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Continuous Association of *Escherichia coli* Single-Stranded DNA Binding Protein with Stable Complexes of *rec*A Protein and Single-Stranded DNA†

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ABSTRACT: The single-stranded DNA binding protein of *Escherichia coli* (SSB) stimulates *rec*A protein promoted DNA strand exchange reactions by promoting and stabilizing the interaction between *rec*A protein and single-stranded DNA (ssDNA). Utilizing the intrinsic tryptophan fluorescence of SSB, an ATP-dependent interaction has been detected between SSB and *rec*A–ssDNA complexes. This interaction is continuous for periods exceeding 1 h under conditions that are optimal for DNA strand exchange. Our data suggest that this interaction does not involve significant displacement of *rec*A protein in the complex by SSB when ATP is present. The properties of this interaction are consistent with the properties of SSB-stabilized *rec*A–ssDNA complexes determined by other methods. The data are incompatible with models in which SSB is displaced after functioning transiently in the formation of *rec*A–ssDNA complexes. A continuous association of SSB with *rec*A–ssDNA complexes may therefore be an important feature of the mechanism by which SSB stimulates *rec*A protein promoted reactions.

The steps in homologous genetic recombination mediated by the *rec*A protein of *Escherichia coli* have been investigated through the study of DNA strand exchange reactions promoted by this enzyme in vitro. One of these in vitro systems has been especially informative. In the presence of ATP, the *rec*A protein promotes the exchange of complementary strands between homologous circular single-stranded and linear duplex DNA molecules derived from bacteriophage, forming nicked circular heteroduplex and linear single-stranded species as products. Strand exchange occurs in three distinguishable phases: (1) *presynapsis*, the cooperative binding of *rec*A protein to single-stranded DNA (ssDNA)[1] in the presence of

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ATP (Cox & Lehman, 1982; Radding et al., 1983); (2) *synapsis* or D-loop formation, the alignment of homologous sequences and formation of a nascent heteroduplex joint between the reactant molecules (McEntee et al., 1979; Shibata et al., 1979; Cox & Lehman, 1981a; DasGupta & Radding, 1982); and (3) *polar branch migration*, the unidirectional extension of the heteroduplex region, coupled to the hydrolysis of ATP (Cox & Lehman, 1981a,b; Kahn et al., 1981; West et al., 1981). This system provides an in vitro model for the homologous pairing (synapsis) and heteroduplex extension (branch migration) phases of homologous recombination events that occur in the cell.

The single-stranded DNA binding protein (SSB) of *Escherichia coli* enhances both the rate and final extent of heteroduplex formation in the strand exchange reaction (Cox & Lehman, 1981a, 1982; Wu et al., 1982). This effect is intriguing in light of the demonstrated importance of SSB in homologous recombination (Johnson, 1977; Glassberg et al., 1979; Vales et al., 1980) and in DNA replication and repair processes in vivo (Glassberg et al., 1979; Whittier & Chase, 1981). Previous studies have shown that SSB enhances strand exchange by stabilizing the presynaptic *rec*A–ssDNA complex (Cox et al., 1983a; Radding et al., 1983). The resulting complex has several properties that distinguish it from complexes formed in the absence of SSB. *rec*A–ssDNA complexes formed in the presence of SSB have apparent dissociation half-times of greater than 90 min (Cox et al., 1983a), a stability 20–30 times that observed for these complexes in the presence of ATP alone (McEntee et al., 1981a; Bryant et al., 1985). With the SSB-stabilized *rec*A–ssDNA complexes as intermediates, branch migration replaces synapsis as the rate-limiting step of strand exchange, resulting in a higher rate and efficiency of heteroduplex formation. While SSB has a modest effect on the *rate* of the branch migration step promoted by *rec*A protein, the *efficiency* of this step with respect to the stoichiometry of ATP hydrolysis is greatly enhanced by SSB (Cox et al., 1983b). In the absence of SSB, several thousand molecules of ATP are hydrolyzed per heteroduplex base pair formed during branch migration. This ratio improves to 100–200 ATP molecules per base pair when SSB is present.

The mechanism by which SSB participates in strand exchange has been difficult to determine, primarily because intact *rec*A–ssDNA complexes containing SSB have not been isolated or detected. Although Tsang et al. (1985) recently isolated complexes containing both proteins, the amounts of *rec*A protein and SSB present appeared to be inversely proportional, suggesting that partial dissociation of *rec*A–ssDNA complexes had allowed some SSB to bind. As a result, the two models for the action of SSB proposed to date involve only a transient role for SSB in the formation of the *rec*A–ssDNA complex. In one model, the stabilization of *rec*A–ssDNA interactions is a result of the helix-destabilizing activity of SSB. It is proposed that SSB melts out secondary structure in ssDNA, allows *rec*A protein to bind, and is then displaced by the *rec*A protein (Flory et al., 1984; Muniyappa et al., 1984). Therefore, in this model SSB acts passively via the ssDNA, without interacting with *rec*A protein. A second model involves SSB as a specific assembly factor promoting the formation of stabilized *rec*A–ssDNA complexes through interactions of the SSB and *rec*A protein molecules prior to the displacement of SSB (Griffith et al., 1984; Register & Griffith, 1985). Muniyappa et al. (1984) reported that conditions that alternately promoted or inhibited the formation of secondary structure in ssDNA affected the *rec*A protein dependent formation of D loops in reciprocal fashion. The strong correlation they observed between secondary structure and the rate of *rec*A protein promoted synapsis in the absence of SSB supported the first (passive) model of SSB action. Muniyappa et al. (1984) also reported that, like SSB, both the phage T4 gene 32 protein and the β protein of phage λ increase the rate and extent of D-loop formation. The ability of three different helix-destabilizing proteins to accelerate this reaction argued against specific interactions between *rec*A protein and SSB as a means of *rec*A–ssDNA complex stabilization. Evidence for *rec*A–SSB interactions in the second model was provided by electron micrographic observations (Griffith et al., 1984).

Results from a number of other studies are not easily explained by a model involving only a transient participation of SSB in complex formation. First, the order of addition of *rec*A protein and SSB has a remarkable effect on the rate of strand exchange (Cox & Lehman, 1982; West et al., 1982). When SSB was mixed with ssDNA prior to the addition of *rec*A protein, a large time lag occurred in D-loop formation, indicating that the kinetically slow dissociation of the preformed SSB–ssDNA complex was necessary before SSB-primed *rec*A–ssDNA complex formation could begin (Cox & Lehman, 1982). This order of addition effect is not unique to the strand exchange reaction of *rec*A protein; hydrolysis of ATP and dATP in the absence of homologous duplex is strongly inhibited when SSB is added to solutions of ssDNA prior to the addition of *rec*A protein or nucleotide (McEntee et al., 1980; Cohen et al., 1983). Although *rec*A protein is capable of binding very tightly to ssDNA under conditions similar to those used for strand exchange (Morrical & Cox, 1985; Bryant & Lehman, 1985), these results indicate that displacement of cooperatively bound SSB from ssDNA by *rec*A protein is very slow. Second, the strong enhancement of the rate of DNA strand exchange by SSB is observed even in the presence of large excesses of *rec*A protein (Cox et al., 1983a), and stoichimetric levels of SSB are required even when excess *rec*A protein is utilized. The enhancement of the efficiency of branch migration in the presence of SSB suggests that this requirement may be continuous throughout strand exchange.

It is conceivable that the previous inability to detect SSB in intact *recA*–ssDNA complexes is a result of the techniques applied (electron microscopy and centrifugation), which insert several potentially disruptive sample handling steps between the formation of complexes and data acquisition. We therefore sought more sensitive and less disruptive techniques to study the interaction between SSB and *rec*A–ssDNA complexes. The SSB protein contains four tryptophan residues per 18.9-kDa monomer (Horii et al., 1980; Sancar et al., 1981) and shows strong tryptophan fluorescence in solution. Tryptophan fluorescence is strongly quenched upon binding of SSB to ssDNA; this property has been used to characterize the SSB–ssDNA interaction (Lohman & Overman, 1985; Lohman et al., 1986). Fluorescence therefore appeared to be a potentially useful probe for studying SSB interactions with *rec*A–ssDNA complexes. In this study, we present SSB tryptophan fluorescence data that indicate (1) that SSB is continuously associated with *rec*A–ssDNA complexes in the presence of ATP during and following their assembly from components, (2) that the binding of SSB to these complexes

[1] Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; SSB, *Escherichia coli* single-stranded DNA binding protein; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); ϕX174(+), circular single-stranded genome of bacteriophage ϕX174; M13*mp*8(+), circular single-stranded genome of bacteriophage M13*mp*8; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; kDa, kilodalton(s); kb, kilobase(s).

does not involve significant displacement of *rec*A protein when ATP is present, and (3) that therefore the effects of SSB on *rec*A-promoted strand exchange cannot be entirely attributed to the passive helix-destabilizing activity of this protein or to a transient role of SSB in *rec*A–ssDNA complex formation.

Experimental Procedures

Materials. *E. coli* *rec*A protein was purified as previously described (Cox et al., 1981) and stored frozen at −70 °C in buffer A [20 mM Tris, 80% cation (pH 7.5), plus 1 mM DTT, 0.1 mM EDTA, and 10% glycerol]. The concentration of *rec*A protein in stock solutions was determined by the absorbance at 280 nm, using an extinction coefficient of $\epsilon_{280} = 0.59\ A_{280}$ mg^{-1} mL (Craig & Roberts, 1981). *E. coli* SSB protein was purified by a modification of a published procedure (Weiner et al., 1975) and stored frozen at −70 °C in buffer B [50 mM imidazole, 72% cation (pH 6.5), plus 0.15 M NaCl, 1 mM EDTA, 1 mM DTT, and 20% glycerol]. SSB stock concentrations were determined by the absorbance at 280 nm, using an extinction coefficient of $\epsilon_{280} = 1.5\ A_{280}$ mg^{-1} mL (Lohman & Overman, 1985). ϕX174(+) circular ssDNA (5.4 kb) was prepared by a published procedure (Cox & Lehman, 1981a). M13*mp*8(+) circular ssDNA (7.2 kb) was prepared by a modification of the procedure of Messing (1983). All ssDNA solutions were stored at 0 °C in 10 mM Tris and 1 mM EDTA, pH 7.5. ssDNA stock concentrations were determined by the absorbance at 260 nm, using 36 $\mu g\ mL^{-1}\ A_{260}^{-1}$ as a conversion factor. All ssDNA concentrations are expressed in nucleotides. ATPγS was purchased from Boehringer Mannheim; all other biochemicals and enzymes were purchased from Sigma.

Instrumentation. All fluorescence measurements were obtained on an SLM Instruments 8000 series scanning fluorometer equipped with a thermojacketed cuvette holder attached to a constant-temperature water circulator, and with an externally controlled magnetic stirring motor for the sample chamber. This instrument has a focal length of 2 in. (lens to sample) and mean beam path lengths of 128 cm (source to sample) and 105 cm (sample to photomultiplier). Signal acquisition time was fixed at 1 s in all experiments; cell path length and band-pass were equal to 1 cm and 2 nm, respectively.

Absorbance and turbidity measurements were obtained on a Perkin-Elmer Lambda 7 double-beam recording spectrophotometer equipped with a thermojacketed cuvette holder attached to a constant-temperature water circulator. Cell path length and band-pass were equal to 1 cm and 2 nm, respectively, in all experiments.

Fluorescence Measurements. Protein fluorescence spectra were obtained at an excitation wavelength of 295 nm and emission wavelengths of 320–420 nm. SSB spectra were taken at 37 ± 0.1 °C in a standard reaction buffer consisting of 20 mM Tris, 80% cation (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 5% glycerol. SSB emission is maximal at 349 nm and is greatly enhanced with respect to an equal amount (4 SSB monomer equiv) of free tryptophan. The nucleotides ATP, ADP, and ATPγS have no effect on SSB fluorescence. Fluorescence emission spectra of the *rec*A protein were taken in 40 mM rather than 20 mM Tris to avoid light-scattering interference with fluorescence measurements due to *rec*A filament formation, which occurs at the lower ionic strength in the absence of nucleotides (Morrical & Cox, 1985; Cotterill & Fersht, 1983a). *rec*A emission is maximal at 345 nm in this buffer system, and at 355 nm is slightly quenched with respect to an equal amount (2 *rec*A monomer equiv; Sancar et al., 1980) of free tryptophan; 1 mM ATP, 1 mM ADP, and 0.2 mM ATPγS each produce a small (less than 5% at 355 nm) quenching effect on *rec*A fluorescence. Quenching effects by nucleotides are included in control values described below and under Results. The effect of ssDNA on the *rec*A fluorescence spectrum was determined in standard reaction buffer containing either 1.0 mM ATP or 0.2 mM ATPγS.

All other fluorescence studies were carried out under the following conditions, unless specifically noted. Excitation and emission wavelengths were fixed at 295 and 355 nm, respectively. A constant temperature of 37 ± 0.1 °C was maintained. Standard reaction buffer consisted of 20 mM Tris, 80% cation (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 5% glycerol. Standard reaction mixtures contained *rec*A protein and SSB at various concentrations, 6.75 μM ssDNA [either ϕX174(+) or M13*mp*8(+)], and 1.0 mM ATP in a total volume of 2.0 mL. In all cases, duplicate experiments involving different ssDNA species gave identical results. Additions of all components were made to temperature-equilibrated cuvettes containing rapidly stirring reaction buffer to ensure fast mixing. The order of addition of reaction components used was generally ATP, ssDNA, *rec*A protein, and SSB. When the time course for complex formation was monitored by fluorescence, zero time was taken as the instant of addition of the last component (usually SSB). Variations in the composition of reaction mixtures and changes in the order of addition are specifically noted under Results and in figure legends.

Reverse titrations of SSB with ssDNA were carried out in standard reaction buffer at 37 ± 0.1 °C. Small aliquots of ssDNA stock solutions (concentration 0.9–1.2 mM) were added sequentially to solutions of 0.45 μM SSB, with the resulting fluorescence intensity recorded after each addition. The total change in reaction volume resulting from titration additions was less than 1%; therefore, no correction was made for dilution. The estimation of equivalence points from these titrations is described in the text.

Treatment of Data. Relative fluorescence intensities were obtained as fluorometer output corrected for background (the signal obtained from a blank solution of standard reaction buffer). SSB emission spectra and reverse titration curves were corrected for residual fluorescence in SSB storage buffer (buffer B). The residual level was approximately 11% of the fluorescence signal of SSB when monitored at 355 nm. This residual is included in the control values F_0 and F_Q in all other experiments. All fluorescence data reported in the text are normalized with respect to the fluorescence of 0.45 μM SSB at standard conditions, unless specifically noted. Data points in all figures are average values from at least two separate experiments. Line- and curve-fitting procedures are described in the text. The relative fluoresence of *rec*A protein was determined in each reaction mixture by recording fluorescence intensity prior to the addition of SSB. Where order of addition changes made this impossible, *rec*A fluorescence was measured independently in a separate solution. According to these procedures, F_{recA} incorporates any small quenching effects by nucleotides. The fluorescence of SSB in the presence and absence of ssDNA was determined independently in all experiments. The control value F_0 was determined by adding the relative fluorescence of *rec*A protein to that of free SSB for experiments at fixed concentrations of *rec*A and SSB. This value is taken as the initial fluorescence intensity (at time zero) in all time courses. When *rec*A and/or SSB concentration was varied in an experiment, F_0 was determined independently at each protein concentration as the relative fluorescence of the *rec*A–SSB mixture in the absence of ssDNA. In all cases,

the F_0 calculated from the relative fluorescence intensities of separate solutions of *rec*A and SSB was equal to the F_0 observed in solutions containing both SSB and *rec*A. The control value F_Q was calculated by adding the relative fluorescence of *rec*A protein to the independently determined fluorescence of SSB in the presence of ssDNA.

Effects of Photobleaching, Light Scattering, and Absorption on Fluorescence Measurements. Observed fluorescence intensity can be affected by photobleaching of the chromophore or by absorption or scattering of the excitation light by other molecules in the solution. The system under investigation is a complex mixture of components, so detailed controls were carried out to evaluate the effect of the above factors.

Two approaches were taken to determine what fraction, if any, of fluorescence quenching effects was attributable to photobleaching of *rec*A protein or SSB fluorescence. (1) Continuous exposure of solutions of *rec*A protein and SSB in the absence of ssDNA to the excitation beam for periods of time up to 60–70 s resulted in no detectable loss of fluorescence intensity. This was true at all concentrations of *rec*A protein and SSB used in these experiments, and in no experiment was a sample continuously exposed to the excitation beam for more than 70 s. (2) NaCl was added to samples quenched as a result of protein–ssDNA complex formation to dissociate the complexes and determine if the original fluorescence intensities could be recovered. Quenched and unquenched samples (with and without ssDNA, respectively) were exposed to the excitation beam for 60 s and then diluted with concentrated NaCl to a final NaCl concentration of 2 M. In all cases, the final fluorescence intensities of the two samples were identical. We could detect no significant photobleaching of samples in any case under the conditions of these experiments. As a general precaution against photobleaching, exposure of all samples to the source beam was held to 10 s or less when acquiring data, except during the first minute of time courses when more rapid data acquisition was required.

The sensitivity of fluorescence signals to changes in the turbidity of reaction mixtures was tested by measuring SSB fluorescence in solutions of known turbidity. Turbid solutions of *rec*A filaments were generated as described by Morrical and Cox (1985). *rec*A protein (0.5–2.0 μM) was incubated with 0.45 μM SSB for 15 min at 37 ± 0.1 °C in 10 mM Tris, 80% cation (pH 7.5), and 10 mM $MgCl_2$. The apparent turbidities of these solutions and of solutions containing *rec*A–ssDNA complexes formed in the presence of SSB were measured as the absorbance at 355 nm. The highest turbidity observed for *rec*A filaments produced an A_{355} of 0.12 at 2.0 μM *rec*A protein. Relative fluorescence intensities of the same *rec*A protein solutions containing SSB and of control solutions containing *rec*A protein but lacking SSB were also measured as described above. Fluorescence due to *rec*A protein alone was subtracted to give apparent SSB fluorescence in the turbid solutions. The observed fluorescence of SSB was independent of solution turbidity over the A_{355} range of 0–0.12. The highest turbidity measured for solutions of *rec*A protein–ssDNA formed in the presence of ATP and SSB was approximately 0.03 under standard reaction conditions. Since the turbidity of our reaction mixtures falls within the lower part of the A_{355} range determined above, changes in fluorescence intensity observed in mixtures of *rec*A, SSB, and ssDNA cannot be attributed to light scattering by the system.

The excitation wavelength, 295 nm, was chosen to minimize interference by nucleotide or nucleic acid absorption (inner filter effects). Decreases in fluorescence intensity due to inner filter effects were evaluated in the following way. Titrations of *N*-acetyl-L-tryptophanamide solutions were carried out with the nucleic acid or nucleotide of interest (or both). No changes in fluorescence intensity were detected under standard experimental conditions with the highest concentrations of ssDNA used in these experiments. Decreases of approximately 3% per millimolar unit of nucleotides ATP, ADP, and ATPγS were observed; however, since the experimental control values utilized included the effects of nucleotides initially present on protein fluorescence, correction for primary absorption by nucleotides was necessary only when additional ATP, ADP, or ATPγS was added to a reaction mixture. Using the method of Birdsall et al. (1983), a correction of approximately 9% resulted at the highest nucleotide concentration used in these experiments.

ATP Regeneration and ATPase Assay. An ATP regenerating system consisting of 2.24 units/mL pyruvate kinase, 2.31 mM phosphoenolpyruvate, and 0.44 mM KCl was included in some reaction mixtures; 2.24 units/mL lactate dehydrogenase and 1.0 mM NADH were added to the regenerating system to form a coupled spectrophotometric assay system for *rec*A ATPase activity. Decreases in NADH concentration as a function of time were monitored by the absorbance at 370 nm at a constant temperature of 37 ± 0.1 °C. NADH absorbance was measured at 370 nm rather than 340 nm (the absorbance maximum) in order to accommodate the high concentration of NADH needed for the coupled assay while remaining within the linear absorbance range of the spectrophotometer. Initial rates expressed as micromolar ADP produced per minute were calculated from $-\Delta A_{370}$ per minute data by using an extinction coefficient of $\epsilon_{370} = 2.64$ mM^{-1} cm^{-1} for NADH. All time courses were linear following an initial induction (τ) time of 1–2 min. Slightly longer lag times were observed in the presence of SSB. Initial velocities were calculated from the linear portions of time courses since ATP is regenerated by the coupling system and $[ATP] = [ATP]_0$ in the steady state. Doubling the concentration of pyruvate kinase and lactate dehydrogenase in the reaction mixtures had no effect on the observed rate of NADH oxidation; therefore, ATP hydrolysis by the *rec*A protein was strictly rate limiting in the coupled assay. Reported K_m values for ATP in *rec*A protein promoted ATP hydrolysis range from 20 to 90 μM in the presence or absence of SSB (Weinstock et al., 1981a; Cox et al., 1983b). Doubling the ATP concentration from 1 mM to 2 mM had no significant effect (<3% increase) on the rate of ATP hydrolysis, so that ATP hydrolysis is being measured under maximum velocity conditions in all experiments reported. Reaction volumes of 0.91 mL were contained in 1-mL cuvettes. ATP hydrolytic reactions were started by the addition of *rec*A protein to cuvettes containing buffer, ATP, ssDNA, and coupling system. SSB was added last, 1 min after the addition of *rec*A protein. All other reaction conditions were as described for the fluorescence experiments.

RESULTS

Interaction of SSB with ssDNA in the Absence or Presence of recA Protein

Fluorescence Properties of SSB and recA Protein. The effects of ssDNA on the fluorescence spectra of SSB and *rec*A protein in standard reaction buffer are presented in Figure 1. SSB fluorescence is strongly quenched by ssDNA. At low binding density (excess ssDNA), the extent of quenching exceeds 80% when monitored at 355 nm (Figure 2). With 1.0 mM ATP present, the emission spectrum of *rec*A protein in the presence of stoichiometric amounts of ssDNA was indistinguishable from the spectrum in the absence of ssDNA.

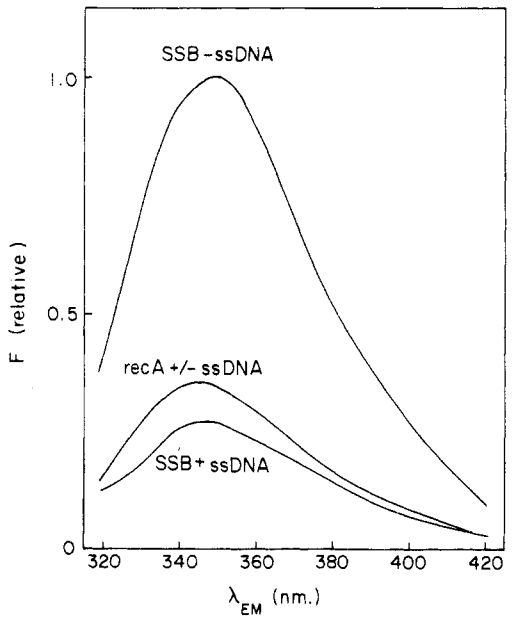


FIGURE 1: Fluorescence spectra of SSB and *rec*A protein in the presence and absence of ssDNA. Excitation wavelength was 295 nm. *rec*A protein spectra were obtained in the presence of 1 mM ATP. All other conditions were as described under Experimental Procedures. The concentrations of *rec*A protein and SSB were 1.69 and 0.45 μM, respectively. When present, ssDNA concentration was 6.75 μM.

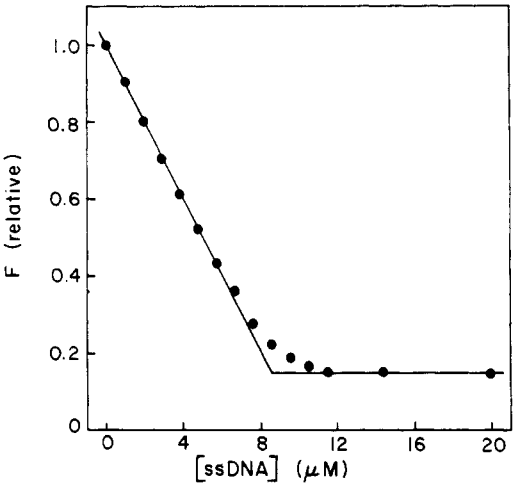


FIGURE 2: Reverse titration of SSB with ssDNA. Titrations were carried out as described under Experimental Procedures; saturation of SSB with ssDNA was monitored by fluorescence at 355 nm. The equivalence point estimated from the titration is indicated by the intersection of asymptotic lines drawn on the plot.

Identical results were obtained with 0.2 mM ATPγS replacing ATP in the mixture. Dombroski et al. (1983) have previously reported a lack of tryptophan fluorescence quenching in *rec*A–dsDNA complexes formed in the presence of ATPγS.

At equal concentrations of monomers, the fluorescence intensity of SSB at 355 nm is much higher than that of *rec*A at the same wavelength, due to the 2-fold higher tryptophan content of SSB and the large fluorescence enhancement effect in the SSB tryptophan emission spectrum. In the absence of ssDNA, the 355-nm fluorescence intensities of *rec*A and SSB were found to be completely additive over the entire range of concentrations used in these experiments (see Figures 6 and 9). This strict additivity of the fluorescence intensities of SSB and *rec*A protein was a feature of the entire fluorescence emission spectra of the two proteins and protein mixtures (data not shown). Therefore, neither protein has an observable quenching effect on the other in the absence of ssDNA. Because of the relatively high fluorescence of SSB, and because of the additivity of SSB and *rec*A fluorescence, changes in the SSB signal brought about by quenching can be easily detected above a high background of *rec*A fluorescence.

$MgCl_2$ Induces the High-Salt Binding Mode of SSB. Lohman and Overman (1985) observed that SSB may assume one of at least two distinct ssDNA binding modes, depending on the NaCl concentration of the solution. The "high-salt" binding mode is characterized by a ssDNA binding site size of 65 ± 5 nucleotide residues per SSB tetramer (16 ± 1 residues per monomer) and 83 ± 3% quenching of tryptophan fluorescence in the ssDNA-bound protein. This binding mode exclusively is observed at NaCl concentrations greater than 0.1–0.2 M. The "low-salt" binding mode is characterized by a ssDNA binding site size of 33 ± 3 nucleotide residues per SSB tetramer (8 ± 1 residues per monomer) and 51 ± 3% quenching of tryptophan fluorescence in the ssDNA-bound protein. This binding mode exclusively is observed at NaCl concentrations less than 10 mM. It is postulated that $(SSB)_{33}$ (the low-salt form) arises through binding of ssDNA to two SSB monomers in a tetramer, while in $(SSB)_{65}$ (the high-salt form) ssDNA is wrapped to interact with all four monomers of the tetramer, giving twice the apparent binding site size and a higher degree of fluorescence quenching. The transition between the two forms at intermediate salt concentrations appears to be correlated with a decrease in the cooperativity parameter (ω) for SSB as the salt concentration is increased (Ruyechan & Wetmur, 1976; Lohman & Overman, 1985; Lohman et al., 1986).

Under the conditions employed in this study (particularly with 10 mM $MgCl_2$ replacing NaCl), and in the absence of *rec*A protein, SSB interacts with ssDNA in its high-salt binding mode. Figure 2 shows a reverse fluorescence titration performed by adding incremental amounts of M13*mp*8(+) ssDNA to a constant total concentration of SSB. Identical results were obtained with ϕX174(+) ssDNA. The calculated apparent binding site size of SSB, n_{app}, is equal to the SSB concentration divided by the concentration of ssDNA obtained at the intersection of a line drawn through the "plateau" region of the binding curve (low binding density, SSB saturated with ssDNA) and the best line drawn through the linear descending portion of the curve. Five separate titrations gave an average value of 17 ± 2 nucleotide residues per SSB monomer (68 ± 8 residues per tetramer) for n_{app}, which is very close to the value obtained by Lohman and Overman (1985) under high-salt conditions.[2] In addition, quenching in the saturated mixture averaged 85 ± 2%, again well within experimental error of the value obtained by Lohman and Overman (1985) at high [NaCl]. These results establish that relatively low concentrations of $MgCl_2$ induce a binding mode of SSB approximately equivalent to that observed at high NaCl concentrations and demonstrate that interactions between SSB and free ssDNA occur via this binding mode under conditions routinely used in the study of *rec*A protein promoted DNA strand exchange. These conclusions are consistent with results obtained elsewhere (T. Lohman, personal communication).

Control Values for Fluorescence Measurements. All protein fluorescence data reported in subsequent sections were compared to two control values, determined independently for each

[2] A binding site size of 45 nucleotides per SSB tetramer is observed under these conditions when a poly(dT) lattice is utilized. The difference may reflect secondary structure unavailable to SSB binding in the polynucleotide employed here. The extent of fluorescence quenching observed is consistent with the "high-salt" binding mode for both M13*mp*8(+) and poly(dT) (T. Lohman, personal communication).

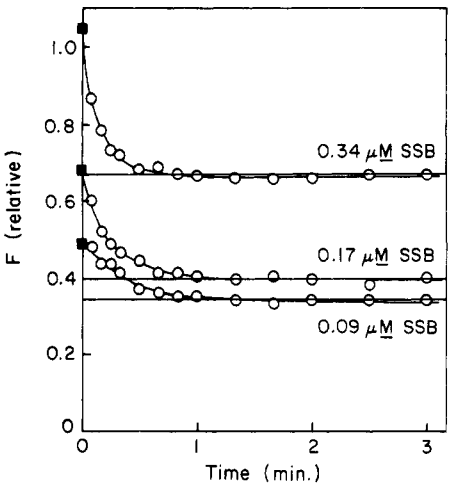


FIGURE 3: Interaction of SSB with *rec*A–ssDNA complexes formed in the absence of ATP. The indicated amounts of SSB were added to preincubated mixtures of 1.0 μM *rec*A protein and 3.38 μM ssDNA in 40 mM Tris, 80% cation (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 5% glycerol. All other conditions were as described under Experimental Procedures. The horizontal lines drawn represent the values of F_Q, and solid squares (■) denote F_0 values determined at the indicated SSB concentrations.

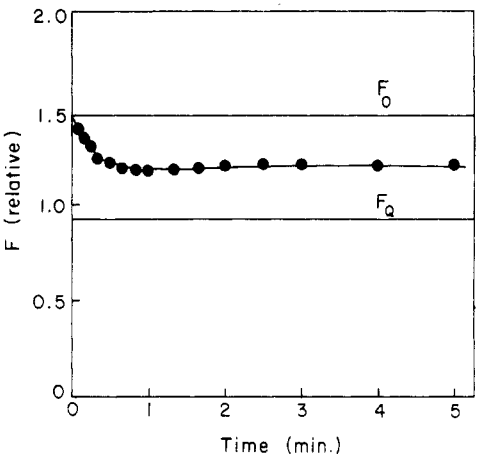


FIGURE 4: Interaction of SSB with *rec*A–ssDNA complexes formed in the presence of ATP. SSB (0.45 μM) was added at time zero to a solution containing 2.0 μM *rec*A, 6.75 μM ssDNA, and 1.0 mM ATP. All other conditions were as described under Experimental Procedures. The control values F_0 and F_Q are described in the text.

experiment. The first control value will be referred to as "total fluorescence control", or simple F_0. This value represents the relative fluoresence intensity from a solution containing given concentrations of SSB and *rec*A protein in the presence of all other reaction components except ssDNA. F_0 therefore includes any small quenching effects of nucleotides on *rec*A protein and is equal to the sum of the intrinsic fluorescence of SSB (in the absence of ssDNA) and the fluorescence of *rec*A protein in a reaction mixture. The second control value will be referred to as "quenched fluorescence control", or simply F_Q. This value represents the *calculated sum* of the fluorescence of SSB in the presence of ssDNA and the fluorescence of *rec*A protein, both of which are determined independently in each experiment in the presence of all other non-protein reaction components. In practice, F_Q is the minimum possible fluorescence intensity in a given reaction mixture (assuming no further quenching effects on *rec*A protein), attained when the SSB is maximally bound to the ssDNA under the conditions of the experiment. F_0, on the other hand, is the maximum possible fluorescence intensity in a given reaction mixture, attained when all SSB is displaced from ssDNA.

SSB and recA Protein Bind ssDNA Competitively in the Absence of ATP. On addition of SSB to *rec*A–ssDNA complexes formed in the absence of ATP, fluorescence decreases with time to the level of F_Q (Figure 3), suggesting that SSB has displaced a stoichiometric amount of *rec*A protein from the ssDNA and coated the single strand as $(SSB)_{65}$. Final fluorescence intensities are equivalent to F_Q at both saturating and subsaturating concentrations of SSB in these experiments. These experiments were carried out at a relatively high buffer concentration (40 mM Tris and 10 mM $MgCl_2$, pH 7.5) to block *rec*A filament formation and thereby prevent competition for *rec*A monomers by aggregation reactions (Morrical & Cox, 1985). Under these conditions, it has been shown that *rec*A protein binds to ssDNA with high affinity but at a reduced number of sites (1 *rec*A per 9–10 nucleotide residues) compared to the 1 *rec*A monomer per 4 nucleotide stoichiometry measured at lower buffer or $MgCl_2$ concentrations (Morrical & Cox, 1985). The difference in apparent stoichiometries is probably attributable to increased secondary structure in ssDNA at the higher ionic strength, which appears to be inaccessible to *rec*A protein binding (Muniyappa et al., 1984; Flory et al., 1984). Our results indicate that $(SSB)_{65}$ has access to all of its normal binding sites under conditions where *rec*A binding may be restricted, whether or not *rec*A protein is present. The SSB–ssDNA interaction appears to be much stronger than the *rec*A–ssDNA interaction in the absence of nucleotide effectors. These and other results (S. Morrical and J. Lee, unpublished results) indicate that SSB and *rec*A protein compete for ssDNA binding sites in the absence of ATP.

Binding of SSB to recA–ssDNA Complexes in the Presence of ATP. When SSB is added to a preincubation mixture containing ssDNA, ATP, and excess *rec*A protein, a time-dependent decrease in net fluorescence intensity is observed (Figure 4). Taking F_0 as the initial point, fluorescence decreases until leveling off at a value roughly midway between F_0 and F_Q, a quenching effect of approximately 50% the magnitude observed in the absence of *rec*A protein. The rate at which the fluorescence intensity decreases is also much slower than that observed in the absence of *rec*A protein (see Figure 7), requiring nearly 60 s to reach its final quenched value. This quenching effect is dependent on ssDNA, as no similar decrease occurs when SSB is added to *rec*A protein and ATP (i.e., in the total fluorescence control). Partial quenching is also dependent on ATP since, as shown in the previous section, SSB fluorescence is quenched to the same extent in the presence or absence of *rec*A protein when ATP is deleted from the system. Controls described under Experimental Procedures demonstrate conclusively that the decreases in signal intensity observed when SSB is added to *rec*A–ssDNA complexes cannot be attributed to photobleaching or to interference due to absorption or scattering of the excitation beam. Instead, they are solely the result of quenching effects on protein fluorescence. Fluorescence assays performed in the presence of a pyruvate kinase–phosphoenolpyruvate ATP regenerating system revealed tryptophan fluorescence quenching effects identical with those observed in reactions without a regenerating system (data not shown). Under these conditions, the final partially quenched fluorescence intensity observed was stable for more than 1 h. Thus, the ATP-dependent quenching effect on protein fluoresence we have observed is continuously maintained while the ATP/ADP ratio remains high.

In our reaction mixtures, decreases in fluorescence intensity could be brought about through quenching of SSB tryptophan

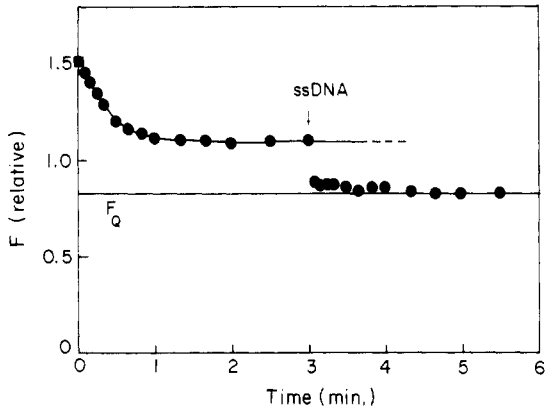


FIGURE 5: Challenge of preformed complex with excess ssDNA. ssDNA (6.75 μM) was added to stable *rec*A–ssDNA complexes formed in the presence of SSB and ATP as described in the text, and the effect on protein fluorescence was monitored. *rec*A protein and SSB concentrations were 2.0 and 0.34 μM, respectively; all other reaction components were present at their standard concentrations. The value of F_Q was determined relative to the fluorescence of 0.34 μM SSB in this experiment. (■) denotes the value of F_0 in this experiment.

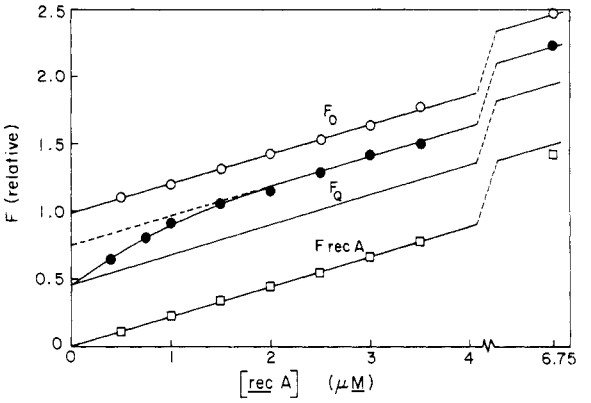


FIGURE 6: Fluorescence of complex at variable *rec*A protein concentration. Reactions were initiated by the addition of 0.45 μM SSB to solutions containing 6.75 μM ssDNA, 1.0 mM ATP, and the indicated amount of *rec*A protein. All F_0 values were measured in standard reaction buffer containing 1.0 mM ATP. All other conditions were as described under Experimental Procedures. (●) denotes final fluorescence intensity attained after a 5-min incubation of all components; (○) denotes F_0; (□) denotes F_{recA}, the fluorescence of *rec*A protein alone in the presence of 1.0 mM ATP at the indicated [*rec*A]. The control values F_0 and F_Q, as well as methods used in their determination, are defined in the text. The extrapolation of control lines to 6.75 μM *rec*A protein is based on linear regression fits of control data at the 0.5 through 3.5 μM *rec*A data points.

fluorescence, *rec*A tryptophan fluorescence, or both. The three simplest explanations for our observations can be stated as follows: (1) Both *rec*A protein and SSB interact normally with ssDNA in the complexes we observe, with quenching of SSB fluorescence as observed in $(SSB)_{65}$ and with no quenching effect on *rec*A fluorescence. Binding of the two proteins in this case is mutually exclusive, so that the decreased magnitude of fluorescence quenching seen would result from SSB binding at a reduced number of sites (those not occupied by *rec*A). (2) The interaction of SSB with a *rec*A–ssDNA complex involves a specific conformation of SSB unique to this interaction, producing a qualitatively different fluorescence quenching effect on SSB. In this case, SSB is not competing with *rec*A protein for ssDNA binding sites but is binding to the intact *rec*A–ssDNA complex in a manner that is not necessarily analogous to its normal interaction with ssDNA. (3) All or part of the quenching effect is due to quenching of *rec*A fluorescence by some novel mechanism in the presence of SSB, ssDNA, and ATP, while SSB fluoresence remains largely unquenched (presumably SSB is almost completely displaced from the ssDNA after a transient participation in complex formation). Several observations argue against case number 3. The insensitivity of *rec*A fluorescence to quenching by nucleotides, ssDNA, or dsDNA and the high susceptibility of SSB fluorescence to quenching by single-stranded polynucleotides argue against *rec*A and for SSB as the protein species undergoing fluorescence quenching effects in stabilized *rec*A–ssDNA complexes. In some experiments, the extent of fluorescence quenching is greater than the total intrinsic fluorescence of *rec*A protein in the experiment. Finally, if *rec*A protein fluorescence is quenched in the complex and most or all of the SSB is free in solution, it should be possible to obtain a net fluorescence intensity significantly lower than F_Q by adding excess ssDNA to completely quench the fluorescence of the free SSB in its high-salt ssDNA binding form. Excess ssDNA was added to a *rec*A–ssDNA complex preformed with ATP and SSB and exhibiting the typical partial quenching of fluorescence shown in Figure 4. In this experiment, fluorescence decreased rapidly to a level *equal* to F_Q, not less than F_Q (Figure 5). F_Q again represents the fluorescence intensity expected if $(SSB)_{65}$ is saturated with ssDNA and *rec*A protein is unquenched. Our inability to generate fluorescence intensities lower than F_Q in experiments of this type, combined with the results described above, argues strongly against quenching of *rec*A fluorescence in an SSB-free *rec*A–ssDNA complex as a viable explanation for the observed fluorescence quenching in this system. Therefore, the fluorescence quenching we have described is the result of a continuous association of SSB with a *rec*A–ssDNA complex. Experiments described in the next section were carried out to distinguish between cases 1 and 2. The experiment presented in Figure 5 may imply that the association of SSB with *rec*A–ssDNA complexes is relatively loose. This possibility will be discussed below in another context.

Characterization of the ATP-Dependent Interaction between SSB and recA–ssDNA Complexes

As described previously the partial quenching of SSB fluorescence due to the continuous association of SSB with *rec*A–ssDNA complexes in the presence of ATP could result from (1) a lower number of $(SSB)_{65}$–ssDNA interactions due to mutually exclusive binding of *rec*A protein and SSB or (2) the interaction of SSB in an altered conformation with an intact *rec*A–ssDNA complex. In an attempt to distinguish between these two possibilities, experiments were carried out to determine the stoichiometries of *rec*A protein and SSB in the complex. All complexes described below were formed in the presence of ATP.

Effect of recA Protein Concentration. *rec*A protein binds to ssDNA at a stoichiometry of 1 *rec*A monomer per 3.5–4.0 nucleotide residues in both the absence (Menetski & Kowalczykowski, 1985; Morrical & Cox, 1985; Bryant & Lehman, 1985) and presence (Tsang et al., 1985; Neuendorf & Cox, 1986) of SSB under conditions in which such binding is not inhibited by secondary structure in the ssDNA. Minor differences in the reported binding site size can be attributed entirely to differences in the extinction coefficient used to determine *rec*A protein concentration in work carried out in different laboratories.

If binding of *rec*A protein and SSB is mutually exclusive, the extent of fluorescence quenching of SSB due to ssDNA binding should be inversely proportional to the concentration of *rec*A protein. The results presented in Figure 6 are not consistent with this prediction. At *rec*A concentrations greater

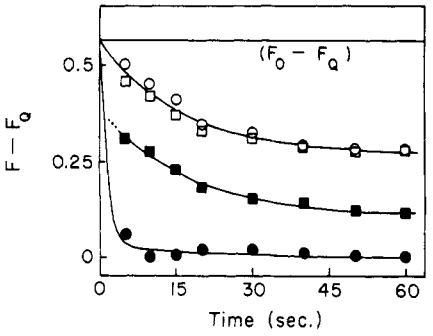


FIGURE 7: Time course for complex formation; effect of *rec*A protein concentration. Reactions were started by addition of 0.45 μM SSB to solutions containing 6.75 μM ssDNA, 1.0 mM ATP, and the indicated amount of *rec*A protein. All other conditions were as described under Experimental Procedures. *rec*A protein concentrations were (●) 0, (■) 0.5, (○) 2.0, and (□) 4.0 μM. The value of F_Q was subtracted from all data and controls. Thus, the fluorescence of *rec*A protein (which increases as [*rec*A] is increased) has been subtracted out, and the magnitude of the SSB-dependent fluorescence quenching effect in each case is plotted relative to the [*rec*A]-independent control value ($F_0 - F_Q$).

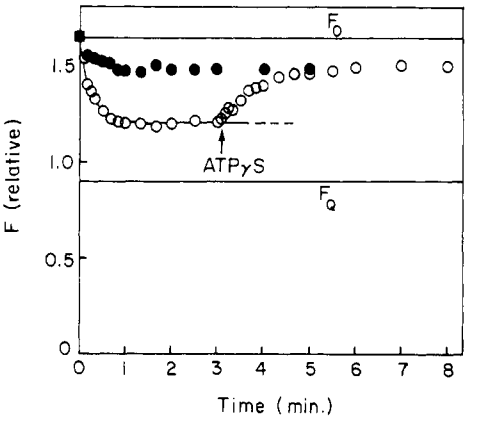


FIGURE 8: SSB fluorescence quenching effects in ATPγS-treated *rec*A–ssDNA complexes. SSB, *rec*A protein, and ssDNA concentrations were 0.45, 2.0, and 6.75 μM, respectively. ATPγS (0.2 mM) either replaced ATP in the initial reaction mixture (●) or was added to a complex preformed in 1.0 mM ATP (○). (■) denotes the value of F_0 in this experiment. All other conditions were as described under Experimental Procedures.

than 1.7 μM (one *rec*A monomer per four nucleotides in these reaction mixtures), the extent of quenching of the SSB fluorescence is completely independent of *rec*A protein concentration. This is true even when a 4-fold excess of *rec*A protein is added. At levels of *rec*A protein insufficient to saturate the ssDNA ([*rec*A] < 1.7 μM at 6.75 μM ssDNA), the observed extent of fluorescence quenching is greater due to the direct interaction of SSB with ssDNA in regions not coated by *rec*A protein. The inability to decrease the fluorescence quenching effect with a large excess of *rec*A protein suggests that we are observing two distinct SSB binding modes. One corresponds to $(SSB)_{65}$ in the absence of *rec*A protein and the other to a new binding mode that is observed only in the presence of *rec*A protein.

The time course of fluorescence quenching observed in these experiments supports the same interpretation. The *rate* of SSB association with *rec*A–ssDNA complexes as monitored by this fluorescence change is also independent of [*rec*A] at saturating *rec*A protein levels (Figure 7). When *rec*A protein is in excess, the fall in fluorescence intensity from the initial value of F_0 to the final value of approximately $(F_0 + F_Q)/2$ occurs with an approximate $t_{1/2}$ of 15 s. In contrast, binding of $(SSB)_{65}$ to ssDNA in the absence of *rec*A protein is essentially complete within 5 s after addition of SSB to the ssDNA solution (Figure 7). The methods employed in these experiments do not permit accurate data acquisition in the period 0–5 s. At subsaturating *rec*A concentrations, time courses are obtained that are apparently biphasic within the limits of our measurements, with a fast phase consistent with the rapid binding of $(SSB)_{65}$ to ssDNA not coated by *rec*A protein, and with a slow phase representing the qualitatively different association of SSB with *rec*A–ssDNA complexes observed and described above.

The observation that both the rate and extent of fluorescence quenching due to SSB association with *rec*A–ssDNA complexes (+ATP) exhibit saturation behavior with respect to *rec*A concentration argues against a competition between *rec*A protein and SSB for ssDNA binding sites. Since saturation occurs at approximately one *rec*A monomer per four ssDNA nucleotide residues, a concentration sufficient to bind all of the ssDNA with *rec*A protein alone, our data suggest that SSB is interacting with the *rec*A–ssDNA complex rather than with ssDNA that is free of *rec*A protein.

Effect of ATPγS. When SSB is added to mixtures of *rec*A protein, ssDNA, and ATPγS, a decrease in fluorescence intensity similar to that seen in the presence of ATP is observed (Figure 8). The final magnitude of fluorescence quenching is reduced, however, with only 20–25% of the quenching effect produced by free ssDNA observed. Addition of ATPγS to a complex preformed in the presence of ATP causes fluorescence intensity to increase to an equivalent level (Figure 8). The difference in the magnitudes of quenching observed with ATPγS vs. ATP could reflect a loss of SSB binding sites in the presence of ATPγS due to the binding of additional *rec*A protein or could be due to a reduced affinity of SSB for the complex in the presence of ATPγS brought about by a conformational change in the *rec*A protein. The stoichiometry of *rec*A protein binding to ssDNA at saturation in the presence of ATPγS and SSB is one *rec*A monomer per four nucleotides (Neuendorf & Cox, 1986). This value is identical with the apparent stoichiometry of *rec*A protein binding in the complexes studied in this work. It is therefore unlikely that significant amounts of additional *rec*A protein are bound upon the addition of ATPγS. Instead, this observation represents a second piece of evidence suggesting a direct interaction between *rec*A–ssDNA complexes and SSB. Evidence that ATPγS produces a conformational change in *rec*A protein has been presented elsewhere (Menetski & Kowalczykowski, 1985).

ATPγS induces irreversible binding of *rec*A protein to ssDNA (McEntee et al., 1981b; Weinstock et al., 1981b). This leads to the formation of long filaments containing ssDNA uniformly coated with *rec*A protein that are easily visualized by electron microscopy (DiCapua et al., 1982; Flory & Radding, 1982). The observation of smooth, continuous filaments of *rec*A protein and ssDNA indicates that binding of *rec*A protein to ssDNA in the presence of ATPγS is complete and independent of secondary structure. Thus, it is important that a reduced but significant SSB-dependent fluorescence quenching effect is observed even in the presence of ATPγS. This result again suggests that SSB interacts continuously with a complex saturated with *rec*A protein and supports the argument that this interaction cannot be a normal binding interaction between SSB and regions of ssDNA inaccessible to *rec*A protein binding.

Effect of SSB Concentration. If SSB binds to a reduced number of sites on ssDNA when *rec*A protein and ATP are present, normal levels of fluorescence quenching should be observed at lower, subsaturating concentrations of SSB. The maximum extent of quenching will simply be attained at a

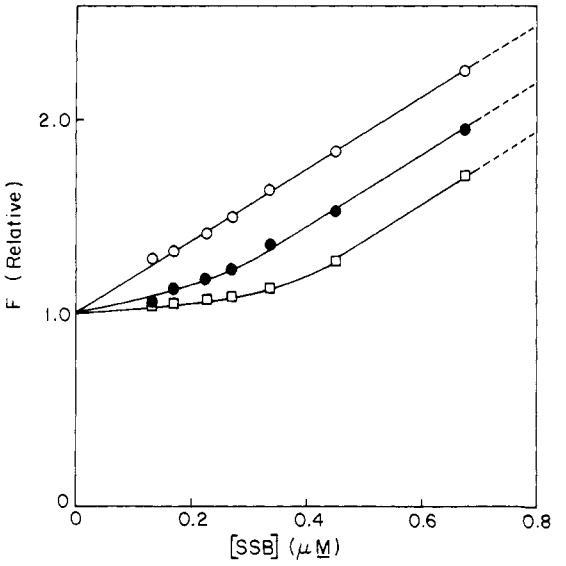


FIGURE 9: Effect of SSB concentration. Reactions were initiated by the addition of the indicated amount of SSB to solutions containing 6.75 μM ssDNA, 1.0 mM ATP, and 3.38 μM *rec*A protein. All other conditions were as described under Experimental Procedures. (●) denotes final fluorescence intensity attained after a 5-min incubation of all components. (○) denotes F_0, measured in each case in standard reaction buffer containing 1.0 mM ATP. (□) denotes F_Q, which in this experiment is the sum of the quenched fluoresence of SSB–ssDNA at a given [SSB] and the fluorescence of 3.38 μM *rec*A protein in 1.0 mM ATP. All data and control values were normalized relative to the fluorescence of 3.38 μM *rec*A protein, which was determined independently in this experiment. Data obtained at 1.35 μM SSB fall directly on an extension of the dashed lines (not shown).

lower concentration of SSB, since less ssDNA is available for binding. High concentrations of SSB may compete with *rec*A protein for binding sites in this case, so that the extent of the quenching effect should be dependent on SSB concentration. To test this hypothesis, we varied the concentration of SSB in reaction mixtures containing excess *rec*A protein and compared the final fluorescence intensities to those from equivalent solutions lacking *rec*A protein. The results of this experiment are shown in Figure 9. The lower curve (F_Q) in this figure is essentially a forward titration of ssDNA with increasing amounts of SSB. Below the equivalence point (approximately 1 SSB monomer per 18 nucleotide residues), fluorescence increases little with increasing [SSB], since SSB is saturated and fully quenched by ssDNA. Above the equivalence point, ssDNA is completely coated by $(SSB)_{65}$, and subsequent additions of SSB rapidly increase the net fluorescence of the mixture. The final fluorescence intensities from reactions with all components (including *rec*A) are significantly greater than the control values at *all* concentrations of SSB used in the reactions (Figure 9). At high [SSB], the magnitude of quenching in these reactions is approximately half that seen in the absence of *rec*A protein, a value that is independent of SSB concentration above the equivalence point. Since the magnitude of fluorescence quenching is independent of SSB concentration at high concentrations of SSB, these results again argue against competitive binding of *rec*A protein and SSB and argue for case number 2 as described above. Normal extents of fluorescence quenching expected for SSB binding to ssDNA are not observed even at very low SSB concentrations. This in turn suggests that sites large enough for normal binding of SSB to ssDNA are rare or nonexistent in these complexes. Neuendorf and Cox (1986) have shown that a stoichiometric ratio of approximately 1 SSB monomer per 20 nucleotide residues is required for stable *rec*A–ssDNA complex formation. This is approximately equivalent to the SSB concentration required to obtain the maximum extent of fluorescence quenching observed in the presence of *rec*A protein and ATP.

Time courses for fluorescence quenching obtained at various SSB concentrations resemble that in Figure 4, except at very high [SSB], where an interesting phenomenon occurs. The initial rate of fluorescence decrease was essentially independent of SSB concentration throughout the range examined (data not shown). This suggests that a rearrangement of *rec*A protein on ssDNA or a conformational change in *rec*A protein is the rate-limiting step in complex formation. At high [SSB], fluorescence intensity goes through a minimum before the final magnitude of approximately $(F_0 + F_Q)/2$ is attained (data not shown). The effect was most noticeable at 1.35 μM SSB, the highest SSB concentration examined, and was easily detectable at 0.675 μM SSB. Occasionally, a slight minimum was observed when working at 0.45 μM SSB, though the effect was irregular, varying with reaction conditions and protein preparations. We speculate that at high concentrations of SSB, some direct binding of SSB to ssDNA can occur during the relatively slow assembly of the stable *rec*A–ssDNA complex, producing a transiently higher magnitude of fluorescence quenching due to the interaction of $(SSB)_{65}$ with ssDNA. This directly bound SSB is slowly displaced by a complex containing *rec*A protein, ssDNA, and SSB in continuous association, restoring the lower level of fluorescence quenching we attribute to this complex. The transient phase we observe in some experiments may reflect the SSB interaction during complex assembly observed by Register and Griffith (1985) by electron microscopy.

recA Protein Promoted ATP Hydrolysis in the Presence and Absence of SSB. ATP hydrolysis by the *rec*A protein is dependent on the interaction of *rec*A protein and single-stranded DNA under the conditions employed in these studies; therefore, factors that prevent or inhibit binding of ssDNA by *rec*A protein should simultaneously prevent or inhibit ATP hydrolysis. If SSB and *rec*A protein compete for binding sites on ssDNA, SSB should then be expected to inhibit the ATPase activity of *rec*A protein by decreasing the number of sites available for *rec*A protein binding. This type of inhibition was in fact observed by Cohen et al. (1983) when the substrate dATP was the last reaction component added to the reaction mixture. Cohen and co-workers preincubated *rec*A protein and SSB with ssDNA in the absence of dATP before starting the reaction with dATP. As described above, SSB competitively displaces *rec*A protein from ssDNA in the absence of nucleotide substrate; therefore, the SSB concentration dependent decreases in the rate of dATP hydrolysis observed by Cohen et al. (1983) reflect the loss of ssDNA-bound *rec*A protein due to this competition. This order of addition also strongly inhibits the strand exchange reaction of *rec*A protein (Cox & Lehman, 1982).

We have measured the effect of SSB on *rec*A-catalyzed ATP hydrolysis when SSB is the last component added (the order of addition in which DNA strand exchange is optimal). In both the presence and absence of SSB, the initial velocity is dependent on *rec*A protein concentration at constant [ssDNA]; however, as shown in Figure 10, SSB has a strong stimulatory effect, rather than an inhibitory effect, on ATP hydrolysis at high *rec*A protein concentrations when SSB is added last. A stimulation of 2.5–3-fold is observed at saturating concentrations of *rec*A protein. The partial quenching of SSB fluorescence described above is observed for more than 1 h in the presence of an ATP regenerating system (see above). Therefore, ATP hydrolysis catalyzed by *rec*A protein is stim-

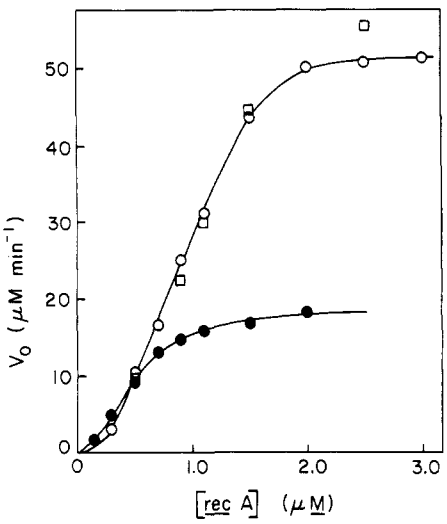


FIGURE 10: *rec*A protein promoted ATP hydrolysis in the presence and absence of SSB. Coupled assays for ATP hydrolysis were carried out as described under Experimental Procedures. Reaction mixtures contained 5 μM ssDNA, 1 mM ATP, and variable amounts of *rec*A protein. (●) denotes reactions carried out in the absence of SSB. (○) denotes reactions carried out in the presence of 0.33 μM SSB, which was added 1 min after the reaction was initiated with *rec*A protein. (□) denotes reactions carried out in the presence of 0.67 μM SSB added in the same fashion.

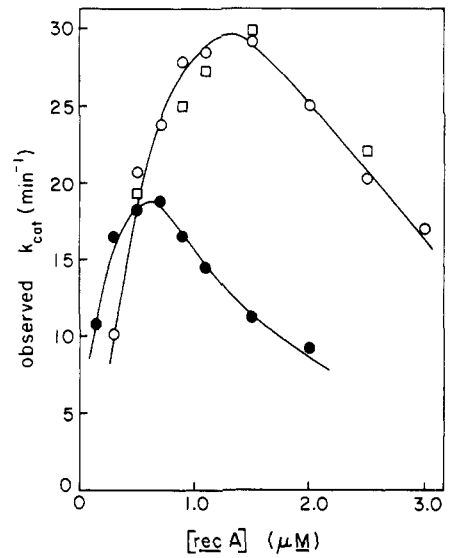


FIGURE 11: SSB-induced shift in the optimum turnover number for *rec*A protein promoted ATP hydrolysis. Apparent k_{cat} values were calculated from the data presented in Figure 10 as described in the text. (●) denotes reactions carried out in the absence of SSB. (○) and (□) denote reactions carried out in the presence of 0.33 and 0.67 μM SSB, respectively, added as described.

ulated by SSB under conditions in which SSB is known to interact continuously with the *rec*A–ssDNA complex.

With ATP saturating, the apparent value of k_{cat} at various concentrations of *rec*A protein and ssDNA is given by the ratio $v_0/[recA]$ (in units of min^{-1}). The "true" (optimal) value of k_{cat} should occur at a *rec*A protein concentration just sufficient to saturate the single-stranded DNA, since further increases in [*rec*A] do not lead to corresponding increases in v_0 when *rec*A protein is in excess. Therefore, by measuring k_{cat} as a function of [*rec*A], we could estimate the apparent binding stoichiometry of *rec*A protein to ssDNA in the presence and absence of SSB. These results are shown in Figure 11. In the absence of SSB, k_{cat} is optimal at a *rec*A/ssDNA ratio of 1 monomer per 10 nucleotide residues, with a maximum k_{cat} of approximately 19 min^{-1}. This 1/10 apparent stoichiometry of binding is similar to that observed previously in the absence of ATP at 10 mM $MgCl_2$ and relatively high buffer concentration (Morrical & Cox, 1985) and may reflect a decrease in the total amount of ssDNA available for *rec*A binding due to secondary structure (Muniyappa et al., 1984; Flory et al., 1984). In the presence of SSB (added last), k_{cat} is optimal at a *rec*A/ssDNA ratio of approximately 1/4, with a maximum k_{cat} of approximately 29 min^{-1}. Identical results were obtained at a 2-fold higher concentration of SSB (Figure 11). Two observations are important here: First, at constant [ssDNA], k_{cat} is optimal at a higher *rec*A concentration in the presence of SSB than in its absence. The optimal k_{cat} is observed at a *rec*A/ssDNA ratio of 1/4 in the presence of SSB, a value in good agreement with reported stoichiometries of *rec*A protein to ssDNA at saturation. This again suggests that the associated SSB is not displacing *rec*A protein but rather is present in addition to the normal saturating complement of *rec*A protein. The fact that the optimal *rec*A/ssDNA ratio does not change when SSB concentration is doubled is another strong indication that *rec*A protein and SSB do not compete for binding sites in these complexes. Second, the maximum k_{cat} value measured was more than 50% higher in the presence of SSB than in its absence. While less ssDNA is available to *rec*A protein binding in the absence of SSB, the same maximum k_{cat} would be expected when the available ssDNA was saturated. This indicates that the observed stimulation of *rec*A ATPase activity by SSB is not entirely due to an increase in the amount of *rec*A protein bound to ssDNA. There is an additional stimulatory effect, which suggests a direct interaction between *rec*A protein and SSB. These results again support the notion that SSB is associated with *rec*A–ssDNA complexes (+ATP) in a novel binding mode that does not involve displacement of significant amounts of *rec*A protein.

ADP and Order of Addition Effects. Previous studies have revealed two important characteristics of the *rec*A–ssDNA complex active in strand exchange that should be shared by our SSB-containing complexes if they are chemically competent for the DNA strand exchange reaction. First, Cox et al. (1983b) showed that levels of ADP on the order of 50–60% of the total nucleotide pool caused SSB-stabilized *rec*A–ssDNA complexes to dissociate, halting the strand exchange reaction. Second, preincubation of SSB with ssDNA prior to the addition of *rec*A protein (an order of addition inverse to that used in the experiments described above) strongly inhibited both the initial rate and final extent of D-loop formation by blocking the formation of the active *rec*A–ssDNA complex (Cox & Lehman, 1982; West et al., 1982). The results of these studies allowed us to make several predictions concerning the fluorescence behavior of our system in response to inhibitory levels of ADP and to a reversed order of protein addition.

If the complex observed by fluorescence is the same complex active in strand exchange, we would expect to see dissociation of this complex in a mixture of 50% ADP and 50% ATP. In our fluorescence-monitored system, dissociation would be detectable as a decrease in fluorescence intensity, since dissociation of *rec*A protein would open up regions of free ssDNA for direct binding by $(SSB)_{65}$, producing the normal fluorescence quenching effect. The complex was preformed in 100% ATP, and then an equivalent amount of ADP was added to quickly raise the ADP content of the solution to 50%. Figure 12 shows that the predicted decrease in fluorescence intensity was actually observed. The decrease was relatively slow, but a final intensity equal to F_Q (the value expected if all *rec*A

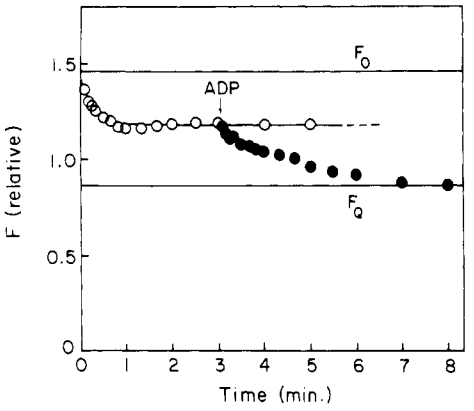


FIGURE 12: ADP-induced dissociation of the *rec*A–ssDNA complex. *rec*A–ssDNA complexes were formed as described in the text using the standard order of addition. Reaction mixtures contained 2.0 μM *rec*A protein, 6.75 μM ssDNA, 0.45 μM SSB, and 1.0 mM ATP. (○) denotes the time course for the normal reaction without added ADP. (●) denotes the fluorescence time course following the addition of 1.0 mM ADP to a standard reaction mixture at the time indicated.

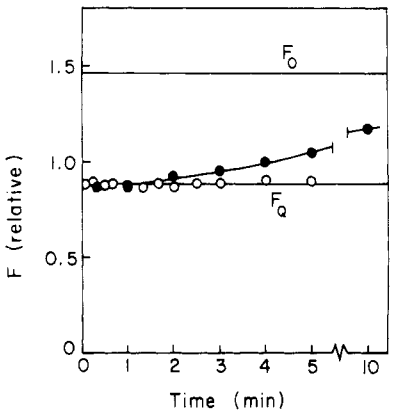


FIGURE 13: Effect of reverse order of addition (SSB added prior to *rec*A protein). SSB (0.45 μM) was preincubated with 6.75 μM ssDNA prior to the addition of 2.0 μM *rec*A protein in the presence of either 1.0 mM ATP (○) or 0.2 mM ATPγS (●).

is dissociated) was reached within 6–8 min after addition of ADP. Thereafter, the signal was stable at F_Q. When the ADP concentration was rapidly increased from 0% to 75% in a similar experiment, a much faster drop ensued in which F_Q was reached within 40 s (data not shown). Thus, the complex we have studied is dissociated by levels of ADP known to disrupt the active, SSB-stabilized complex of *rec*A protein and ssDNA in a manner consistent with published results.

We also predicted that a reversed order of addition of *rec*A protein and SSB would inhibit complex formation. Here, complex formation would be detected as an increase in fluorescence intensity, since we are starting with ssDNA fully coated by $(SSB)_{65}$, which must be displaced in the reaction. When SSB was preincubated with ssDNA and the reaction started by addition of *rec*A protein, we saw no significant increase in fluorescence intensity from F_Q [the level expected for ssDNA fully coated by $(SSB)_{65}$] during a 5-min monitoring period (Figure 13). A slow increase in fluorescence intensity was subsequently observed (data not shown); however, the level of fluorescence typically seen with complexes described in this work was never attained within a 30-min monitoring period. This observation is consistent with the long time lags in and decreased extents of DNA strand exchange reactions observed when SSB is added prior to *rec*A protein (Cox & Lehman, 1982). When the same experiment was repeated with ATPγS replacing ATP in the mixture, a somewhat faster increase in fluorescence intensity was observed (Figure 13), but at a rate that was still much slower than the rate of SSB association measured for the normal order of addition. [Note that this rate of increase is also considerably slower than the rate observed when ATPγS is added to a complex preformed in ATP (Figure 8).] Obviously, the addition of SSB to ssDNA prior to *rec*A protein strongly inhibits the formation of this complex, even in the presence of ATPγS, which causes *rec*A protein to bind rapidly and irreversibly to ssDNA. These results are consistent with a scheme in which $(SSB)_{65}$–ssDNA must dissociate before an active complex of *rec*A protein, ssDNA, and SSB can form, and further identifies the complex studied in these experiments with the stable complex of *rec*A protein and ssDNA active in DNA strand exchange.

Discussion

Our principal conclusion is that SSB is associated with *rec*A–ssDNA complexes in a form that is detectable by tryptophan fluorescence quenching effects. The observed quenching effects are a property of SSB in the system and cannot be attributed to quenching of *rec*A fluorescence in a complex that lacks SSB. The association occurs under conditions that are optimal for *rec*A protein promoted DNA strand exchange. The interaction of SSB with *rec*A–ssDNA complexes revealed in this study has several important characteristics: (1) It is continuous, with no detectable displacement of SSB for up to 60 min at any concentration of *rec*A protein. (2) It is ATP dependent. In the absence of ATP, our data are consistent with an interaction in which SSB and *rec*A protein compete for ssDNA binding sites and SSB always exhibits properties consistent with the $(SSB)_{65}$ binding conformation. When ATP is present, the interaction is demonstrably altered to one that is noncompetitive; i.e., stoichiometric amounts of *both* *rec*A protein and SSB now appear to be associated with the complex. (3) It appears to be distinct from the normal binding interactions of SSB with ssDNA as determined by kinetic properties.

The interaction of SSB with *rec*A–ssDNA complexes in the presence of ATP does not involve significant displacement or exclusion of *rec*A protein from ssDNA binding sites brought about by the normal binding of $(SSB)_{65}$ to those sites. The evidence that the interaction is instead a novel one takes several forms. First, both the rate and extent of the observed quenching of SSB fluorescence are lower than those observed when $(SSB)_{65}$ binds directly to ssDNA. Second, both the rate and extent of this fluorescence quenching effect are independent of either *rec*A protein or SSB concentration above saturating levels. If *rec*A protein and SSB competed for ssDNA binding sites, the amount of SSB bound as monitored by fluorescence quenching should be dependent upon the relative concentration of the two proteins. Third, this effect exhibits saturation at *rec*A protein levels equivalent to those required to saturate ssDNA with *rec*A protein, i.e., one *rec*A monomer per four nucleotide residues. The k_{cat} or turnover number for *rec*A protein promoted ATP hydrolysis also reaches a maximum at the same stoichiometry in the presence of SSB; doubling the SSB concentration does not alter this observed stoichiometry. These results indicate that there is not significant displacement of *rec*A protein by SSB in these complexes. Fourth, this fluorescence quenching effect exhibits saturation with respect to SSB concentration at levels similar to those required to saturate ssDNA alone with SSB. This suggests that stoichiometric levels of *both* SSB and *rec*A protein are continuously present in this complex. Even at very low SSB concentrations, we find no evidence for a normal association of SSB with ssDNA when saturating levels of *rec*A

protein and ATP are present. Fifth, some quenching of SSB fluorescence is observed even when ATPγS is present and *rec*A protein irreversibly forms unbroken filaments on ssDNA. Combined, these data argue that SSB associates with an intact *rec*A–ssDNA complex in a novel manner.

Several additional experimental observations suggest, but do not demonstrate, that a direct interaction occurs between *rec*A protein and SSB. The maximum turnover number for *rec*A protein promoted ATP hydrolysis is enhanced by greater than 50% in the presence of SSB. In addition, the extent of fluorescence quenching of SSB in these complexes is rapidly reduced when ATPγS is added. Binding and hydrolysis of nucleotides can only occur with *rec*A protein, and one simple interpretation of these results is that SSB and *recA* protein interact in these complexes. Lohman and Overman (1985) reported that at least two conformations of SSB with significantly different tryptophan fluorescence properties can be induced by changes in the ionic media in which ssDNA binding reactions take place. It is interesting to speculate that a direct interaction between *rec*A protein and SSB may induce a similar conformational change in SSB, causing SSB to interact with the *rec*A–ssDNA complex in a manner distinct from its regular high-salt interaction with ssDNA.

We have observed that the stoichiometry of *rec*A protein binding to ssDNA is reduced under conditions that favor the formation of ssDNA secondary structure, consistent with results reported elsewhere (Muniyappa et al., 1984; Flory et al., 1984). It is also clear that this barrier is removed in the presence of SSB. The data reported here, however, suggest that this effect is not simply a result of the helix-destabilizing capability of SSB. SSB is not displaced after playing a transient role in *rec*A–ssDNA complex formation but instead remains associated with the complex. Thus, experimental observations that seemed to reflect helix destabilization by SSB (Muniyappa et al., 1984) may instead reflect the properties of joint complexes of *rec*A protein and SSB.

The SSB-containing complexes we have detected by fluorescence exhibit properties consistent with those expected for the presynaptic complexes characterized in *rec*A protein promoted DNA strand exchange reactions. ADP dissociates these complexes, and a reverse order of addition of proteins inhibits their formation. The reaction between SSB and *rec*A–ssDNA complexes monitored by fluorescence quenching is also kinetically competent to be the first step in the strand exchange reaction. Complex formation proceeds with a $t_{1/2}$ of approximately 15 s, while the half-times for synapsis with homologous duplex and for the subsequent extension of heteroduplex are approximately 45 s and 10 min, respectively (Cox et al., 1983b); therefore, complex formation in our reaction mixtures occurs faster than the rate-limiting step of DNA strand exchange and fast enough to account for the rate of homologous pairing in the strand exchange pathway. These facts are consistent with a model in which the continuous interaction of SSB with *rec*A–ssDNA is an integral part of the presynaptic phase of DNA strand exchange, and not an unproductive side reaction.

Our observation that SSB stimulates ATP hydrolysis when it is the last component added is consistent with earlier reports of stimulatory effects of SSB on ATP hydrolysis in the presence or absence of homologous duplex DNA (Cox & Lehman, 1982; Cox et al., 1983b). By using a coupled assay for ATP hydrolysis employing pyruvate kinase and lactate dehydrogenase, we have demonstrated that the same stimulatory effect occurs under conditions in which ATP concentration is maintained at high levels and ADP concentration at minimal levels throughout the reaction period. Other workers have reported inhibition of *rec*A–ssDNA-catalyzed ATP and dATP hydrolysis by SSB (McEntee et al., 1980; Cohen et al., 1983). As shown in this study, this inhibition is the result of a different order of addition of reaction components. When SSB is added to ssDNA in a reaction mixture before either ATP or *rec*A protein, a very stable SSB–ssDNA complex forms that almost completely blocks the binding of *rec*A in the presence or absence of ATP, thereby inhibiting ATP hydrolysis as well as *rec*A-dependent recombination activities.

The properties of the joint SSB–*rec*A–ssDNA complex revealed in this study also suggest explanations for the inability to detect this interaction in previous studies. SSB appears to be transferred rapidly to ssDNA added as a challenge after complex formation. This suggests that the interaction of SSB with the complex is relatively weak and might be disrupted in the steps required to fix *rec*A–ssDNA complexes for electron microscopy or centrifugation (Griffith et al., 1984; Soltis & Lehman, 1983). ATPγS, which is often used to stabilize *rec*A–ssDNA complexes in these experiments, may have an adverse effect on SSB interactions with *rec*A–ssDNA. SSB represents 10% or less of the total protein mass in the complexes defined in these experiments and may not be observed in electron micrographs if, as we suspect, it is bound in a form distinct from its normal mode of binding to ssDNA. Direct observation of a complex containing both SSB and *rec*A protein may be elusive for one or more of these reasons. Utilization of fluorescence quenching for these studies is convenient and nondisruptive. The fluorescence quenching results we observe clearly reflect a previously undetected effect of SSB.

These results do not provide information about the structural details of the joint SSB–*rec*A–ssDNA complex. We cannot rigorously eliminate models in which *rec*A protein and SSB are interspersed on the ssDNA in a specific pattern that may change somewhat under different ionic conditions. On the basis of available data on the stoichiometries of the proteins in the complex, we prefer a model in which SSB interacts directly with *rec*A filaments complexed to ssDNA, or to very short regions of ssDNA exposed because of some structural feature of the *rec*A filaments bound to them. It is tempting to speculate that the continuous association of SSB reflects a continuous role for SSB in *rec*A protein promoted DNA strand exchange, but this also remains to be rigorously demonstrated. Virtually all of the many effects of SSB on *rec*A protein promoted DNA strand exchange could reflect properties of *rec*A complexes acquired in the presynapsis or complex formation phase of the reaction.

While the detailed mechanism remains to be elucidated, SSB does appear to affect all stages of *rec*A protein promoted DNA strand exchange (Cox & Lehman, 1981a, 1982; Radding et al., 1982; Wu et al., 1982; Cox et al., 1983b). SSB also affects *rec*A protein promoted proteolysis in a manner suggesting that the complexes described in this report are also the active species in the proteolytic reaction (Resnick & Sussman, 1982). It has previously been suggested, on the basis of the stoichiometries of *rec*A protein and SSB required for optimal DNA strand exchange, that both proteins are bound to ssDNA in the active complex in a novel structure (Cox & Lehman, 1982; Julin et al., 1986). The experiments reported herein support this view.

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Registry No. ATP, 56-65-5; ATPγS, 35094-46-3; ADP, 58-64-0.

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