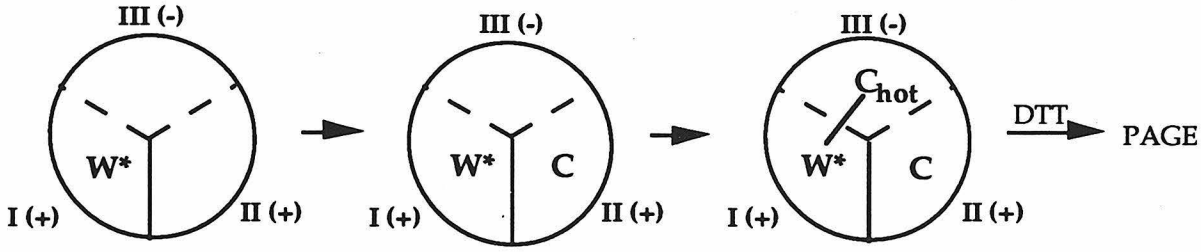

Figure A.3. Schematic illustration of experiment 1 (table A.1) which is one of six experiments proposed to investigate the relative orientation of DNA strands bound to the RecA protein. The RecA protein is represented by a circle partitioned into three DNA binding sites (I, II, and III). The relative orientation of DNA bound to each of the sites as predicted by Norden and coworkers[17] is indicated by (+) or (–). The oligonucleotides are added sequentially at equivalent concentrations in the order **W***, **C**, and then $\mathbf{C_{hot}}$ (oligonucleotide sequences shown in figure A.1). Predicted Watson-Crick base-pairing in the final complex is indicated by a solid line between **W*** and $\mathbf{C_{hot}}$. Following complex formation, dithiothreitol (DTT) is added to initiate afffinity cleavage. The products are resolved by denaturing polyacrylamide gel electrophoresis (PAGE).

References

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