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Isolation and visualization of active presynaptic filaments of recA protein and single-stranded DNA

(nucleoprotein filaments/electron microscopy/general recombination/single-stranded DNA binding protein)

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ABSTRACT **In the presence of ATP, recA protein forms a presynaptic complex with single-stranded DNA that is an obligatory intermediate in homologous pairing. Presynaptic complexes of recA protein and circular single strands that are active in forming joint molecules can be isolated by gel filtration. These isolated active complexes are nucleoprotein filaments with the following characteristics: (*i*) a contour length that is at least 1.5 times that of the corresponding duplex DNA molecule, (*ii*) an ordered structure visualized by negative staining as a striated filament with a repeat distance of 9.0 nm and a width of 9.3 nm, (*iii*) approximately 8 molecules of recA protein and 20 nucleotide residues per striation. The widened spacing between bases in the nucleoprotein filament means that the initial matching of complementary sequences must involve intertwining of the filament and duplex DNA, unwinding of the latter, or some combination of both to equalize the spacing between nascent base pairs. These experiments support the concept that recA protein first forms a filament with single-stranded DNA, which in turn binds to duplex DNA to mediate both homologous pairing and subsequent strand exchange.**

Purified recA protein catalyzes the pairing of single-stranded or partially single-stranded DNA with homologous duplex DNA, with concomitant hydrolysis of ATP (for a review see ref. 1). This *homologous pairing reaction* is related to the essential role of recA protein in general recombination (2, 3). Much evidence shows that single-stranded DNA plays a special role in the reaction promoted by recA protein *in vitro*: The pairing reaction requires that one of the substrates be at least partially single-stranded and further requires a stoichiometric amount of recA protein that is largely determined by the amount of single-stranded DNA present (4–6); recA protein binds to single-stranded DNA more strongly than to duplex DNA (5, 7–9); and the binding of single-stranded DNA to recA protein stimulates the binding and unwinding of duplex DNA (5, 10–13). In the absence of *Escherichia coli* single-stranded DNA binding protein (SSB), the association of recA protein with single-stranded DNA can be rate limiting; under such conditions, preincubation of single-stranded DNA with recA protein and ATP eliminates a lag in the reaction and increases the initial rate (14). In the presence of SSB, preincubation of single strands with a limiting amount of recA protein and ATP excludes participation of subsequently added single strands (15, 16). Furthermore, conditions that favor secondary structure in single-stranded DNA impede the binding of recA protein and consequently can completely block homologous pairing (ref. 17 and unpublished observations). All of these observations support the conclusion that a stoichiometric complex of recA protein and single-stranded DNA is an essential early intermediate in homologous pairing; indeed, they support the inference that the first step in the reaction is the stoichiometric coating of single-stranded DNA by recA protein.

Electron microscopic studies have shown directly that recA protein forms a nucleoprotein filament with single-stranded DNA (8, 18, 19). The participation of this filament in the formation of joint molecules has been directly demonstrated by micrographs that show the filament to be part of a joint molecule (20). Such nucleoprotein filaments are about 10 nm wide, but depending upon the conditions of formation and mounting for electron microscopy their contour lengths vary widely in relation to the corresponding duplex DNA with whose bases those in the filament must pair (8, 18, 19). In this report, we describe the visualization and characterization of filaments of recA protein and single-stranded DNA that have been formed and isolated in the presence of ATP, that are active in forming joint molecules, and that have not been treated with fixatives or ATP analogs.

METHODS

Proteins. recA protein was prepared as described previously (21). ^{35}S-labeled recA protein was prepared by E. Azhderian by essentially the same procedure, which will be described in detail elsewhere. Concentrations of recA protein were determined spectrophotometrically, using an $A_{1cm}^{1\%}$ of 4.6 at 280 nm. SSB was a generous gift of John Chase of the Albert Einstein College of Medicine.

Preparation and Isolation of Presynaptic Complexes. (*i*) *Complexes formed without SSB.* The standard reaction mixture contained 5.8 μM recA protein, 8.0 μM phage M13 circular single-stranded DNA, 1.3 mM ATP, creatine kinase (Sigma) at 10 units/ml, 3 mM phosphocreatine, 2 mM dithiothreitol, 33 mM Tris·HCl at pH 7.5, and 1 mM $MgCl_2$. The standard procedure involved prewarming all components except recA protein for 2 min at 37°C before adding recA protein. After a 10-min incubation at 37°C, the mixture was chromatographed on a small column of Sepharose 2B-300 at room temperature. The column buffer contained 33 mM Tris·HCl at pH 7.5, 1 mM $MgCl_2$, and 1 mM ATP. Immediately after chromatography, we added $MgCl_2$ to the column fractions to a final concentration of 13 mM. The chromatography required approximately 7 min, and we applied the samples to the grids within 2 min after chromatography.

(*ii*) *Complexes prepared with SSB.* The incubation mixture contained 0.58 μM SSB and 13 mM $MgCl_2$. In the standard procedure, the SSB was added to the mixture 30 sec after recA protein. The column buffer was also modified so that it contained 13 mM $MgCl_2$.

Electron Microscopy. Grids were prepared by a modification of the polylysine technique of Williams (22) as described previously (8). Samples were applied to the grids either di-

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Abbreviations: SSB, single-stranded DNA binding protein; ATP[γS], adenosine 5′-[γ-thio]triphosphate.

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rectly as droplets or by microinjection of sample into a droplet of buffer (33 mM Tris·HCl pH 7.5/13 mM $MgCl_2$/1.3 mM ATP, except as noted) which had been placed on the grid. After 1 min, the droplets were aspirated from the grids, and the grids were rinsed with one droplet of the same buffer used above. Either of two methods of contrast enhancement was used: (*i*) *Shadowing*. Grids were stained for 30 sec with 5% uranyl acetate, rinsed with three droplets of 30 mM ammonium acetate, and rotary shadowed with tungsten (22). (*ii*) *Negative Staining*. Grids were stained for 5 sec with 1% uranyl formate or uranyl acetate.

We examined specimens in a Philips EM201 electron microscope and measured molecular lengths by projecting the negatives in a device utilizing a Hewlett-Packard 9825 calculator-digitizer and programs provided by Jack Griffith. We express contour lengths as percentages of the average contour length of nicked circular duplex M13 DNA (6407 nucleotide pairs), which is 1950 nm (±60 nm SD, n = 55). This value was measured from shadowed specimens of DNA mounted from buffer (33 mM Tris·HCl, pH 7.5/13 mM $MgCl_2$). The 87.5-Å spacing of catalase crystals photographed at the same magnification was used as a calibration standard.

RESULTS

Isolation of Active Presynaptic Complexes. Large aggregates form when recA protein, single-stranded DNA, and ATP are incubated under conditions that are optimal for the formation of joint molecules, conditions that include $MgCl_2$ at a concentration greater than 10 mM (6). Aggregation can be avoided by reducing the concentration of $MgCl_2$ to 1 mM or by adding SSB. Under either of these conditions, presynaptic complexes can be isolated by gel filtration in a form that contains 1 molecule of recA protein per 2–3 nucleotide residues and that is more active in forming joint molecules than a mixture of free reagents at the same concentrations. The results for complexes formed in 1 mM $MgCl_2$ are shown in Fig. 1, and similar results have been obtained with complexes formed in the presence of SSB (data not shown).

Since the optimal formation of stable joint molecules requires a concentration of $MgCl_2$ greater than 10 mM (6), we added $MgCl_2$ to complexes isolated in 1 mM $MgCl_2$ to bring the concentration to 13 mM just prior to preparing specimens for microscopy. In this way, we adsorbed the complexes to grids under conditions in which they are active for homologous pairing. Aggregates were not observed among complexes prepared by using this procedure.

In the following, we describe complexes formed and isolated in 1 mM $MgCl_2$ without SSB and complexes formed in 13 mM $MgCl_2$ in the presence of SSB.

Active Presynaptic Complexes. When we visualized complexes of recA protein and circular single-stranded DNA prepared without SSB, most of the structures on the grids were extended striated circular filaments (Fig. 2*a*). Similar results were obtained with shadowed preparations, although shadowing did not resolve the striations (not shown). The distribution of contour lengths for a random sample of shadowed complexes is shown in Fig. 3*a*. The average length is 146% of the length of the corresponding duplex DNA molecule (Table 1). The distribution is skewed, however, by a "tail" of shorter complexes. If the "tail" of complexes shorter than 140% of the duplex length is excluded, the average rises to 151% of the duplex length.

A regular pattern of striations inclined with respect to the filament axis is apparent over most of each filament in negatively stained specimens, although some segments have a zig-zag appearance (Fig. 2*a*). Helical structures frequently exhibit this type of behavior in negative staining (25). Preferential staining of the "near" or "far" side of the helix results

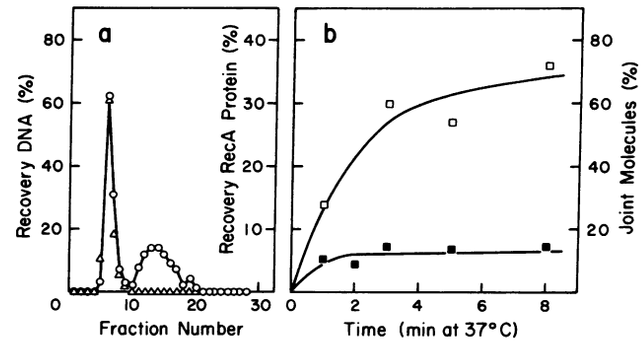

FIG. 1. Isolation of active presynaptic complexes of recA protein and single-stranded DNA. (*a*) Elution profiles of complexes formed with ^{3}H-labeled DNA (△) or ^{35}S-labeled recA protein (○). Presynaptic complexes were formed in 1 mM $MgCl_2$ without SSB and chromatographed as described in *Methods*, except that the column buffer also contained 1 mM dithiothreitol and bovine serum albumin at 100 μg/ml. (*b*) Homologous pairing activity of isolated presynaptic complexes. Complexes isolated as in *a*, using ^{32}P-labeled single-stranded DNA, were supplemented with an ATP regeneration system (3 mM phosphocreatine and creatine kinase at 10 units/ml) and warmed at 37°C for 2 min. ^{3}H-labeled homologous linear duplex DNA (3 μM M13 duplex DNA linearized with *Hin*cII) was added, and the formation of joint molecules during further incubation at 37°C (□) was monitored (23). In a separate experiment, recA protein and single-stranded DNA were mixed at the same final concentrations as in the isolated complexes used above (0.8 μM and 2 μM, respectively) and incubated for 10 min at 37°C under standard conditions in 1 mM $MgCl_2$. The $MgCl_2$ concentration was increased to 13 mM, 3 μM ^{3}H-labeled homologous linear duplex DNA was added, and the formation of joint molecules at 37°C was followed as above (■).

in a series of bars slanted in either of two orientations with respect to the helix axis, and equal staining of both surfaces results in a zig-zag image. While our data strongly suggest a helical structure, other structures cannot be excluded. We are unable to determine the handedness of the putative helix because the images of slanted bars could result from preferential staining of either surface of the helix. Unfortunately, shadowing, which should visualize only the "near" surface of the filament, did not resolve the striations.

The simplest way to determine the average repeat distance in these filaments would be to divide the total number of striations in each complex by its total contour length. Since the striations in some portions of each complex were unclear, we counted striations over as much of each complex as possible and divided by the sum of the lengths of the counted segments. The repeat distance, 8.9 nm, corresponds approximately to 22 nucleotides (Table 2). By calculation from the stoichiometry of 2.5 nucleotides per recA protein monomer in the complex as isolated in 1 mM Mg^{2+}, we estimate that each repeat also contains approximately 8 monomers of recA protein (Table 2). The width of these filaments is 9.3 nm (Table 2), and the average contour length in negatively stained specimens is 152% of the duplex length (Table 2), which is close to the average observed for shadowed complexes (Table 1).

In the presence of SSB, presynaptic complexes can be formed and isolated in 13 mM Mg^{2+}, a concentration that is sufficient to support the formation of joint molecules in the homologous pairing reaction. Under these conditions, striated nucleoprotein filaments form (Fig. 2 *b* and *f*) that are very similar in contour length, width, and repeat distance to those formed in the absence of SSB (Figs. 2 *a* and *e* and 3*b*; Tables 1 and 2). We did not detect either any effects of SSB on the appearance of the filaments or any segments of the complexes with the characteristic appearance of single-stranded DNA coated with SSB (8, 28).

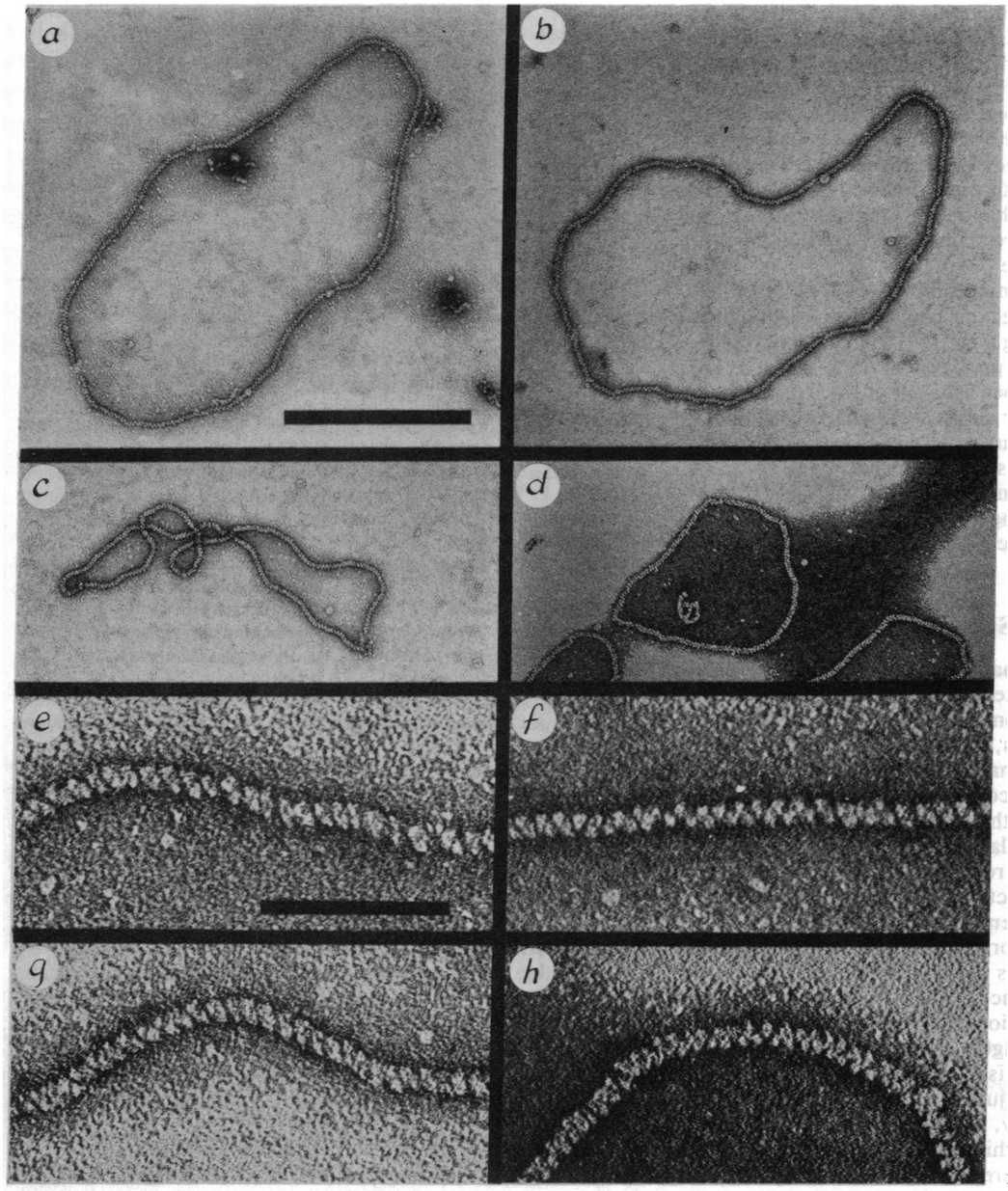

FIG. 2. Complexes of recA protein and single-stranded DNA formed in the presence of ATP and visualized by negative staining. (*a*) Presynaptic complex without SSB. We formed and purified complexes at 1 mM $MgCl_2$, added $MgCl_2$ to a final concentration of 13 mM, and then prepared specimens. (*b*) Presynaptic complex formed with SSB. We formed complexes in 13 mM $MgCl_2$ in the presence of SSB and maintained the $MgCl_2$ concentration at 13 mM during purification and mounting. (*c*) Presynaptic complexes formed without SSB—structure at 1 mM $MgCl_2$. The procedure was identical to that in *a* except that the $MgCl_2$ concentration remained at 1 mM throughout the procedure. (*d*) Effect of removing ATP from complexes formed in the presence of SSB. The procedure was identical to that in *b*, except that we omitted ATP from the buffer used in Sepharose 2B chromatography. (*e–h*) Portions of the complexes in *a–d*, respectively, shown at higher magnification. Frames *a–d* are all at the same magnification, and the bar in *a* indicates 500 nm. The contour lengths are *a*, 155% of the length of duplex M13 DNA; *b*, 157%; *c*, 137%; and *d*, 71%. Frames *e–h* are all at the same magnification, and the bar in *e* indicates 100 nm.

Factors That Affect the Structure of Presynaptic Complexes. ATP was apparently required to maintain the extended structure of the filament. Omission of ATP from the column buffer during the purification of presynaptic complexes formed in the presence of ATP and SSB resulted in striated circular filaments with a much shorter contour length and smaller repeat distance (Figs. 2 *d* and *h* and 3*d*). The average contour length decreased by nearly half, to 78% of the duplex length (Table 1), and the repeat distance decreased to 6.9 nm (Table 2). The width of the filament was unchanged, however, at 9.3 nm (Table 2). When we omitted ATP from the reaction entirely, the complexes formed were indistinguishable from those formed by SSB and single-stranded DNA in the absence of any recA protein (not shown).

The concentration of Mg^{2+} also affected the contour length and repeat distance, although the effects were smaller. If we did not increase the $MgCl_2$ concentration from 1 to 13 mM after the purification of complexes formed without SSB, we found circular filaments with a shorter contour length (Figs 2*c* and 3*c* and Table 1), a more disperse distribution of lengths (Fig. 2*c*), and a shorter repeat distance of 7.7 nm (Table 2 and Fig. 2*g*). In addition, some filaments con-

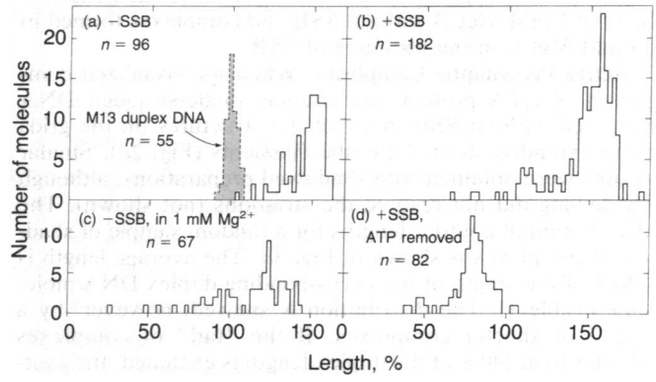

FIG. 3. Distribution of contour lengths of complexes of recA protein and single-stranded DNA visualized by shadowing. In *a–d* we measured the contour lengths of random samples of complexes prepared as described in the legend to Fig. 2 *a–d*, respectively, but visualized by shadowing. Lengths are expressed as % of the mean length of M13 duplex DNA. In *a*, we have superimposed the distribution of contour lengths of M13 duplex DNA (shaded area).

Table 1. Contour lengths of complexes of recA protein and single-stranded DNA visualized by shadowing

Complex	Mean length,* %	$n^{\dagger}$
Presynaptic complexes formed in 1 mM $MgCl_2$ in the absence of SSB (Fig. 2*a*), mounted in 13 mM $MgCl_2$	146 ± 11	96
Presynaptic complexes formed in 13 mM $MgCl_2$ in the presence of SSB (SSB added last) (Fig. 2*b*)	147 ± 19	182
Requirements for presynaptic filament structure:		
Complexes formed without SSB		
Prepared and mounted in 1 mM $MgCl_2$ (Fig. 2*c*)	112 ± 24	67
Complexes formed with SSB		
Order of addition of components during formation of complexes:		
ATP, then SSB	144 ± 24	29
DNA last	141 ± 26	44
ATP last	104 ± 33	13
ATP removed during purification (Fig. 2*d*)	78 ± 14	82
Effect of fixation		
Formed in triethanolamine buffer and fixed with formaldehyde	91 ± 11	45
Formed in triethanolamine buffer but not fixed	151 ± 12	39

The reaction conditions for the first three entries have been described in the legend to Fig. 2. The next three entries concern the effects of changing the order of addition of recA and SSB proteins, ATP, and DNA to the reaction mixture. The standard procedure was to incubate the mixture of buffer, ATP, ATP regeneration system, and DNA for 2 min at 37°C before adding recA protein, followed 30 sec later by SSB protein. In "ATP, then SSB," we incubated all components except ATP and SSB for 2 min at 37°C before adding ATP, followed 30 sec later by SSB. In "DNA last," we incubated all components except DNA for 2 min at 37°C before adding the DNA. In "ATP last," we incubated all components except ATP together for 2 min at 37°C before adding ATP. In the fixation experiment, we substituted 30 mM triethanolamine·HCl, pH 7.5, for Tris at all steps of the procedure. We fixed complexes prepared in triethanolamine with 1% formaldehyde (Polysciences EM grade) for 15 min at 0°C (24) before purifying them.

*Expressed as percent of the mean length (±SD) of duplex M13 DNA.

†Number of complexes measured.

sisted of a circle with one or occasionally two short arms projecting from them (not shown). The width of the filaments, 9.1 nm, was unchanged (Table 2).

Fixation also reduced the contour length of presynaptic complexes. When complexes formed in the presence of SSB were fixed with formaldehyde before purification, the contour length of the filaments decreased to 91% of the duplex length (Table 1). Fixation with both formaldehyde and glutaraldehyde (24) had a similar effect (not shown). The reactivity of Tris towards the fixatives make it necessary to use triethanolamine as a buffer in these experiments, but unfixed complexes formed in triethanolamine buffer had the same contour length as those formed in Tris (Table 1).

The order in which we added the components to the incubation mixture also affected the contour length of the complexes that formed. The normal procedure for these experiments involved the warming of all components except recA protein and SSB at 37°C. We then added recA protein, followed 30 sec later by SSB. If we warmed both proteins with the DNA before adding ATP, we observed shorter filaments with an average contour length 104% of the duplex length (Table 1). The contour length did not change significantly, however, when DNA was the final component added, or when ATP and SSB were added last (Table 1). These results suggest that the two proteins may compete for the DNA, with recA protein having a higher affinity than SSB in the presence of ATP and a lower affinity in the absence of ATP. The contour length of complexes of SSB and single-stranded DNA is 30% of the duplex length (28), so that a shorter contour length might be expected if SSB binds to the DNA in the absence of ATP and cannot be completely dislodged by recA protein after ATP is added. This explanation requires one to suppose that in these complexes SSB is bound to DNA in segments that are too small to detect.

DISCUSSION

In the experiments described above, we formed presynaptic complexes of recA protein and single-stranded DNA under conditions that minimize secondary structure in the DNA, and we isolated them at room temperature in the presence of ATP, without any fixation. By this approach, we isolated complexes that are active in forming joint molecules. Given their instability, such complexes are not necessarily fully saturated with recA protein despite their extended contour length. Indeed, the observations of Muniyappa *et al.* (17) demonstrate that in the absence of SSB some localized sites of secondary structure in the single-stranded DNA may not be disrupted by recA protein.

There are several implications of the observation that ac-

Table 2. Dimensions of complexes of recA protein and single-stranded DNA measured from negatively stained specimens

recA protein complex	$MgCl_2$ during mounting, mM	Contour length, % of duplex	Width, nm	Repeat distance, nm	Nucleotides per repeat	Nucleotides per recA protein monomer
(*a*) −SSB	13	152 ± 5 (10)	9.3 ± 0.9 (25)	8.9 ± 0.2 (10)	22	2.5*
(*b*) +SSB	13	160 ± 3 (10)	9.3 ± 0.9 (27)	9.1 ± 0.2 (10)	21	2.5
(*c*) −SSB	1	110 ± 13 (11)	9.1 ± 0.7 (31)	7.7 ± 0.2 (11)	26	2.5
(*d*) +SSB, ATP removed	13	78 ± 6 (10)	9.3 ± 0.8 (25)	6.9 ± 0.5 (10)	33	4.3
Duplex DNA, ATP[γS]†	10	150	11	9.1	18.6‡	2.9

Rows *a–d* correspond to panels *a–d* in Fig. 2. Mean values for contour length, width, and repeat distance are given ± SD, followed in parentheses by the number of complexes measured. The ratios of nucleotide residues to recA protein were estimated from measurements in parallel experiments of [^{35}S]recA protein and [^{3}H]DNA in the peak that contains the complexes (see Fig. 1*a*). Total recoveries of DNA and protein were uniformly better than 90%, and each value is an average of at least two determinations. The stoichiometries reported here correspond to the stoichiometry reported by DiCapua *et al.* (26) for filaments formed on duplex DNA in the presence of adenosine 5′-[γ-thio]triphosphate (ATP[γS]) but differ from that reported by Chrysogelos *et al.* (27) for these structures.

*Assumes that the stoichiometry does not change when the $MgCl_2$ is increased from 1 mM to 13 mM after the complexes have been purified.

†Data taken from DiCapua *et al.* (26).

‡Nucleotide pairs rather than nucleotides for complexes formed on duplex DNA in the presence of ATP[γS].

tive filaments are at least 1.5 times as long as duplex DNA: The extended contour length appears to exclude a structure like that of chromatin (29, 30) or of the nucleoprotein filament formed by SSB and single strands (28) in which the DNA is wrapped around beads of protein. Furthermore, the extended contour length leads one to wonder how the internucleotide distances in the single-stranded nucleoprotein filament and in duplex DNA are equalized to permit base pairing. We presume either that the nucleoprotein filament and duplex DNA must be interwound to produce a structure with common internucleotide distances or that the duplex DNA must be unwound such that, in the limiting case, pairing might occur with no interwinding. Previous observations have shown that homologous pairing occurs perfectly well even when neither the nucleoprotein filament nor the duplex DNA has free ends, a circumstance under which strand exchange and the formation of truly intertwined heteroduplex DNA is not possible (31). The formation of such a synaptic structure is accompanied by extensive unwinding of the duplex DNA, and the incoming single strand can be topologically linked to its complementary strand by *E. coli* topoisomerase I (12, 31, 32). Thus experimental data show that the synaptic matching of complementary bases is accompanied by unwinding of the duplex DNA.

The active presynaptic complexes of recA protein and single-stranded DNA formed in the presence of ATP are strikingly similar in structure to the complexes of recA protein and duplex DNA formed in the presence of ATP[γS]. Both are nucleoprotein filaments with similar appearance, contour length, repeat distance, stoichiometry and width (refs. 18 and 26 and Table 2). However, even in the absence of DNA, ATP[γS] causes the formation of long striated filaments by recA protein (refs. 7 and 8; unpublished data); and when either circular single-stranded or circular duplex DNA is present, the striated filament formed in the presence of ATP[γS] follows the contour of the DNA (refs. 18 and 26; unpublished data). The ATP–recA protein filament formed on single-stranded DNA is an obligatory intermediate in homologous pairing (ref. 14; unpublished data), but additional observations are required to evaluate the significance of the ATP[γS]–recA protein filament that is formed on duplex DNA. No report has described the finding of an extended recA filament on duplex DNA in the presence of ATP, but there is evidence of extensive recA-dependent unwinding of duplex DNA that follows the formation of a joint molecule, even when the invading single strand has dissociated (12, 13, 32). Furthermore, once a joint molecule has formed, the subsequent reaction of recA protein in promoting strand exchange resists concentrations of ADP 10-fold greater than required to dissociate presynaptic complexes (ref. 12 and 14; unpublished data). Since duplex DNA is unwound in the ATP[γS] filament (27, 33) and since the distance between bases in the latter matches that in the essential presynaptic filament described here, we favor the view that a common structure formed initially on single-stranded DNA mediates both homologous pairing and strand exchange; but the nature of the ADP-resistant structure that mediates strand exchange has not yet been determined.

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