Active nucleoprotein filaments of single-stranded binding protein and recA protein on single-stranded DNA have a regular repeating structure

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ABSTRACT

When *E. coli* single-stranded DNA binding protein (SSB) coats single-stranded DNA (ssDNA) in the presence of 1 mM $MgCl_2$ it inhibits the subsequent binding of recA protein, whereas SSB binding to ssDNA in 12 mM $MgCl_2$ promotes the binding of recA protein. These two conditions correspond respectively to those which produce 'smooth' and 'beaded' forms of ssDNA-SSB filaments. By gel filtration and immunoprecipitation we observed active nucleoprotein filaments of recA protein and SSB on ssDNA that contained on average 1 monomer of recA protein per 4 nucleotides and 1 monomer of SSB per 20 – 22 nucleotides. Filaments in such a mixture, when digested with micrococcal nuclease produced a regular repeating pattern, approximately every 70 – 80 nucleotides, that differed from the pattern observed when only recA protein was bound to the ssDNA. We conclude that the beaded ssDNA-SSB nucleoprotein filament readily binds recA protein and forms an intermediate that is active in the formation of joint molecules and can retain substantially all of the SSB that was originally bound.

INTRODUCTION

Escherichia coli recA protein is a DNA-dependent ATPase which regulates an inducible SOS pathway and plays an indispensable role in general genetic recombination (1). By utilizing linear duplex DNA and ssDNA, various laboratories have noted three clearly separable phases in homologous pairing: (i) a presynaptic phase, (ii) synapsis, and (iii) unidirectional strand exchange (1, 2). The product of the *ssb* gene, single strand binding protein (SSB), is essential for replication, and plays an important role in homologous recombination as well as in the induction of the SOS system (3). *In vitro*, SSB potentiates both the formation of joint molecules by recA protein, and subsequent strand exchange (1–3).

The mechanism by which SSB participates, with recA protein, in homologous pairing and strand exchange *in vitro* is complicated however. We found that conditions which favour secondary structure in ssDNA made homologous pairing dependent on the action of SSB (4). However, when nucleoprotein filaments of recA protein-ssDNA were formed at 1 mM $MgCl_2$ without SSB, the initial rate of formation of joint molecules was 75% or more of that observed in the presence of SSB, which shows that SSB is not essential for the initial formation of the recA nucleotprotein filament (5). On the other hand, Cox and Lehman and their colleagues made a series of observations which show that the presence of SSB stabilizes recA protein-ssDNA nucleoprotein filaments (2); and more recently, Morrical *et al.* (6) have reported the occurrence of a mixed nucleoprotein filament with a defined stoichiometry.

Further complexity was revealed by observations on the binding of SSB to ssDNA, and on the effects of order of addition of SSB and recA protein (2). The cooperative association of SSB with ssDNA is determined by solution variables, most notably the cations. Recent evidence suggests that the mode of binding of SSB to ssDNA is modulated by its density as well as cation concentration (7–12). Lohman and his colleagues, by physicochemical methods, have demonstrated the occurrence of at least three major binding modes as $(SSB)_n$, n = 35, 56 and 65 nucleotides per tetramer, on ssDNA, and further suggested that these different binding modes may selectively facilitate processes such as replication, recombination and repair (12). It is not clear, however, which of these binding modes support the formation of joint molecules when recA protein is added. The binding of recA protein to ssDNA is also cooperative (2, 13, 14) and one monomer of recA protein in the presence or in the absence of SSB binds to about 3.6–4.0 nucleotides (5, 15). Observations from a number of laboratories have indicated that the order of addition of recA protein and SSB affects the hydrolysis of ATP and formation of joint molecules promoted by recA protein (1, 2). When a saturating concentration of SSB was incubated with ssDNA prior to recA protein, it inhibited the formation of joint molecules and hydrolysis of ATP (2). However, at stoichiometric concentrations, SSB stimulated the initial rates as well as the extent of ADP production and the

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formation of joint molecules by recA protein (1, 2, 4, 16).

We show here that the inhibition by SSB of the pairing function of recA protein is associated with the $(SSB)_{35}$ mode whereas the $(SSB)_{65}$ form stimulates the reaction. We also show that the final presynaptic complex of recA protein and circular ssDNA, formed in the presence of 12 mM $MgCl_2$, retains a stoichiometric amount of SSB as an integral component. To get information on the spatial distribution of SSB and recA protein on circular ssDNA, we used an enzymatic probe, micrococcal nuclease, which generates a characteristic repeating pattern of fragments of DNA, approximately every 70–80 nucleotides.

EXPERIMENTAL PROCEDURES

Enzymes, proteins and DNA. RecA protein was purified as described by the method of Shibata *et al.* (17) and its concentration was determined as described (5). The preparation of [^{3}H]SSB and [^{35}S]recA protein has been described (5). [^{3}H]SSB had a specific radioactivity of 4.4×10^4 cpm/μg protein. Ascites fluid containing monoclonal antibodies to recA protein was kindly provided by John Flory of our Department. Monoclonal antibodies were purified by B.J.Rao on Protein A-Sepharose as suggested by the supplier. Circular duplex DNA and ssDNA from phage M13 were prepared and their concentration was determined as described (18).

Assay for joint molecules. The assay based on the method of Beattie *et al.* (19) which measures the retention on nitrocellulose filters of duplex [^{3}H]DNA that has become partially single-stranded and the procedure has been described (4).

Isolation of complexes of recA protein-ssDNA-SSB and their products gene rated by micrococcal nuclease by gel-infiltration on Sepharose 2B-300. The standard reaction mixtures contained (90 μl) 33 mM Tris-HCl (pH 7.5), 1.5 mM dithiothreitol, 1.5 mM ATP, 12 mM $MgCl_2$, 2 mM $CaCl_2$, 88 μg/ml of bovine serum albumin, 6 mM phosphocreatine, 10 U/ml creatine phosphokinase, and 8 μM ^{32}P-labelled ssDNA. The reaction mixtures were incubated at 37°C for 2 min, before the addition of 0.5 μM SSB. After 5 min with SSB, we added 2.7 μM recA protein and incubated for 10 min. These complexes were digested with micrococcal nuclease for the times indicated. An aliquot (70 μl) was applied on a column (0.8×18 cm) of Sepharose 2B-300 which had been equilibrated with a buffer (20 ml) containing 33 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 0.1 mg/ml of bovine serum albumin and 1 mM $MgCl_2$. The column was eluted with the same buffer and fractions of 220 μl were collected at a flow rate of 0.5 ml/min.

RESULTS

Conditions that determine the interactions of recA protein and SSB with ssDNA in the formation of joint molecules. Recent studies have suggested that, depending on the concentration of cation SSB binds to ssDNA in at least three binding modes resulting in $(SSB)_{35}$, $(SSB)_{56}$ and $(SSB)_{65}$ forms of ssDNA-SSB (12). The $(SSB)_{35}$ mode, which occurs at low salt conditions, exhibits a metastable high degree of cooperativity, whereas $(SSB)_{56}$ or $(SSB)_{65}$ modes which occur at high salt conditions show a low degree of cooperativity (12). Furthermore, it has been suggested that the 'beaded' structures observed by Griffith *et al.* (7) in the electron microscope are likely to represent either

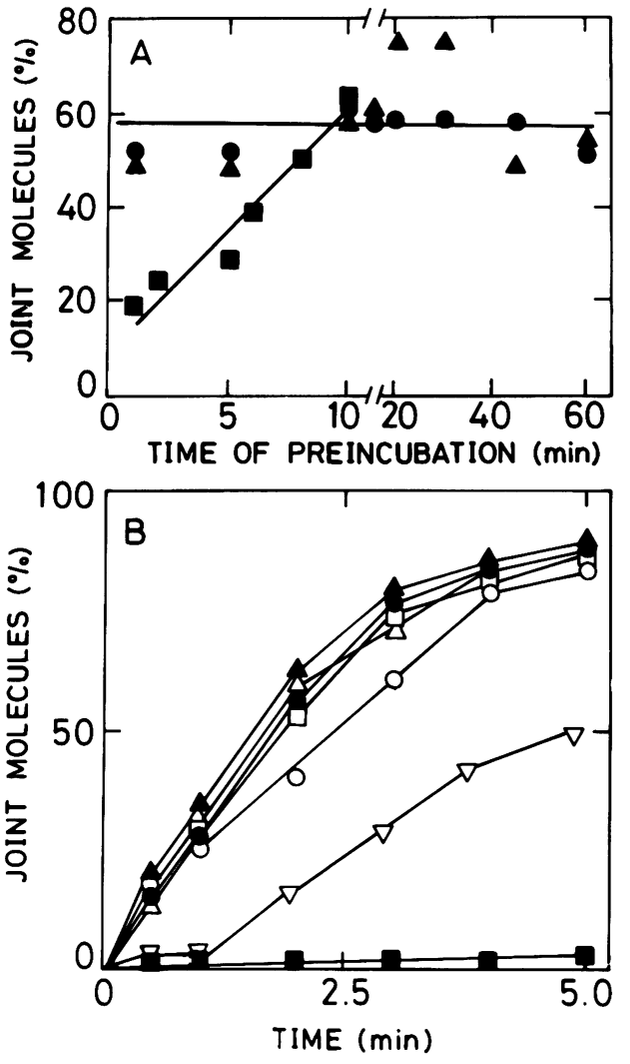

Figure 1. Conditions that determine the formation of joint molecules by recA protein in the presence of SSB. **A.** Effect of time by preincubation of SSB. Reaction mixture (30 μl) in a standard assay buffer containing 8 μM M13 ssDNA, 1 μM SSB, 12 mM $MgCl_2$ and an ATP-regeneration system, were preincubated for the times indicated, after which we added 2.7 μM recA protein and continued incubation for 10 min. The formation of joint molecules was initiated by the addition of 4 μM M13 linear duplex [^{3}H]DNA (Superhelical duplex DNA linearized with HpaI), and the reaction was terminated after 5 min of incubation. (○), (▲), results of two independent experiments. In a separate experiment (□), we incubated 0.8 μM SSB with 8 μM ssDNA for 5 min, added recA protein for various periods of time as indicated, then added duplex DNA and assayed the yield of joint molecules 5 min later. **B.** Effect of varying concentrations of SSB. Circular ssDNA (8 μM) was preincubated for 5 min at 37°C with various concentrations of SSB in a standard assay buffer containing 12 mM $MgCl_2$, followed by 2.7 μM recA protein for 10 min at 37°C. Joint molecule formation was initiated by the addition of 4 μM M13 linear duplex [^{3}H]DNA (superhelical DNA linearized with HpaI). At the indicated times aliquots of 10 μl were added to 0.3 ml of 25 mM EDTA (pH 9.0) at 0°C. Concentrations of SSB were: (○) 2 μM; (●) 1 μM; (□) 0.8 μM; (▲) 0.57 μM; (△) 0.45 μM. In another experiment (■) ssDNA were preincubated with SSB (0.8 μM) and 1 mM instead of 12 mM $MgCl_2$; (▽) recA protein alone. Joint molecule formation was measured in the presence of 12 mM $MgCl_2$.

$(SSB)_{56}$ or $(SSB)_{65}$ binding modes. Similarly previous studies have demonstrated the action of SSB in reactions promoted by recA protein can be either stimulatory or inhibitory, depending upon the conditions (20). To correlate the major binding modes

Table 1. Relative amounts of bound [^{35}S] recA protein versus input concentrations of SSB

Input Amounts (μM) [^{35}S]recA	SSB	ssDNA	Amount of Bound [^{35}S] recA Protein (μM) 1 mM $MgCl_2$	12 mM $MgCl_2$
Expt.A				
4.0	0.54	8.0	0.63	
4.0	0.45	8.0	0.72	
4.0	0.32	8.0	0.86	
4.0	0.20	8.0	1.00	
Expt.B				
4.0	0.50	8.0	2.40	2.50
4.0	–	8.0	1.00	0.68

Binding reactions and isolation of presynaptic complexes were done essentially as described (5). In experiment A, binding and gel filtration were done in buffers containing 1 mM $MgCl_2$, whereas in experiment B, the binding buffer contained 12 mM $MgCl_2$ and gel filtration buffer contained either 1 mM or 12 mM $MgCl_2$.

with stimulation or inhibition of the formation of joint molecules by recA protein, we did the following experiments.

We incubated SSB with ssDNA in a standard assay buffer containing 12 mM $MgCl_2$, followed by incubation with a saturating amount of recA protein. In one set of experiments we varied the time of incubation of SSB with ssDNA, in the presence of 12 mM $MgCl_2$, followed by incubation with recA molecules 5 min later (Fig. 1A). After different times of incubation of ssDNA with a saturating amount of SSB, the yield of joint molecules was about 60% suggesting that the prior incubation of SSB with ssDNA, even for a prolonged time, had no adverse effect.

We also examined the time required for recA protein to convert SSB-coated ssDNA into active presynaptic nucleoprotein filaments. We incubated ssDNA with a saturating concentration of SSB for 5 min, added recA protein and assayed at various times for the ability of these complexes to make joint molecules. This conversion was relatively slow; it took 10 min of formation of SSB-ssDNA filaments with recA protein to reach the control level of activity (Fig. 1A).

To examine the effect of the concentration of SSB, we incubated ssDNA with various concentrations of SSB at 12 mM $MgCl_2$ for 5 min. After 10 min of incubation with recA protein, we measured joint molecules as a function of time. As illustrated in Fig. 1B, even at a 2-fold excess concentration of SSB, the initial rates of formation and the yields of joint molecules were similar to those seen at lower concentrations of SSB.

By contrast with the observations just described, a striking inhibition of formation of joint molecules was observed when the concentration of $MgCl_2$ was 1 mM during the incubation of SSB with ssDNA (Fig. 1B). This condition promotes the metastable high cooperative binding of SSB to ssDNA and produces a smooth nucleoprotein filament (7–12). This inhibitory effect did not require a saturating concentration of SSB, but was also evident when a third as much SSB was incubated with ssDNA (data not shown). To relate the lack of activity of these complexes to the binding of recA protein to ssDNA, we estimated the amount of bound recA protein by gel filtration. In a standard assay buffer, which however contained 1 mM $MgCl_2$ we incubated ^{32}P-labelled ssDNA for 10 min with one monomer of SSB per 15, 18, 25 and 40 nucleotide residues: after a subsequent 20 min of incubation with 4 μM [^{35}S] recA protein, the isolated complexes contained only one monomer of [^{35}S] recA protein per 12.75, 11.0, 9.3 and 8.0 nucleotides, respectively which is to be compared with the optimal binding of 1 monomer of recA protein per 3–4 nucleotides for maximal activity (Table I).

We conclude that, in 1 mM $MgCl_2$, SSB prevents the binding of recA protein to ssDNA and therefore inhibits homologous pairing whereas in 12 mM $MgCl_2$, SSB facilitates the binding of recA protein and therefore favours the formation of joint molecules. The data in Fig. 1A suggests that inhibitory effects observed when SSB is added first in 12 mM $MgCl_2$, can be explained by the relatively slow uptake of recA protein by ssDNA already coated with SSB (20). These results are in agreement with those of Griffith *et al.* (7) who found that recA protein-ssDNA nucleoprotein filaments formed in the presence of various concentrations of SSB resulted in 'smooth' and 'beaded' forms and it was the latter which was active in cleaving lexA repressor.

The stoichiometry of binding of recA protein and SSB to ssDNA in 12 mM $MgCl_2$. Previous experiments from this laboratory have shown that, when isolated by gel filtration, complexes of ssDNA with recA protein and SSB contained variable ratios of SSB and recA protein, consistent with mutually exclusive binding of these proteins to ssDNA under a variety of conditions (5). When recA protein-ssDNA-SSB complexes were formed in 12 mM $MgCl_2$ and isolated by gel filtration at 12 mM $MgCl_2$, the isolated complexes contained 1 recA monomer per 4 nucleotides, and less than 1 monomer of SSB per 40 nucleotides (5). Subsequent experiments, however, showed that complexes which were formed in 12 mM $MgCl_2$ and then isolated by gel filtration in 1 mM $MgCl_2$ contained 1 monomer of SSB per 22 nucleotides and 1 monomer of recA protein per 4 nucleotides. The same stoichiometry was found when complexes formed in 12 mM $MgCl_2$ and 1 mM ATP were treated with ATPrS (adenosine 5'-O-(thiotriphosphate)) and isolated by gel filtration in 1 mM $MgCl_2$ (data not shown).

Since ATPrS causes virtually irreversible binding of recA protein to DNA (21), the experiments just described indicated that the large amounts of bound SSB found when gel filtration was carried out in 1 mM $MgCl_2$ did not require the displacement of recA protein by SSB.

Therefore, we sought a milder method to distinguish bound from unbound SSB. We used monoclonal antibodies to recA protein to precipitate the presynaptic complexes containing recA protein and [^{3}H]SSB. We incubated ^{32}P-labelled ssDNA with [^{3}H]SSB in a standard buffer containing 12 mM $MgCl_2$ and an ATP-regeneration system for 5 min. Complexes of ^{32}P-labelled ssDNA-[^{3}H] SSB were then incubated with saturating concentrations of recA protein. At the times indicated in Fig. 2, we added purified monoclonal antibodies directed against recA protein. After 5 min of incubation with monoclonal antibodies,

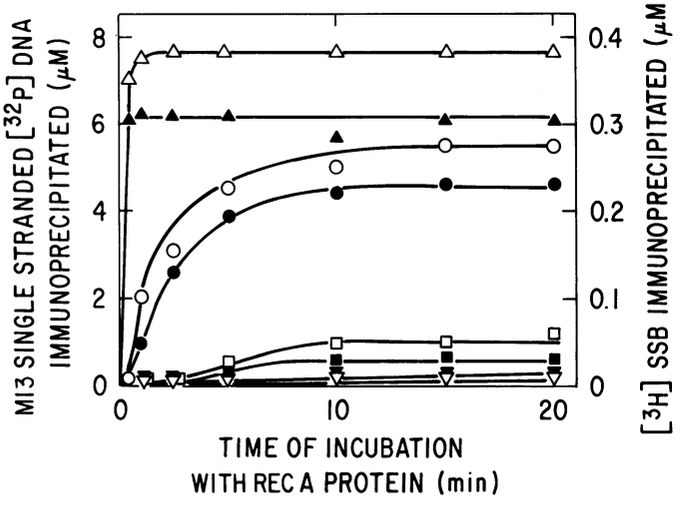

Figure 2. Immunoprecipitation of complexes of recA protein-ssDNA-SSB by monoclonal antibodies to recA protein. ^{32}P-labelled ssDNA (8 μM) was preincubated with 0.5 μM [^{3}H]SSB in a standard assay buffer containing either 1 mM or 12 mM $MgCl_2$ and an ATP-regeneration system. After 5 min of incubation with SSB, we added 2.7 μM recA protein and incubated for various times indicated, which was followed by an additional 5 min of incubation in the case of ATPrS. Then the complexes were reacted with monoclonal antibodies (2 μM) to recA protein for 5 min and the immunocomplexes were sedimented by centrifugation in an Eppendorf centrifuge at 15,600 g for 5 min. The radioactivity in the immunoprecipitate was quantitated. (a) Control reaction done with SSB at 12 mM $MgCl_2$; (▽)^{32}P-labeled ssDNA; (▼) [^{3}H]SSB; (b) Reaction done with ^{3}[H]SSB and recA protein at 1 mM $MgCl_2$; (□)^{32}P-labelled ssDNA; (■) [^{3}H]SSB; (c) Reaction done as in (b), but at 12 mM $MgCl_2$; (○)^{32}P-labelled single-stranded DNA; (●) [^{3}H]SSB; (d) Reactions done as in c, but incubated with ATPrS for 5 min before the addition of monoclonal antibody; (△)^{32}P-labelled ssDNA; (▲) [^{3}H]SSB.

the immunoprecipitate was sedimented by centrifugation for 5 min at 15,600 ×g. As a function of the time of incubation with recA protein, increasing amounts of ^{32}P-labelled ssDNA and [^{3}H]SSB were precipitated by antibody to recA protein. At all indicated times, the immunoprecipitate contained one monomer of [^{3}H]SSB per 20−22 nucleotides of ssDNA. Under these conditions, control experiments showed lack of sedimentation of complexes of recA protein-ssDNA-SSB, in the absence of monoclonal antibodies to recA protein.

We also prepared complexes of SSB-ssDNA in the presence of an ATP regenerating system, added recA protein and after the time indicated in Fig. 2, we added 1 mM ATPrS and incubated for 5 min. Incubation with monoclonal antibodies to recA protein and sedimentation of complexes was done essentially as above. As shown in Fig. 2, the precipitation of complexes that were treated with ATPrS for 5 min was rapid and was independent of the time of further incubation with recA protein. These complexes contained one monomer of SSB per 20−22 nucleotides, in agreement with Neuendorf and Cox (22) who also observed stoichiometric complexes of recA protein-ssDNA-SSB in the presence of ATPrS.

The pattern of digestion of recA protein-ssDNA-SSB nucleoprotein filaments by micrococcal nuclease. We incubated ssDNA with SSB for 5 min in a standard buffer containing 12 mM $MgCl_2$ plus 2 mM $CaCl_2$, and then with recA protein at 1 recA monomer/2 nucleotides. After 20 min of incubation with recA protein, the complexes were digested with micrococcal nuclease for various time intervals (Fig. 3A, lanes e−k). For comparison,

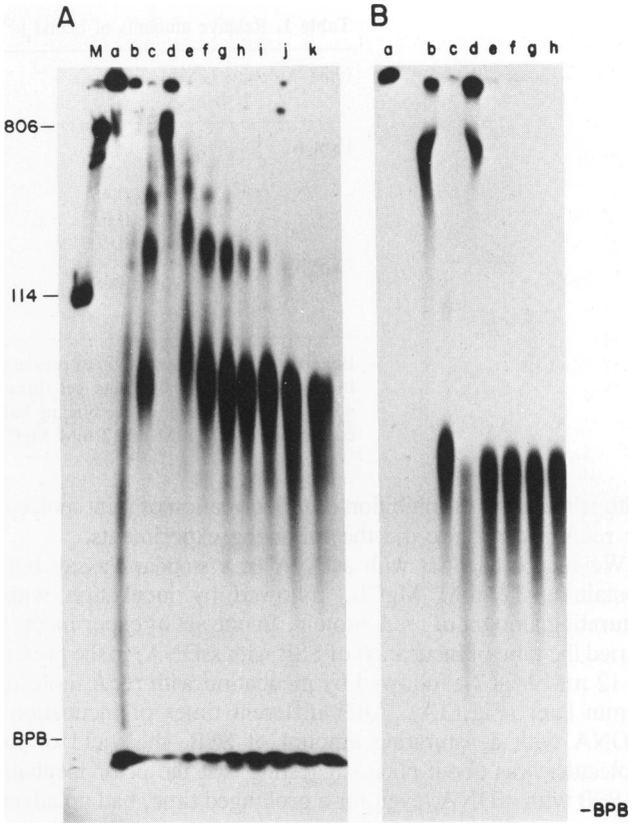

Figure 3. Repeating pattern of fragments of DNA regenerated by micrococcal nuclease in complexes of recA protein-ssDNA-SSB. **A.** Complexes formed by incubating ssDNA with SSB first, then recA protein. ^{32}P-labelled ssDNA (16 μM) was preincubated with 1 μM SSB in a standard assay buffer (90 μl) containing 12 mM $MgCl_2$, 2 mM $CaCl_2$ and an ATP-regeneration system at 37°C. After 5 min with SSB, 8 μM recA protein was added (except as indicated below) and incubated for 20 min. The DNA was digested by 0.01 units of micrococcal nuclease for various times, as indicated. Micrococcal nuclease digestion was terminated by the addition of EDTA and EGTA, each to a final concentration of 10 mM. The DNA was extracted with phenol, chloroform: isoamyl alcohol (25:24:1) and was precipitated by ethanol at −20°C for 16 h. The precipitated DNA was collected by centrifugation. The pellet was washed several times with 70% ethanol (cold) and resuspended in loading buffer. Electrophoresis was done on a 7% polyacrylamide gel containing 7 M urea at 1150 V for 2.5 h. The gel was analyzed by autoradiography with Kodak XRP-1 film. Lane M, Markers: denatured restriction fragments of double-stranded φX174 DNA; lane a, ssDNA incubated in the absence of proteins and micrococcal nuclease. All other samples were digested with micrococcal nuclease. Lane b, in the absence of recA protein or SSB for 5 min; c, SSB alone digested for 1 min; d, recA protein incubated with ssDNA at 1 mM $MgCl_2$ for 20 min, but digested in the presence of 12 mM $MgCl_2$ and 2 mM $CaCl_2$ for 1 min; e−k, contained complexes of recA protein-ssDNA-SSB; lane e, digested for 1 min; f, 2 min; g, 4 min; h, 6 min; i, 8 min; j, 10 min; k, 15 min.

B. Complexes formed by incubating ssDNA with recA protein first followed by SSB. Circular ssDNA (16 μM) was preincubated with 5.4 μM recA protein in a standard buffer containing an ATP-regeneration system and 1 mM $MgCl_2$ for 20 min. After incubation with recA protein, the concentrations of $MgCl_2$ and $CaCl_2$ were adjusted to 12 mM and 2 mM respectively, which was followed by incubation with 1 μM SSB for the times indicated. The complexes were digested by micrococcal nuclease for 5 min and the DNA was prepared for electrophoresis as described above. Lane a, ssDNA incubated in the absence of SSB, recA protein and micrococcal nuclease; all other samples were digested in micrococcal nuclease; Lane b, ssDNA incubated with recA protein; c, as a control, ssDNA was first incubated with 1 μM SSB for 5 min in standard buffer containing 12 mM $MgCl_2$ and 2 mM $CaCl_2$, followed by 5.4 μM recA protein for 20 min as in part A lanes e−k; d, ssDNA preincubated with recA protein at 1 mM $MgCl_2$ and with SSB for 0.5 min at 12 mM $MgCl_2$ and 2 mM $CaCl_2$; e, same as d but incubation with SSB for 1 min; g, same as e, but incubation with SSB for 2.5 min; f, same as e, but incubation with SSB for 5 min; h, same as g, but incubation with SSB for 10 min.

we incubated recA protein with ssDNA in an identical buffer containing 1 mM $MgCl_2$ to promote optimal binding of recA protein (4, 5), we then added $MgCl_2$ to 12 mM and $CaCl_2$ to 2 mM before subjecting the complexes to digestion with micrococcal nuclease for 1 min (Fig. 3A, lane d). Similarly, SSB was incubated with ssDNA in a standard buffer containing 12 mM $MgCl_2$ and 2 mM $CaCl_2$ and the complexes were digested with micrococcal nuclease for 1 min (Fig. 3A, lane c). After digestion with micrococcal nuclease, the reaction was terminated by the addition of EGTA and EDTA to 10 mM, the DNA was prepared and subjected to electrophoresis on 7% polyacrylamide gels containing 7 M urea.

When recA protein and SSB were absent, digestion with micrococcal nuclease produced a smear of DNA fragments (Fig. 3A, lane b). Partial digestion of recA protein-ssDNA-SSB complexes revealed a characteristic repeating pattern of fragments of DNA, approximately every 70–80 nucleotides (Fig. 3A, lanes e–f). However, prolonged digestion converted the repeating pattern into one intense species of DNA of about 70–80 nucleotides (Fig. 3A, lanes j and k). Limited digestion of complexes of ssDNA-SSB with micrococcal nuclease generated a repeating pattern of fragments (Fig. 3A, lane c), which was similar to the pattern seen upon digestion of recA protein-ssDNA-SSB complexes. In a separate experiment we confirmed the size of the repeating pattern of fragments of DNA as multiples of 70–80 nucleotides by using a series of markers ranging from 69 to 2527 nucleotides (data not shown). By contrast, micrococcal nuclease digestion of recA protein-ssDNA complexes reduced much of the DNA into heterogeneous products while a significant fraction resisted digestion (Fig. 3A, lane d). The pattern of digestion was similar whether recA protein was initially incubated with ssDNA in 1 mM $MgCl_2$ (Fig. 3A, lane d) or in 12 mM $MgCl_2$ (not shown) prior to the addition of micrococcal nuclease, $CaCl_2$, and any additional $MgCl_2$ needed to raise the concentration to 12 mM. The addition of ATPrS to stoichiometric complexes of recA protein-ssDNA-SSB abolished the characteristic repeating pattern and rendered the complexes resistant to digestion by micrococcal nuclease (data not shown).

In a complementary experiment (Fig. 3B) we first formed recA protein-ssDNA complexes in 1 mM $MgCl_2$ to promote optimal binding (5), then increased the concentration of $MgCl_2$ to 12 mM, added 2 mM $CaCl_2$ and SSB. Digestion of these complexes with micrococcal nuclease produced a pattern of digestion similar to that of SSB-ssDNA complexes. Thus, the addition of SSB to preformed recA protein-ssDNA nucleoprotein filaments converted the digestion pattern from that seen for ssDNA-SSB or recA protein-ssDNA-SSB. The presence of 2 mM $CaCl_2$ influenced neither the rate of formation of nucleoprotein filaments nor their final activity (data not shown).

Because of variable recoveries of labelled nucleotides when the digestion products were prepared for electrophoresis, we also quantitated the products by gel filtration. Control experiments established that undigested recA protein-ssDNA-SSB complexes appeared in the void volume, whereas the radioactivity from a digest of naked DNA was eluted at the end of the profile (Fig. 4). The digestion products of recA protein-ssDNA complexes appeared throughout the distribution of products bounded at one end by undigested DNA and at the other end by extensively digested DNA, in agreement with the observations made by gel electrophoresis. Digested complexes of ssDNA-SSB appeared as a single peak centred about 3/4 of the way between undigested and digested DNA. The material of interest was the product of

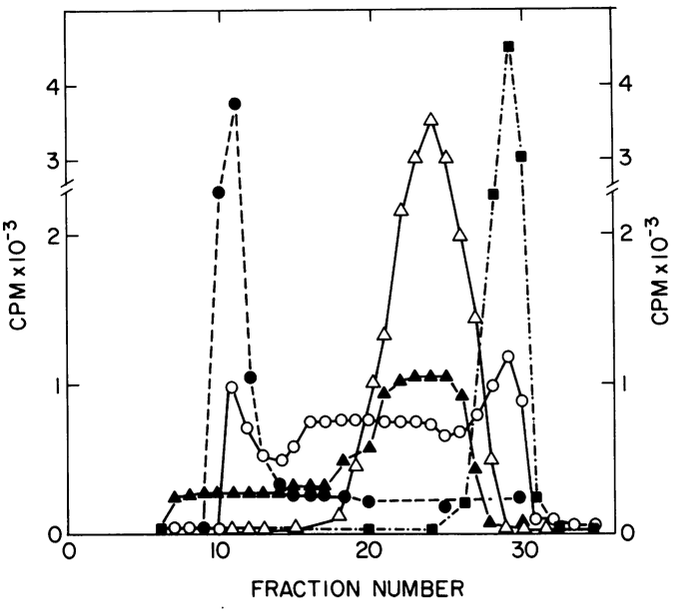

Figure 4. Resolution of products generated by micrococcal nuclease from ssDNA, complexes of recA protein-ssDNA, SSB-ssDNA, and recA protein-ssDNA-SSB on Sepharose 2B-300. The complexes were formed and digested with micrococcal nuclease as described in the Experimental Procedures. An aliquot was chromatographed on a column of Sepharose as describe in Methods. (□) control, in the absence of SSB and recA protein, digested with micrococcal nuclease for 5 min; (●) complexes of recA protein-ssDNA-SSB, not treated with micrococcal nuclease; (▲) complexes of recA protein-ssDNA-SSB, digested with micrococcal nuclease for 10 min; (○) complexes of recA protein-ssDNA digested with micrococcal nuclease for 10 min; (△) complexes of ssDNA-SSB digested with micrococcal nuclease for 10 min.

digestion of recA protein-ssDNA-SSB, of which 2/3 appeared in a peak that coincided with the peak of digested ssDNA-SSB. The remaining radioactivity was evenly distributed from this peak to the void volume. The gel filtration experiments, in which all of the labelled products were recovered, show that the majority of DNA in the recA protein-ssDNA-SSB complexes was organized in such a way that its sensitivity to micrococcal nuclease was similar to that in ssDNA-SSB complexes.

DISCUSSION

It was generally believed that the ability of SSB to bind to ssDNA with high degree of cooperativity is central to its function in diverse processes related to DNA metabolism (3). This traditional notion was dispelled by the independent observations of Griffith *et al.* (7) and Lohman and his colleagues (8–12). Griffith *et al.* (7) observed by electron microscopy, two binding modes of SSB to ssDNA depending upon the density of the protein: 'smooth' and 'beaded' forms and it was the latter that supported the binding of recA protein and subsequently active in the cleavage of lexA repressor. On the other hand, Lohman and his colleagues (8–12), by physicochemical methods, demonstrated at least three distinct modes of binding of SSB to ssDNA: $(SSB)_n$ with n = 35, 56 and 65. Further they clearly showed that the transition between these modes is greatly affected by solution variables, most notably the concentration of cations and suggested that different modes of binding of SSB to ssDNA may be used selectively in various processes such as replication, recombination and repair.

In the $(SSB)_{65}$ mode which occurs at high cation

concentrations a tetramer of SSB covers about 65 nucleotides and shows a low degree of cooperativity whereas in the $(SSB)_{35}$ mode which exists under low cation concentrations a tetramer of SSB covers about 35 nucleotides and appears to show a metastable high degree of cooperativity. The inhibitory effect of SSB on the formation of joint molecules by recA protein, as observed in the present experiments correlates with its metastable cooperative binding to ssDNA. This cooperative binding of SSB, even at 3-fold lower concentration relating to the saturating amount, reduces the amount of [^{35}S]recA protein bound to circular ssDNA (data not shown). The lack of inhibitory effect of SSB at 12 mM $MgCl_2$ on the formation of joint molecules draws further support from the *in vitro* experiments of Resnick and Sussman (23) in which cleavage of lambda repressor by recA protein was not inhibited by a large excess of SSB. This is also the case *in vivo* where 60-fold overproduction of wild type SSB does not inhibit lambda prophage induction (24).

In observations described here, we found that when recA protein-ssDNA-SSB complexes were formed at 12 mM $MgCl_2$ and then isolated by gel filtration at 12 mM $MgCl_2$, the complexes contained 1 monomer of SSB per 40 nucleotides. However, when complexes were formed in 12 mM $MgCl_2$ and then isolated by gel filtration in 1 mM $MgCl_2$ we found 1 monomer of SSB per 22 nucleotides, and 1 monomer of recA protein per 4 nucleotides. These observations suggested that weakly bound SSB might have dissociated during gel filtration in 12 mM $MgCl_2$. Immuno-precipitation experiments (Fig. 2) done in conditions containing 12 mM $MgCl_2$ revealed complexes with maximal stoichiometries of both recA protein and SSB, even when the antigen, recA protein, has tightly anchored to the DNA by ATPrS. Our evidence for a stoichiometric complex containing large amounts of recA protein and SSB was further strengthened by micrococcal nuclease digestion experiments which revealed regular repeating pattern of digestion of the recA protein-ssDNA-SSB complexes whether recA protein or SSB were allowed to bind first to the ssDNA.

Recently Morrical *et al.* (6) concluded that there is a continuous and stoichiometric association of SSB with the recA nucleoprotein filament, whereas Kowalczykowski *et al.* (25) concluded that the binding of recA protein and SSB to ssDNA are mutually exclusive. The observations in this paper support the view that under conditions that favour the formation of joint molecules and strand exchange, there is an unusual association of stoichiometric quantities of SSB with the presynaptic nucleoprotein filament. There are, however, conditions under which the binding of SSB or recA protein to ssDNA are mutually exclusive; these conditions include the absence of nucleotide cofactor, and low concentrations of $MgCl_2$, both of which favour the cooperative binding of SSB (5, 6, 25).

Using oligonucleotides of defined length and ATPrS, Leahy and Radding (26) demonstrated that recA protein binds primarily to the phosphate backbone of ssDNA leaving the bases free. It is pertinent here to point out that in addition to ionic interactions (11) nuclear Overhauser measurements of Clore *et al.* (27) suggested that the bases in ssDNA complexed with SSB are in direct contact with the amino acid residues on the surface of SSB whereas the sugar phosphate groups are projected outwards into the solvent. This type of hydrophobic interaction has been postulated to play a major role in the interaction of other single strand binding proteins with DNA (28). These results, which suggest that recA protein interacts with the phosphate backbone and SSB interacts with the bases, may account for the presence of saturating amounts of each protein on the final presynaptic complex.

In the experiments described here, we took an enzymatic approach to analyzing the spatial distribution of recA protein and SSB on ssDNA. Partial digestion of active stoichiometric complexes with micrococcal nuclease produced a characteristic cleavage pattern at intervals of approximately 70−80 nucleotides, in contrast to 145 nucleotides observed by Chrysogelos and Griffith (29). Although, the reason(s) for this difference in cleavage pattern is not clear the limit fragment size of 70−80 nucleotides seems to agree well with the value determined by biophysical methods (8−11). This repeating pattern was similar to that observed from digestion of complexes of SSB-ssDNA. However, when SSB was present with ssDNA at 1 mM $MgCl_2$, the complexes were largely resistant to micrococcal nuclease digestion (data not shown). It is intriguing that there was little apparent difference in the size of the fragments of DNA produced by micrococcal nuclease between the complexes of recA protein-ssDNA-SSB and ssDNA-SSB whether recA protein was loaded first or SSB was loaded first on the DNA. Since we do not know, at present, the precise sites of cutting by micrococcal nuclease, we can only speculate on the possible reasons. Micrococcal nuclease might cleave at sites on the nucleoprotein filament at the junction between SSB and recA protein. As a result of shortening the length of the polynucleotide, recA protein might dissociate more readily, which would enable micrococcal nuclease to degrade the ssDNA further. Alternatively, the hydrolysis of ATP may transiently induce the dissociation of bound recA protein from the ssDNA thus creating sites for micrococcal nuclease digestion. Indeed, the latter may be the case, since when we added ATPrS to such reactions, the ssDNA, which otherwise contained a full complement of SSB and recA protein, was protected from digestion by micrococcal nuclease. Nevertheless, the interspersed positioning of SSB on ssDNA may be important. In addition to removing secondary structure, a kinetic barrier for the polymerization of recA protein (4), the regular, repeated positioning of SSB on ssDNA may make the sequences of the DNA more readily accessible for recA protein and possibly for other proteins.

To think about how SSB facilitates the action of recA protein, it is important to recall the three distinct phases of the recA protein action: presynaptic polymerization on ssDNA, synapsis, which produces a nascent joint molecule, and post-synaptic strand exchange. There appears to be a general agreement that at least some effects of SSB are attributable to the removal of secondary structure (4, 6, 25). Kahn and Radding (30) observed that preincubation of recA protein with ssDNA, which stimulated the initial rate of formation of joint molecules, had no effect on the rate of strand exchange in the subsequent post-synaptic phase, whereas SSB stimulated both the rate of formation of joint molecules and the rate of strand exchange per joint molecule. Since strand exchange is slow, and requires $MgCl_2$ in excess of 10 mM, the continuous presence of SSB may aid that reaction by maintaining the recA protein-ssDNA filament as it does during the presynaptic phase.

The significance of the stoichiometric recA protein-ssDNA-SSB filament and the fundamental mechanism by which SSB aids the reaction are still not certain, but several aspects of the collective observations stand out: SSB removes secondary structure and interacts with ssDNA in subtle ways, involving various modes of binding, some of which favour extensive binding of recA protein even while SSB remains in weak

association with the DNA; and finally, the interchangeability of several other helix destabilizing proteins with SSB (4) strongly suggests that SSB acts via its effect on ssDNA rather than via the specific interactions with recA protein.

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