Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 10393–10397, October 1995
Biochemistry

RecA·oligonucleotide filaments bind in the minor groove of double-stranded DNA

RAMESH BALIGA, JENNIFER W. SINGLETON, AND PETER B. DERVAN†

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125

Contributed by Peter B. Dervan, July 21, 1995

ABSTRACT **_Escherichia coli_ RecA protein, in the presence of ATP or its analog adenosine 5′-[γ-thio]triphosphate, polymerizes on single-stranded DNA to form nucleoprotein filaments that can then bind to homologous sequences on duplex DNA. The three-stranded joint molecule formed as a result of this binding event is a key intermediate in general recombination. We have used affinity cleavage to examine this three-stranded joint by incorporating a single thymidine-EDTA·Fe (T*) into the oligonucleotide part of the filament. Our analysis of the cleavage patterns from the joint molecule reveals that the nucleoprotein filament binds in the *minor groove* of an extended Watson–Crick duplex.**

The RecA protein of *Escherichia coli* (38 kDa) 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 segments of DNA is completely or partially single-stranded (1–4). In the presence of adenosine triphosphate (ATP), or its slowly hydrolyzed analog adenosine 5′-[γ-thio]triphosphate (ATP[γS]), RecA polymerizes on single-stranded DNA forming a right-handed helical nucleoprotein filament with a binding stoichiometry of approximately one RecA monomer for every three bases (5–8). The filament then binds sequence specifically to a site on duplex DNA that is homologous to the sequence of the single strand bound within the filament (9–12). In this synapsis step, a joint molecule is formed that contains the three strands of DNA and numerous RecA monomers. The final step is the release of the products of strand exchange—a displaced single strand and a heteroduplex. In the presence of topological constraints, such as when the 5′ end of the strand to be displaced is at an internal site within duplex DNA, the reaction does not proceed past synapsis and the three strands of DNA remain bound in the joint molecule (13, 14).

Although it is generally accepted that all three strands of DNA are held stably in the form of a joint molecule during synapsis (15–19), two different mechanisms of strand exchange can be envisaged that require different joint molecule structures (20). The first mechanism involves local opening of a region of duplex followed by Watson–Crick base-pairing of the single strand with its complementary strand (21). The outgoing strand either is completely displaced (22) or held associated with the newly formed heteroduplex by non-Watson–Crick interactions in the form of a triple helix (23). For the second mechanism, a fully base-paired duplex makes additional base-specific interactions with the homologous single strand within the RecA-associated complex giving rise to a triple helical complex, which is then processed to give the products of strand exchange (24, 25). Radding and coworkers (26, 27) as well as Camerini-Otero and coworkers (28, 29) have described the RecA-mediated formation of joint molecules, which, after deproteinization, are thermostable and show chemical reactivity consistent with a novel triplex structure (30). These deproteinized joint molecules are proposed to be structurally similar to an intermediate in RecA-mediated strand exchange, and two sets of base triplets have been proposed to explain their stability toward thermal denaturation. The proposed triplets are similar in the placement of the three bases but differ in the strands to which the bases are assigned. Radding and coworkers have proposed a structure where the incoming strand and its complement are base-paired in the Watson–Crick sense and the outgoing strand is associated through contacts in the major groove of the newly formed heteroduplex—a structure that would require local melting of the duplex and insertion of the third strand in the *minor groove* before formation of the joint molecule. On the other hand, Camerini-Otero and coworkers favor an intermediate where the incoming strand is located in the *major groove* of the duplex at the homologous site—a configuration consistent with pairing before strand separation.

Affinity cleavage is a technique wherein a redox-active metal ion tethered to a DNA binding molecule, upon exposure to a reductant, produces a short-lived diffusible oxidant—presumably hydroxyl radicals—to cause localized cleavage of the DNA backbone (for a review on affinity cleaving, see ref. 31). Here, we report evidence for groove location of the incoming third strand in the protein-associated three-stranded joint molecule using affinity cleavage (Fig. 1).

MATERIALS AND METHODS

General. RecA protein was isolated from the strain JC12772 using standard protocols. 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. ATP[γS] purchased from Sigma was found to contain <10% ADP by HPLC analysis 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 Glen Research (Sterling, VA).

Construction of pUCJWII47. This plasmid was prepared by standard methods (32) by annealing two synthetic oligonucleotides, 5′-AATTCAGTTCTCCTCGACGAATTCTTTT-TCTTTCTTCTTTTCTTCGAGTCGAGTCGAG-3′ and 5′-GATCCTCGACTCGACTCGAAGAAAAGAAGAAAGA-AAAAGAATTCGTCGAGGAGAACTG-3′, followed by ligation of the resulting duplex with pUC19 DNA previously digested with *Eco*RI and *Bam*HI; this ligation mixture was used to transform *E. coli* XL1-Blue competent cells (Strat-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

†To whom reprint requests should be addressed.

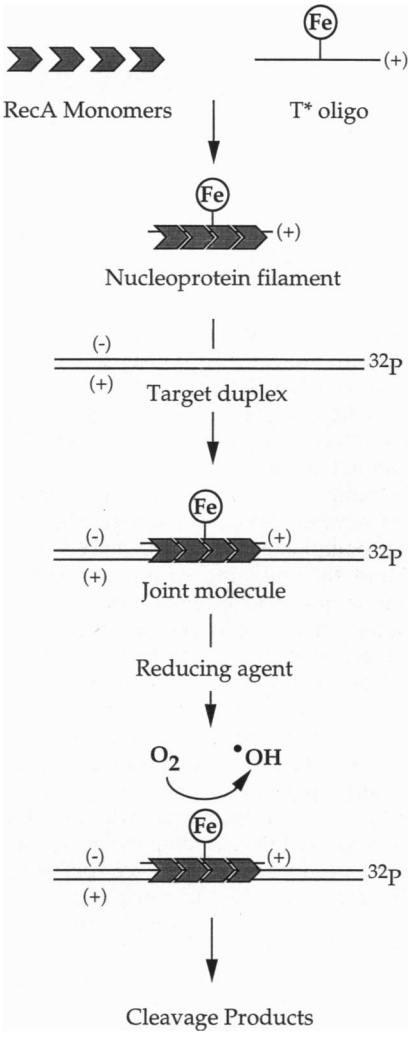

FIG. 1. Sequence-specific cleavage of duplex DNA directed by a RecA nucleoprotein filament formed with an oligonucleotide containing thymidine-EDTA·Fe (T*). In the first step, RecA monomers polymerize on the T* oligonucleotide to form a nucleoprotein filament. Synapsis follows in which the nucleoprotein 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 a reducing agent (sodium ascorbate).

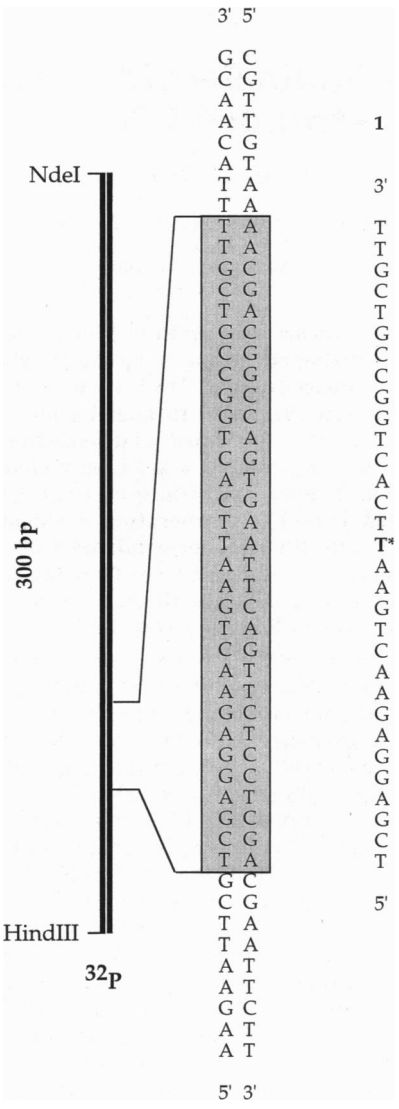

FIG. 2. Duplex target site present on the 300-bp *Hin*dIII/*Nde* I restriction fragment from plasmid pUCJWII47. The expected binding site of the RecA nucleoprotein filament is boxed. The sequence of oligonucleotide is shown, where T* indicates the position of thymidine-EDTA·Fe.

agene). Plasmid DNA from transformants 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 (Chatsworth, CA) 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.

Modified thymidine was incorporated at a central position in oligonucleotide **1** (Fig. 2) using the β-cyanoethyl-*N,N*-diisopropyl chlorophosphoramidite of a thymidine derivative containing an appropriately protected linker-amine (C2-dT, Glen Research). 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 postsynthetically modified with EDTA monoanhydride as described (33) 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.

Purified oligonucleotides were desalted on Pharmacia NAP-5 Sephadex columns. 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 *Hin*dIII/*Nde* I restriction fragment of plasmid pUCJWII47 (Fig. 2) was isolated and labeled at the 5′ or 3′ end on the *Hin*dIII side by standard procedures (32). Adenine-specific sequencing reactions were carried out as described (34).

Affinity Cleavage Reactions. A dried pellet of oligonucleotide-EDTA was dissolved in a solution of aqueous Fe-$(NH_4)_2(SO_4)_2 \cdot 6H_2O$ to produce a solution that was 20 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 of 10× buffer [250 mM Tris acetate/40 mM $Mg(OAc)_2$/1 mM EGTA/5 mM spermidine/8 mM 2-mercaptoethanol, pH 7.5], 30 μl of calf thymus DNA (2 mM in base pairs), and 20 μl of acetylated bovine serum albumin (3 mg/ml). To a given reaction was added 5.5 μl of stock solution, 3 μl of either the oligonucleotide-EDTA·Fe(II) at the appropriate concentration or H_2O [for control reactions, 3 μl of RecA solution (7.4 mg of RecA per ml of RecA storage buffer)] plus RecA storage buffer (20 mM Tris·HCl/0.1 mM EDTA/1 mM dithiothreitol/50% glycerol, pH 7.5) to give the designated 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 ascorbate addition. Following a 1-min incubation at 37°C, nucleoprotein filament formation was initiated by addition of 1 μl of 30 mM ATP[γS]. After 10 min, ≈20,000 cpm (≈1 nM) of 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 μl of 30 mM sodium ascorbate. 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 2-mercaptoethanol, 5% glycerol, 100 μM bp calf thymus DNA, and 1 mM sodium ascorbate (pH 7.5). After 8 h, the cleavage reactions were stopped by phenol/chloroform extraction and 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 of ethanol. The DNA was isolated by centrifugation and removal of the supernatant. The precipitate was dissolved in 20 μl of H_2O, frozen, and lyophilized to dryness. DNA in each tube was resuspended in 5 μl of formamide/TBE (TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) 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 5000 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 1× 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. Cleavage intensities at each nucleotide position were measured using the IMAGEQUANT software and the pixel values obtained were plotted in the form of histograms using KALEIDAGRAPH software.

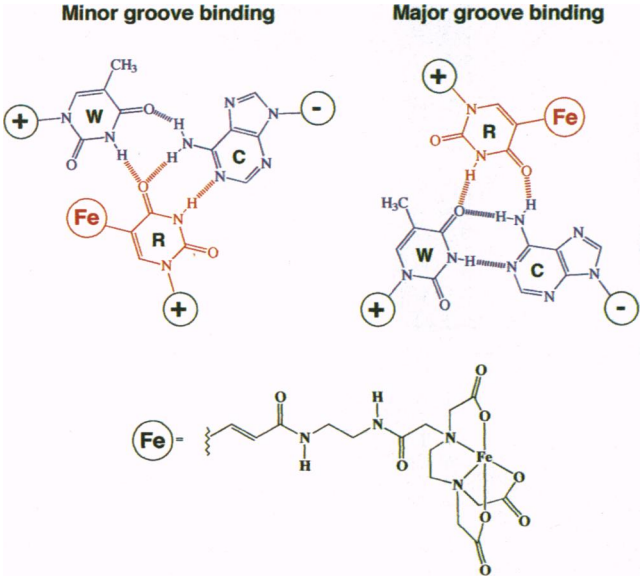

FIG. 3. Location of the coordinated iron atom in each of the two possible intermediates for the three-strand exchange reaction mediated by RecA protein. W, C, and R represent the Watson (homologous), Crick (complementary), and recombinant (incoming) strands, respectively.

RESULTS AND DISCUSSION

Affinity cleavage can be used as a reliable assay for determining the groove location and orientation of a DNA binding ligand. Attachment of EDTA·Fe to a variety of DNA binding molecules—peptides, proteins, and oligonucleotides—provides detailed information about the binding site, groove location, and orientation of these ligands (31, 35–40). A binding mode that places the coordinated metal ion in the major groove of duplex DNA produces a cleavage pattern that is shifted toward the 5′ end on opposite strands, whereas, if the metal ion is located in the minor groove, the cleavage pattern is shifted in the 3′ direction. To study the binding of oligonucleotide third strands in *nonenzymatic* triple helices, a modified thymidine derivative, T*, wherein the DNA cleaving moiety EDTA·Fe is covalently attached at C5 of the thymine heterocycle, was incorporated into oligonucleotides (37, 38, 40). In this case the cleavage pattern generated on opposite strands is shifted toward the 5′ ends. The different groove locations of the coordinated metal atom based on two proposed T·(TA) triplets and the cleavage patterns that are expected for major and minor groove binding of the RecA·oligonucleotide-EDTA·Fe in the three-stranded joint formed are illustrated in Figs. 3 and 4.

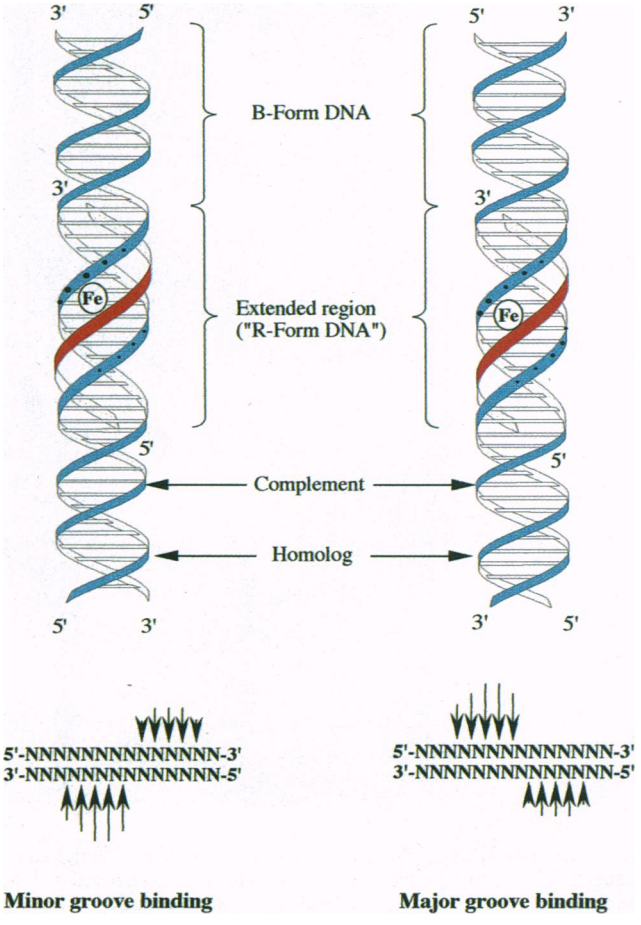

FIG. 4. Expected cleavage patterns for the major and minor groove binding modes of RecA·oligonucleotide (EDTA)·Fe(II) filaments.

The thymidine derivative T* was incorporated at a central position in an oligonucleotide that was homologous to a 31-bp target site in a 300-bp restriction fragment (Fig. 2, **1**). RecA was incubated with oligonucleotide-EDTA·Fe in the presence of ATP[γS] to form a stable nucleoprotein filament after which 5′ or 3′ ^{32}P-labeled 300-bp duplex substrate was added to form the joint molecule (Fig. 1). Sodium ascorbate was then added to initiate the oxidative cleavage reactions. The cleavage products were resolved by denaturing polyacrylamide gel electrophoresis and analyzed by storage phosphor autoradiography. The results from this experiment are shown in Fig. 5*A*. Cleavage is observed on both strands only when RecA and **1**·Fe are present in the reaction. The relative yields of cleavage produced at each nucleotide were measured (lanes 14 and 15) from the storage phosphor autoradiogram (Fig. 5*A*). Remarkably, site-specific cleavage occurs on *both* strands of the target duplex substrate. Moreover, a 3′ shifted cleavage pattern is observed. This cleavage pattern is consistent with an intermediate in which the incoming strand has invaded the target duplex from the minor groove, leaving the outgoing strand in the major groove of the nascent heteroduplex (23). Formation of the products of strand exchange—an interwound heteroduplex and a well-displaced strand—would be expected to give a cleavage pattern on only the complementary strand (37). The fact that cleavage is observed on both strands of the target duplex suggests that the displaced homologous strand is held in close association with the nascent heteroduplex.

The finding that the nucleoprotein filament in the recombination intermediate is lodged in the minor groove of the target duplex is consistent with a number of observations from different laboratories. (*i*) Radding and coworkers (27) found that methylation of functional groups in the major groove—the N4 position of cytosine, N6 of adenine, and N7 of guanine—did not effect the rate or extent of joint molecule formation but reduced the thermal stability of the deproteinized complex. (*ii*) Jain *et al.* (41) have shown that the N7 position of guanine bases in the target duplexes is not involved in the formation of the three-stranded complexes. (*iii*) Kumar and Muniyappa (42) recognized the possibility of minor groove contacts when they observed the inhibition of joint molecule formation by a minor groove binding ligand—distamycin—but not by a major groove binder—methyl green.

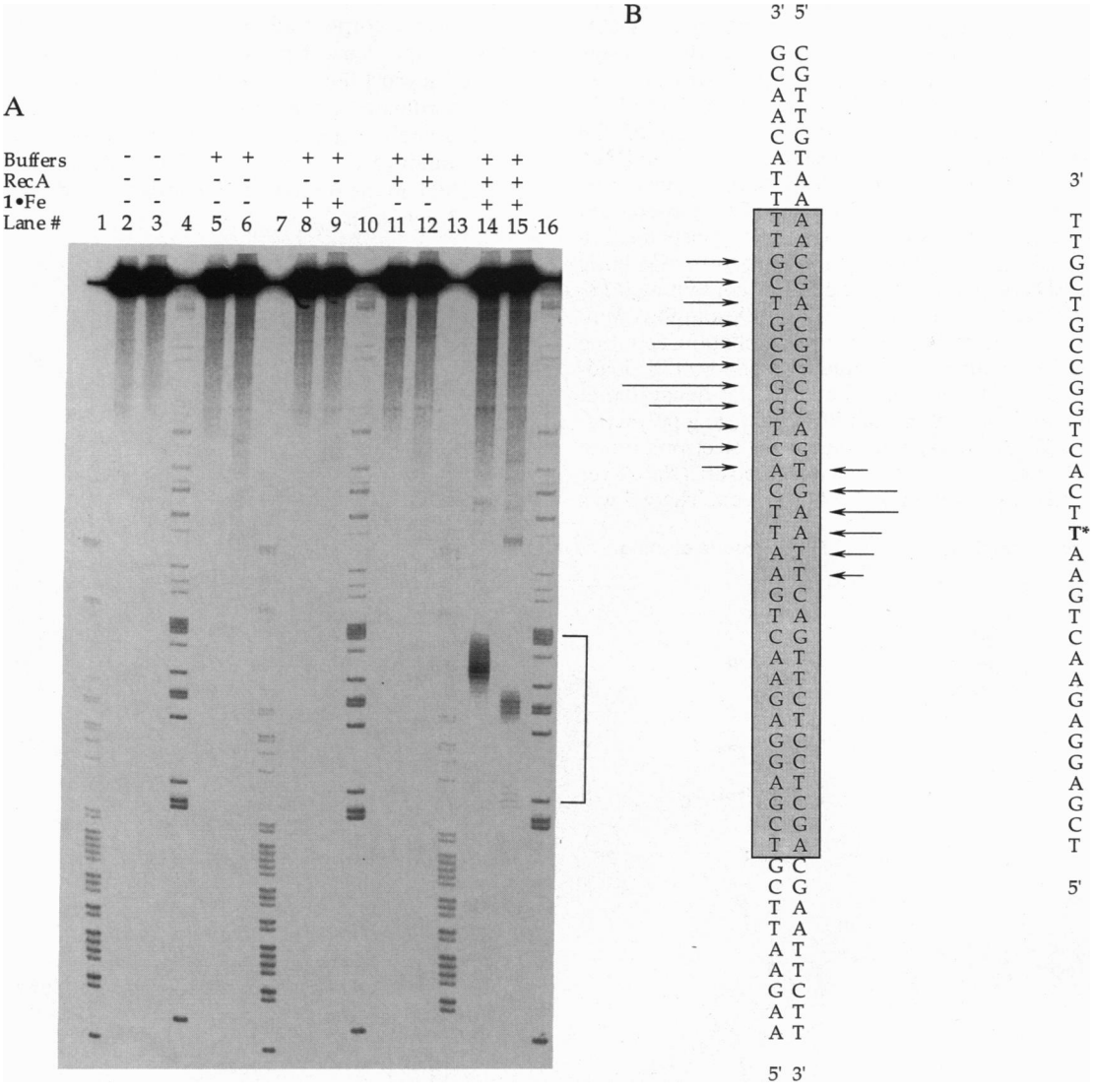

FIG. 5. (*A*) Storage phosphor autoradiogram of an 8% denaturing polyacrylamide gel containing the products of affinity cleavage reactions. Lanes 2, 5, 8, 11, and 14, cleavage products from DNA labeled on the 5′ end at the *Hin*dIII site; lanes 3, 6, 9, 12, and 15, products from DNA labeled on the 3′ end at the *Hin*dIII site; lanes 1, 7, and 13, products of adenine-specific reaction (34) on 5′-end-labeled duplex; lanes 4, 10, and 16, products from 3′-end-labeled duplex. Reaction components for all other lanes were as indicated in the figure under conditions described in *Materials and Methods*. (*B*) Histogram representation of the observed cleavage pattern. The lengths of the arrows represent the relative cleavage intensity at each nucleotide position.

CONCLUSION

We have used affinity cleaving techniques to analyze the structure of the protein-associated joint molecule formed between a RecA·oligonucleotide filament and Watson–Crick double helical DNA using an oligonucleotide with EDTA·Fe attached at a central position. We observed cleavage of both homologous and complementary strands at the site predicted by antiparallel binding of the filament to its complementary sequence. The cleavage pattern was shifted toward the 3′ end on opposite strands, indicating the minor groove location of the incoming strand.

With regard to mechanism, the initial recognition step likely involves insertion of the recA·oligonucleotide filament in the minor groove of an extended unwound double helix with local melting of the double helix for specific base pairing and recognition. We emphasize that our data do not require the existence of specific base triplets (43, 44) but rather only indicate that the three-stranded intermediate formed is a structure wherein the departing strand in the major groove is held in close association with the nascent heteroduplex.

We thank Prof. S. C. Kowalczykowski for providing us with the RecA overexpressing strain JC12772 and a detailed protocol for isolation and purification, Amherst College for a Forris Jewett Moore Fellowship (J.W.S.), and the National Institutes of Health for grant support (GM-51747).

1. Stasiak, A. & Egelman, E. H. (1994) *Experientia* **50,** 192–203.
2. Kowalczykowski, S. C. & Eggleston, A. K. (1994) *Annu. Rev. Biochem.* **63,** 991–1043.
3. West, S. C. (1992) *Annu. Rev. Biochem.* **61,** 603–640.
4. Radding, C. M. (1991) *J. Biol. Chem.* **266,** 5355–5358.
5. Egelman, E. H. & Yu, X. (1989) *Science* **245,** 404–407.
6. Flory, J. & Radding, C. M. (1982) *Cell* **28,** 747–756.
7. Dunn, K., Chrysogelos, S. & Griffith, J. (1982) *Cell* **28,** 757–765.
8. Flory, J., Tsang, S. S. & Muniyappa, K. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7026–7030.
9. Stasiak, A., Egelman, E. H. & Howard-Flanders, P. (1988) *J. Mol. Biol.* **202,** 659–662.
10. Gonda, D. K. & Radding, C. M. (1983) *Cell* **34,** 647–654.
11. Julin, D. A., Riddles, P. W. & Lehman, I. R. (1986) *J. Biol. Chem.* **261,** 1025–1030.
12. Tsang, S. S., Chow, S. A. & Radding, C. M. (1985) *Biochemistry* **24,** 3226–3232.
13. Honigberg, S. M., Gonda, D. K., Flory, J. & Radding, C. M. (1985) *J. Biol. Chem.* **260,** 11845–11851.
14. Menetski, J. P., Bear, D. G. & Kowalczykowski, S. C. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 21–25.
15. Register, J. C., Christiansen, G. & Griffith, J. (1987) *J. Biol. Chem.* **262,** 12812–12820.
16. Umlauf, S. W., Cox, M. M. & Inman, R. B. (1990) *J. Biol. Chem.* **265,** 16898–16912.
17. Jain, S., Cox, M. M. & Inman, R. B. (1995) *J. Biol. Chem.* **270,** 4943–4949.
18. Takahashi, M., Kubista, M. & Norden, B. (1991) *Biochimie* **73,** 219–226.
19. Rao, B. J., Jwang, B. & Dutreix, M. (1990) *Biochimie* **73,** 363–370.
20. Stasiak, A. (1992) *Mol. Microbiol.* **6,** 3267–3276.
21. Howard-Flanders, P., West, S. C. & Stasiak, A. (1984) *Nature (London)* **309,** 215–220.
22. Adzuma, K. (1992) *Genes Dev.* **6,** 1679–1694.
23. Rao, B. J., Chiu, S. K. & Radding, C. M. (1993) *J. Mol. Biol.* **229,** 328–343.
24. Camerini-Otero, R. D. & Hsieh, P. (1993) *Cell* **73,** 217–223.
25. Kim, M. G., Zhurkin, V. B., Jernigan, R. L. & Camerini-Otero, R. D. (1995) *J. Mol. Biol.* **247,** 874–889.
26. Rao, B. J., Dutreix, M. & Radding, C. M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2984–2988.
27. Chiu, S. K., Rao, B. J., Story, R. M. & Radding, C. M. (1993) *Biochemistry* **32,** 13146–13155.
28. Hsieh, P., Camerini-Otero, C. S. & Camerini-Otero, R. D. (1990) *Genes Dev.* **4,** 1951–1963.
29. Hsieh, P., Camerini-Otero, C. S. & Camerini-Otero, R. D. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6492–6496.
30. Muller, B., Burdett, I. & West, S. C. (1992) *EMBO J.* **11,** 2685–2693.
31. Dervan, P. B. (1991) *Methods Enzymol.* **208,** 497–515.
32. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
33. Han, H. & Dervan, P. B. (1994) *Nucleic Acids Res.* **22,** 2837–2844.
34. Iverson, B. L. & Dervan, P. B. (1987) *Nucleic Acids Res.* **15,** 7823–7830.
35. Hertzberg, R. P. & Dervan, P. B. (1982) *J. Am. Chem. Soc.* **104,** 313–315.
36. Schultz, P. G., Taylor, J. S. & Dervan, P. B. (1982) *J. Am. Chem. Soc.* **104,** 6861–6863.
37. Dreyer, G. B. & Dervan, P. B. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 968–972.
38. Moser, H. E. & Dervan, P. B. (1987) *Science* **238,** 645–650.
39. Oakley, M. G. & Dervan, P. B. (1990) *Science* **248,** 847–850.
40. Beal, P. A. & Dervan, P. B. (1991) *Science* **251,** 1360–1363.
41. Jain, S. K., Inman, R. B. & Cox, M. M. (1992) *J. Biol. Chem.* **267,** 4215–4222.
42. Kumar, K. A. & Muniyappa, K. (1992) *J. Biol. Chem.* **267,** 24824–24832.
43. Zhurkin, V. B., Raghunathan, G., Ulyanov, N. B., Camerini-Otero, R. D. & Jernigan, R. L. (1994) *J. Mol. Biol.* **239,** 181–200.
44. Van Meervelt, L., Vlieghe, D., Dautant, A., Gallois, B., Precigoux, G. & Kennard, O. (1995) *Nature (London)* **374,** 742–744.