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## Demonstration of Competitive Binding and the Lack of a Specific Protein–Protein Interaction

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The effect of the *Escherichia coli* single-stranded DNA binding (SSB) protein on the stability of complexes of *E. coli* RecA protein with single-stranded DNA has been investigated through direct DNA binding experiments. The effect of each protein on the binding of the other to single-stranded DNA, and the effect of SSB protein on the transfer rate of RecA protein from one single-stranded DNA molecule to another, were studied. The binding of SSB protein and RecA protein to single-stranded phage M13 DNA is found to be competitive and, therefore, mutually exclusive. In the absence of a nucleotide cofactor, SSB protein binds more tightly to single-stranded DNA than does RecA protein, whereas in the presence of ATP-$\gamma$-S, RecA protein binds more tightly than SSB protein. In the presence of ATP, an intermediate result is obtained that depends on the type of DNA used, the temperature, and the magnesium ion concentration. When complexes of RecA protein, SSB protein and single-stranded M13 DNA are formed under conditions of slight molar excess of single-stranded DNA, no effect of RecA protein on the equilibrium stability of the SSB protein–single-stranded DNA complex is observed. Under similar conditions, SSB protein has no observed effect on the stability of the RecA protein–etheno M13 DNA complex. Finally, measurements of the rate of RecA protein transfer from RecA protein–single-stranded DNA complexes to competing single-stranded DNA show that there is no kinetic stabilization of the RecA protein–etheno M13 DNA complex by SSB protein, but that a tenfold stabilization is observed when single-stranded M13 DNA is used to form the complex. However, this apparent stabilizing effect of SSB protein can be mimicked by pre-incubation of the RecA protein–single-stranded M13 DNA complex in low magnesium ion concentration, suggesting that this effect of SSB protein is indirect and is mediated through changes in the secondary structure of the DNA. Since no direct effect of SSB protein is observed on either the equilibrium or dissociation properties of the RecA protein–single-stranded DNA complex, it is concluded that the likely effect of SSB protein in the strand assimilation reaction is on a slow step in the association of RecA protein with single-stranded DNA. Direct evidence for this conclusion is presented in the accompanying paper.

### 1. Introduction

The RecA protein of *Escherichia coli* has been shown to play an essential role in genetic recombination (for reviews, see McEntee & Weinstock, 1981; Radding, 1982; Dressler & Potter, 1982). An activity of the RecA protein that is thought to play a key role in the enzyme's biological function is its ability to catalyze the exchange of complementary strands of DNA molecules in an ATP-dependent reaction referred to as strand assimilation (Shibata *et al.*, 1979, 1981; McEntee *et al.*, 1979; West *et al.*, 1981; Cox & Lehman, 1981). An additional discovery important to the understanding of the biochemistry of the recombination process is the finding that strand assimilation involving a linear duplex DNA molecule and a circular, single-stranded DNA



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molecule is greatly stimulated by the presence of single-stranded DNA binding (SSB†) protein from either *E. coli* (SSB protein) or from bacteriophage T4 (gene *32* protein) (McEntee *et al.*, 1980; Shibata *et al.*, 1980; Cassuto *et al.*, 1980; West *et al.*, 1982; Cox & Lehman, 1982; Cox *et al.*, 1983*a*).

The specific molecular mechanism by which SSB protein facilitates the RecA-protein-catalyzed strand assimilation reaction is unclear, though a number of proposals for the role of SSB protein have been put forward. Cox & Lehman (1982) demonstrated that SSB protein greatly increased the rate and extent of strand assimilation. Studies of the effect of competing single-stranded DNA on the strand assimilation reaction established that, in the presence of SSB protein, the competing DNA was unable to participate in strand assimilation, whereas, in the absence of SSB protein, the converse was true. From those results it was concluded that, in the presence of SSB protein and ATP, RecA protein formed complexes with single-stranded DNA that did not dissociate for up to 90 minutes, whereas in the absence of SSB protein, the half-time for dissociation was 17 seconds (Cox & Lehman, 1982; Cox *et al.*, 1983*a*). It was inferred that the molecular basis of this effect was a direct protein–protein interaction that resulted in an increased stability of the RecA protein–single-stranded DNA complex (Cox & Lehman, 1982; Cox *et al.*, 1983*b*). Muniyappa *et al.* (1984), however, assayed the effect of SSB protein on joint molecule formation and demonstrated that secondary structure within single-stranded DNA prevented the presynaptic association of RecA protein with the DNA, resulting in an inhibition of joint molecule formation. They concluded that the SSB protein was responsible for removing this inhibitory secondary structure and, therefore, that the effect of SSB protein was primarily at the DNA level rather than due to a specific protein–protein interaction. Finally, on the basis of electron microscopic work, Griffith *et al.* (1984) suggested that SSB protein was important for the formation of stable RecA protein–single-stranded DNA complexes and, therefore, SSB protein could be considered as an assembly factor for binding of RecA protein to DNA.

The experiments presented here are designed to test directly the first hypothesis regarding the function of SSB protein; i.e. that SSB protein interacts directly with, and stabilizes, the RecA protein–single-stranded DNA complex. The expectation is that, if RecA and SSB proteins do form a stable complex when bound to DNA, this would be manifest as an increase in the equilibrium DNA binding affinity of RecA protein (as well as that of SSB protein) and as a decrease in the rate of dissociation of RecA protein from single-stranded DNA. To measure the formation of an SSB protein–DNA complex, we took advantage of the quenching of SSB protein intrinsic fluorescence that occurs upon complex formation (Molineux *et al.*, 1975), and to measure RecA protein–DNA complex formation we utilized the fluorescently modified single-stranded phage M13 DNA referred to as etheno M13 DNA.

Previously, we had characterized the binding of RecA protein to etheno M13 DNA under the experimental conditions of 25°C and 4 mM-magnesium chloride (Menetski & Kowalczykowski, 1985). In this paper, we have examined the effect of SSB protein on the properties of the RecA protein–single-stranded M13 DNA complexes under both these previously characterized experimental conditions and also those conditions more typically required for the strand assimilation reaction (i.e. 37°C and 10 mM-magnesium chloride). In addition to these equilibrium studies, the effect of SSB protein on the lifetime of the RecA protein–single-stranded DNA complex has been investigated directly, through studies of the rate of transfer of DNA-bound RecA protein from one single-stranded DNA molecule to another.

In both the equilibrium and kinetic studies, SSB protein has little or no direct effect on the stability of the RecA protein–single-stranded DNA complex, nor does RecA protein have any effect on the stability of the SSB protein–single-stranded DNA complex. In fact, the binding of RecA and SSB proteins is always observed to be mutually exclusive and, therefore, competitive for DNA sites. Since SSB protein has no effect on the equilibrium affinity of RecA protein for single-stranded DNA and little or no stabilizing effect on the rate of dissociation of RecA protein from single-stranded DNA, we conclude that the effect of SSB protein on the strand assimilation reaction is to catalyze some other rate-limiting step, presumably the association of RecA protein with single-stranded DNA. This conclusion is consistent with that of Muniyappa *et al.* (1984), who suggested that the function of SSB protein is to remove secondary structure from single-stranded DNA, and thereby facilitate the rate of formation of a presynaptic complex of RecA protein and single-stranded DNA. In the accompanying paper (Kowalczykowski & Krupp, 1987) we demonstrate that the effects of SSB protein on the ATPase activity of RecA protein are consistent with the elimination of secondary-structure hypothesis, and consequently, provide additional evidence to support the conclusion drawn from the DNA binding results presented here.

† Abbreviations used: SSB protein, *E. coli* single-stranded DNA binding protein; ATP-$\gamma$-S, adenosine-5′-O′-(3-thiotriphosphate); etheno M13 DNA, single-stranded M13 DNA containing 1,$N^6$-etheno-adenosine and 3,$N^4$-etheno-cytidine residues.

## 2. Materials and Methods

### (a) *Chemicals and buffers*

All chemicals used were reagent grade and all solutions were made in glass-distilled water. ATP and ADP were

purchased from PL Biochemicals, phosphoenol pyruvate from Sigma, and ATP-$\gamma$-S from Boehringer-Mannheim; all were dissolved as concentrated stock solutions at pH 7. The buffer used in all experiments consisted of 20 mM-Tris·HCl (pH 7·5), 0·1 mM-dithiothreitol, and $MgCl_2$ at the concentrations indicated.

(b) *DNA*

Single-stranded M13 phage DNA and etheno M13 DNA were prepared as described (Menetski & Kowalczykowski, 1985). The etheno M13 DNA concentration was determined using an extinction coefficient of 7000/M-nucleotide per cm at 260 nm. This value was determined from phosphate analysis (Menetski & Kowalczykowski, unpublished results) and differs from the value of 12,668/M per cm that was used previously (due to a typographical error, it was reported as 16,268/M per cm: Menetski & Kowalczykowski, 1985). Poly(dA) and poly(dT) were purchased from PL Biochemicals and their concentrations were determined using extinction coefficients at 260 nm of 9400 and 8100/M-nucleotide per cm, respectively.

(c) *Proteins*

RecA protein was purified from *E. coli* strain KM1842 using the procedure described by Cox *et al.* (1981) or from strain JC12772 (Uhlin & Clark, 1981; kindly provided by Dr A. J. Clark of the University of California at Berkeley), with no difference in the results obtained. Protein concentration was determined using an extinction coefficient of $2{\cdot}7 \times 10^4$/M per cm at 280 nm.

SSB protein was isolated from strain RLM 727 using a preparative protocol provided by Dr Roger McMackin of Johns Hopkins University. Protein concentration was determined by using an extinction coefficient of $3{\cdot}0 \times 10^4$/M per cm at 280 nm (Ruyechan & Wetmur, 1975).

Pyruvate kinase was purchased from Sigma as an ammonium sulfate suspension. Working stock solutions were made by centrifuging a homogeneous sample of the suspension, drawing off the supernatant, and then dissolving the protein pellet in buffer at a concentration of 5 units/$\mu$l.

(d) *Fluorescence experiments*

Titrations were carried out as described by Menetski & Kowalczykowski (1985). When fluorescence from the etheno M13 DNA was being monitored, excitation and emission wavelengths of 300 nm and 405 nm, respectively, were used. For experiments in which the SSB protein fluorescence was being monitored, excitation and emission wavelengths of 290 nm and 340 nm were employed. In experiments where native single-stranded M13 DNA is used, the SSB protein intrinsic fluorescence was always monitored; for those experiments involving etheno M13 DNA, the Figure legend indicates whether etheno M13 DNA or SSB protein fluorescence was monitored. Except where indicated, the fluorescence intensity is given in arbitrary units.

## 3. Results

### (a) *RecA protein does not prevent the binding of SSB protein to single-stranded DNA in the absence of a nucleotide cofactor*

The first question to address regarding the binding of both RecA protein and SSB protein to single-stranded DNA is whether these two proteins compete for the same DNA binding sites or whether they share the same sites, perhaps by binding to opposite "sides" of the DNA strand as schematically depicted, for example, by Cox & Lehman (1982). If the binding of these two proteins to single-stranded DNA is competitive, then this competition will be manifest as a change in the apparent stoichiometry (site size) of binding when the competing protein is present.

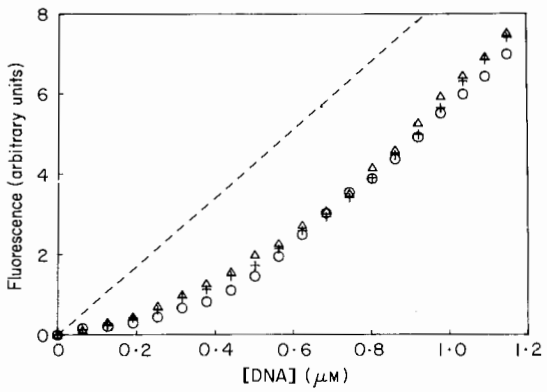


**Figure 1.** Effect of RecA protein on the binding of SSB protein to single-stranded M13 DNA. Titrations were carried out at 25°C and cuvettes contained standard buffer plus 4 mM-$MgCl_2$, 6 $\mu$M-single-stranded M13 and the following amounts of RecA protein: (○) no RecA protein; (△) 0·6 $\mu$M-RecA protein; (+) 1·8 $\mu$M-RecA protein. For wavelengths employed in fluorescence experiments, see Materials and Methods. For the broken line, see the text.

In Figure 1, the effect of RecA protein on the stoichiometry of SSB protein binding to single-stranded DNA is shown. In these experiments, solutions containing single-stranded M13 DNA and various concentrations of RecA protein were titrated with SSB protein while the intrinsic fluorescence of SSB protein was being monitored. Because SSB protein fluorescence is quenched approximately 70% upon formation of a complex with the single-stranded M13 DNA (see also Fig. 2), the initial slope of the fluorescence change upon addition of SSB protein is less than the slope towards the end of the titration. In the absence of DNA, a slope indicated by the broken line is observed. The point at which SSB protein saturates the DNA is defined by the intersection of the two limiting slopes, and, in the absence of RecA protein, is determined to be 13($\pm$1) nucleotides per SSB protein monomer. When RecA protein is added to the cuvette prior to the addition of SSB protein (there are no changes in the intrinsic protein fluorescence of RecA protein upon binding to single-stranded DNA: unpublished results), a slight but detectable decrease in the apparent stoichiometry is observed (Fig. 1). The apparent site size decreases from 13($\pm$1) nucleotides in the absence of RecA protein, to 11($\pm$1) nucleotides per SSB protein

monomer when 1·2 μM (not shown) or 1·8 μM-RecA protein is present. A concentration of 1·2 μM-RecA protein is sufficient to saturate potentially all of the single-stranded DNA present in the cuvette, assuming a RecA protein site size of five nucleotides per RecA protein monomer. If RecA protein were capable of totally displacing SSB protein, then essentially no binding of SSB protein to the DNA would be detected. Since the apparent site size changed by only 15%, two interpretations of these data are possible. Either the binding is competitive and, under these conditions, RecA protein is unable to prevent the binding of SSB protein to single-stranded DNA, or the binding is not competitive, and RecA protein can bind to the SSB protein or the DNA in such a way that the stoichiometry of SSB protein–DNA complex formation is only slightly affected. Although both interpretations are consistent with these data, the fact that, in the presence of ATP-γ-S, RecA protein can prevent the binding of SSB protein (see below) and that a slight alteration of SSB protein-binding stoichiometry is observed in Figure 1, the first possibility is more likely.

In an experiment complementary to that presented in Figure 1, a fixed concentration of SSB protein was titrated with increasing amounts of single-stranded M13 DNA. The fluorescence of SSB protein was measured and, as shown in Figure 2, the addition of single-stranded M13 DNA resulted in a quenching of the intrinsic fluorescence of the SSB protein. In the absence of added RecA protein, a stoichiometry of 11(±1) nucleotides per SSB protein monomer was obtained for the binding of the single-stranded DNA to SSB protein. This binding stoichiometry is slightly lower than that obtained in Figure 1, and may reflect the presence of differing amounts of the two SSB protein DNA binding modes that might be present under these two different experimental conditions (Lohman & Overman, 1985). If increasing amounts of potentially competing RecA protein (0·16, 0·32 and 0·64 μM) are added to the cuvette prior to the DNA titration, the apparent stoichiometry changes to 11·9, 13·0 and 13·7 nucleotides per SSB protein monomer, respectively. Thus, as in Figure 1, these results demonstrate that RecA protein slightly inhibits the binding of SSB protein to single-stranded DNA, and that SSB protein is able to bind to the DNA in the presence of RecA protein. Such results are not limited to data obtained at 25°C, since results identical with those in Figures 1 and 2 are obtained at 37°C in buffers containing either 4 mM or 10 mM-$MgCl_2$ (not shown, see Table 1).

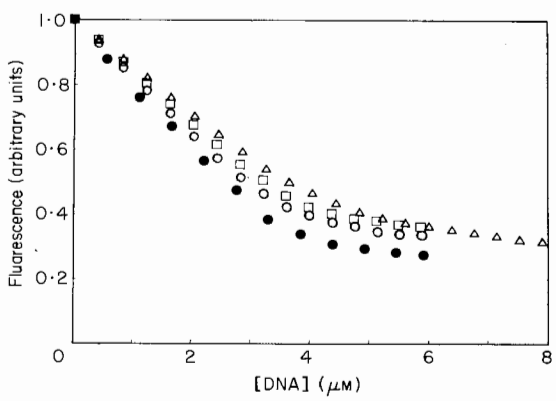


**Figure 2.** Effect of RecA protein on the binding of single-stranded M13 DNA to SSB protein. Titrations were carried out at 25°C and cuvettes contained standard buffer plus 4 mM-$MgCl_2$, 0·3 μM-SSB protein, and the following amounts of RecA protein: (●) no RecA protein; (○) 0·16 μM-RecA protein; (□) 0·32 μM-RecA protein; and (△) 0·64 μM-RecA protein.

### (b) *In the presence of ATP-γ-S, RecA protein prevents the binding of SSB protein to single-stranded DNA*

The data described above demonstrate that, in the absence of a nucleotide cofactor, SSB protein binds to single-stranded DNA, suggesting that if the binding is competitive then SSB protein has a greater affinity for single-stranded DNA than does RecA protein. Since both ATP-γ-S and ATP were shown to induce a high DNA binding affinity form of RecA protein (Weinstock *et al.*, 1981; Menetski & Kowalczykowski, 1985), it was of interest to determine whether this high-affinity form of RecA protein could bind to single-stranded DNA more tightly than did SSB protein.

Figure 3 shows an experiment that is similar to the one presented in Figure 1, except that 100 μM-ATP-γ-S is present. In contrast to the data in Figure 1, it is clear that, when ATP-γ-S is present, increasing concentrations of RecA protein inhibit the binding of SSB protein to single-stranded DNA. At a concentration of 1·2 μM-RecA protein (open triangles), no binding of SSB protein to the DNA can be detected, and at 0·3 μM-RecA protein (open

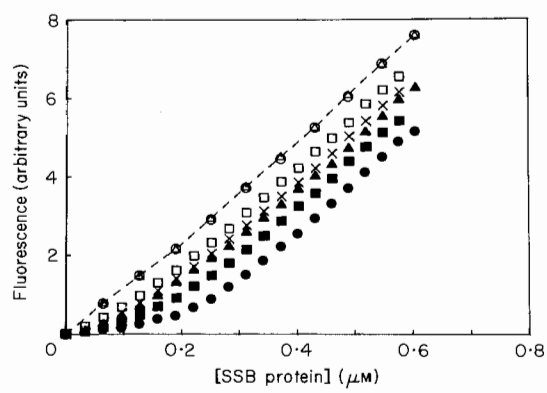


**Figure 3.** Effect of RecA protein on the binding of SSB protein to single-stranded M13 DNA in the presence of ATP-γ-S. Titrations were carried out at 25°C and cuvettes contained standard buffer plus 4 mM-$MgCl_2$, 3 μM-single-stranded M13 DNA, 100 μM-ATP-γ-S, and the following amounts of RecA protein: (●) none; (■) 0·1 μM; (▲) 0·15 μM; (×) 0·2 μM; (□), 0·3 μM; and (△) 1·2 μM. The open circles represent a control titration performed in the absence of single-stranded DNA but containing 0·6 μM-RecA protein.

**Table 1**
*Binding of SSB protein to single-stranded DNA in the presence of RecA protein*

| | | Conditions | | | |
|---|---|---|---|---|---|
| | | 25°C | | 37°C | |
| DNA | Nucleotide cofactor | 4 mM-$MgCl_2$ | 10 mM-$MgCl_2$ | 4 mM-$MgCl_2$ | 10 mM-$MgCl_2$ |
| ssM13 DNA | No cofactor | + | + | + | + |
| | ATP-$\gamma$-S | − | − | − | − |
| | ATP | + | N.D. | +/−† | +/−‡ |
| Etheno M13 DNA | No cofactor | + | + | + | + |
| | ATP-$\gamma$-S | − | − | − | − |
| | ATP | + | + | + | + |

As determined from experiments that measure the quenching of intrinsic fluorescence of SSB protein. All experiments were carried out in standard buffer at the $MgCl_2$ concentration and temperature indicated. The DNA concentration was 3 $\mu$M and the RecA protein concentration was 0·8 $\mu$M, except where indicated. SSB protein was increased by titration to a final concentration of approximately 0·6 $\mu$M. When ATP-$\gamma$-S is present, its concentration is 100 $\mu$M and when ATP is present, its concentration is 1 mM plus 3 mM-phosphoenol pyruvate and 25 units pyruvate kinase/ml. A plus sign indicates that SSB protein is able to bind to the single-stranded DNA in the presence of RecA protein (i.e. is able to displace the RecA protein); a minus sign indicates the SSB protein is unable to bind to the DNA (i.e. is unable to displace the RecA protein); +/− indicates that the results are dependent upon the RecA protein concentration. N.D., not done, ss, single-stranded.

† SSB protein can bind completely in the presence of 0·8 $\mu$M-RecA protein, but at 1·5 and 3·0 $\mu$M-RecA protein only partial binding of SSB protein is observed.

‡ See Fig. 5 for typical results.

squares), only approximately 25% of the SSB protein binding obtained in the absence of RecA protein is observed. From these and similar data, an apparent site size for RecA protein DNA binding, as measured by inhibition of SSB protein binding, can be obtained. This apparent stoichiometry value is 9(±2) nucleotides per RecA protein and is higher than expected from direct DNA-binding studies. However, this may be due to an excluded site effect of RecA protein binding on SSB protein binding, resulting from the fact that, binding as a tetramer, SSB protein requires a DNA binding site of approximately 60 contiguous nucleotides (Lohman & Overman, 1985). The essentially irreversible binding of RecA protein molecules to single-stranded DNA in the presence of ATP-$\gamma$-S may leave protein-free DNA "gaps" of less than 60 nucleotides in length and, therefore, effectively exclude more SSB protein than might be expected on the basis of its true site size. This result, that RecA protein prevents the binding of SSB protein to single-stranded DNA in the presence of ATP-$\gamma$-S, is also independent of both temperature and buffer conditions. When SSB protein titration experiments such as those shown in Figure 3 are carried out in the presence of a saturating amount of RecA protein at either 25 or 37°C in buffers containing either 4 mM or 10 mM-$MgCl_2$, no binding of SSB protein (i.e. no fluorescence quenching) is observed (Table 1).

Thus, it is clear that in the presence of ATP-$\gamma$-S, RecA protein is able to prevent the binding of SSB protein to single-stranded DNA yet, in its absence, essentially all of the SSB protein is able to bind to single-stranded DNA even when RecA protein is present. Taken together, the simplest interpretation of these data is that binding of these two proteins is competitive (i.e. mutually exclusive). In the absence of nucleotide cofactor, SSB protein binds with a higher affinity to single-stranded DNA than does RecA protein, resulting in a displacement of the RecA protein from the DNA; in the presence of ATP-$\gamma$-S, RecA protein has a greater affinity for single-stranded DNA than does SSB protein, consequently preventing the binding of the SSB protein. Any interpretation involving the formation of a specific complex of these two proteins would require the unlikely interpretation that in the presence of ATP-$\gamma$-S, either SSB protein did not bind to the DNA or did not quench its intrinsic fluorescence, but, in the absence of ATP-$\gamma$-S, SSB protein did bind to the DNA. The competitive binding interpretation is more straightforward and is also consistent with other data (see Discussion).

### (c) *Displacement of SSB protein by RecA protein in the presence of ATP-$\gamma$-S*

In section (b), above, it was demonstrated that SSB protein is capable of displacing RecA protein from single-stranded DNA when no nucleotide cofactor is present but that, when ATP-$\gamma$-S is present, SSB protein is unable to do so. Therefore, it was of interest to determine whether, in the presence of ATP-$\gamma$-S, RecA protein was able to displace DNA-bound SSB protein. Thus, an experiment was performed in which SSB protein was first bound to the DNA, resulting in an intrinsic protein fluorescence quenching. RecA protein was then added and the time course of fluorescence change was measured. If SSB protein is displaced by RecA protein, then an increase in fluorescence over that due to the addition of RecA protein should be observed. Figure 4 shows the results of such experiments which indicate that, in the presence of ATP-$\gamma$-S, a slow displacement of

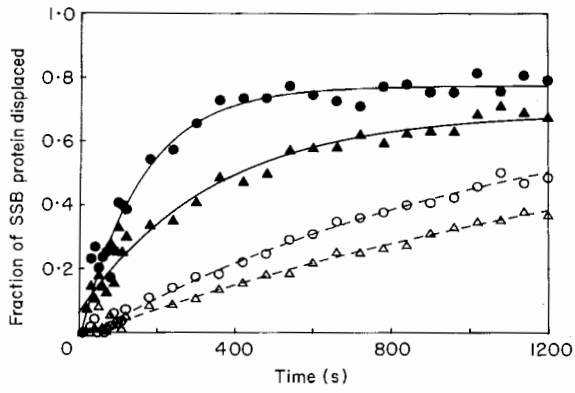


**Figure 4.** Displacement of SSB protein bound to single-stranded M13 DNA by RecA protein in the presence of ATP-$\gamma$-S. Cuvettes contained standard buffer plus the $MgCl_2$ concentrations indicated, 1·5 $\mu$M-RecA protein, and 0·2 $\mu$M-SSB protein at the temperature listed below. Single-stranded M13 DNA (3 $\mu$M) was added and the SSB protein intrinsic fluorescence quenching due to DNA binding was measured. At time zero, 100 $\mu$M-ATP-$\gamma$-S was added and fluorescence was monitored: (●) 4 mM-$MgCl_2$ at 37°C; (▲) 10 mM-$MgCl_2$ at 37°C; (○) 4 mM-$MgCl_2$ at 25°C; and (△) 10 mM-$MgCl_2$ at 25°C. For each curve, the data presented were corrected for a slight time-dependent increase in turbidity due to RecA protein aggregation present in SSB protein-free control experiments carried out under identical experimental conditions. The curves represent a least-squares fit to a single exponential function for each data set.

SSB protein is observed (in the absence of ATP-$\gamma$-S, no displacement is observed). The time courses are not always simple single-exponential curves and so no extensive analysis of these data will be presented. However, qualitatively, these data show that the rate of SSB protein displacement by RecA protein is greater at elevated temperatures, and they clearly demonstrate that the high DNA-binding affinity state of RecA protein induced by ATP-$\gamma$-S is able to displace SSB protein from single-stranded DNA.

### (d) *In the presence of ATP, the outcome of the RecA protein–SSB protein competition is dependent of temperature and magnesium concentration*

The effect of ATP on the binding of RecA protein and SSB protein to single-stranded DNA was also examined. Since ATP is being hydrolyzed continuously under these conditions (see Kowalczykowski & Krupp, 1987), this experiment does not represent true equilibrium conditions but rather some steady-state property of this system. Data obtained at 25°C and 4 mM-$MgCl_2$, in the presence of ATP and a potentially saturating concentration of RecA protein, show no effect on the binding of SSB protein to single-stranded M13 DNA, implying that SSB protein can displace the RecA protein from the DNA (Table 1). Studies of the effect of SSB protein on the ATPase activity of RecA protein, under these identical conditions, show that SSB protein almost completely inhibits the ATPase activity of RecA protein, which is consistent with the interpretation that SSB protein has displaced RecA protein from the single-stranded DNA (Kowalczykowski & Krupp, 1987). Thus, the binding data obtained in the presence of ATP continue to show competitive binding behavior between these two proteins and demonstrate that, at 25°C, the net steady-state favors the binding of SSB protein to the DNA.

Since the RecA protein-catalyzed strand assimilation reaction is normally carried out at 37°C and higher magnesium concentrations, it was of interest to see whether the competitive effects observed at 25°C and 4 mM-$MgCl_2$ would be maintained at higher temperature and magnesium concentrations. In addition, studies of the effects of SSB protein on the single-stranded M13 DNA-stimulated ATPase activity of RecA protein described in the accompanying paper (Kowalczykowski & Krupp, 1987) show clearly that, at low temperature and magnesium concentrations, SSB protein inhibits the ATPase activity, whereas at higher values (i.e. 37°C and either 4 mM or 10 mM-$MgCl_2$), it stimulates this activity. Therefore, experiments were performed to determine whether these effects of SSB protein on ATPase activity were paralleled in the relative DNA binding affinities of these two proteins.

When an SSB protein titration of single-stranded DNA in the presence of RecA protein and ATP is carried out at 37°C and 10 mM-$MgCl_2$ (Fig. 5), a result different from that obtained at 25°C and 4 mM-$MgCl_2$ is observed. Figure 5 shows that, at these elevated temperature and magnesium chloride concentration conditions, the SSB protein titration curves obtained in the presence of RecA protein and ATP lie between the titration data obtained in the absence of RecA protein (filled circles) and the

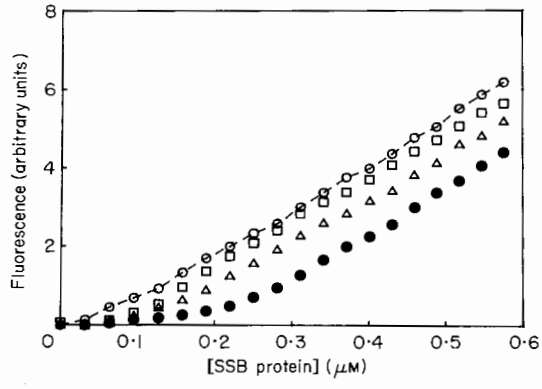


**Figure 5.** Effect of RecA protein concentration on the binding of SSB protein to single-stranded DNA in the presence of ATP at 37°C and 10 mM-$MgCl_2$. Cuvettes contained standard buffer plus 10 mM-$MgCl_2$, 3 $\mu$M-single-stranded M13 DNA, 1 mM-ATP, 3 mM-phosphoenol pyruvate, 25 units pyruvate kinase/ml and: (●) no RecA protein; (△) 0·8 $\mu$M-RecA protein; (□) 1·5 $\mu$M-RecA protein; and (○) control containing 2·5 $\mu$M-RecA protein but without single-stranded DNA.

DNA-free control (open circles). The results demonstrate that, under these conditions, SSB protein is unable totally to displace the DNA-bound RecA protein and that a steady-state exists between DNA-bound forms of RecA and SSB proteins. This effect is particularly obvious at the lower SSB protein concentrations and the higher RecA protein concentrations, where little fluorescence quenching due to SSB protein binding is observed. These results are also completely consistent with the interpretation that even under these conditions the binding of the two proteins is competitive and mutually exclusive. However, in contrast to the results obtained at 25°C, it is clear that at 37°C and 10 mM-$MgCl_2$ the steady-state properties of this system favor the binding of RecA protein to the DNA.

As mentioned previously, the ATPase activity studies described in the accompanying paper (Kowalczykowski & Krupp, 1987) demonstrate that SSB protein inhibits the ATPase activity of RecA protein at 25°C and 4 mM-$MgCl_2$, but not at 37°C and either 4 mM or 10 mM-$MgCl_2$. These results are in qualitative agreement with the binding data in Figure 5 and Table 1, which demonstrate that SSB protein can displace all of the RecA protein at 25°C and 4 mM-$MgCl_2$ (even when RecA protein is present at the higher concentrations of 1·5 and 3·0 $\mu$M (not shown)), whereas, at the higher temperature and magnesium concentration, most of the RecA protein is resistant to displacement by SSB protein. Binding experiments such as those shown in Figure 5 performed at 37°C and 4 mM-$MgCl_2$ also demonstrate that RecA protein is resistant to displacement by SSB protein, though under conditions comparable to the 10 mM-$MgCl_2$ data, approximately 10 to 20% less RecA protein is bound at 4 mM-$MgCl_2$ (not shown). Thus, SSB protein can completely displace ATP–RecA protein complexes from single-stranded DNA at 25°C and 4 mM-$MgCl_2$ but not at 37°C and either 4 mM or 10 mM-$MgCl_2$. Since the ATP hydrolytic properties of RecA protein parallel the effects seen in the RecA protein-binding properties, these results confirm our interpretation that the binding of these two proteins is competitive even in the presence of ATP. However, in contrast to the competition results obtained with no cofactor present or with ATP-$\gamma$-S, the results obtained in the presence of ATP are affected significantly by temperature and magnesium ion concentration.

### (e) *Effect of SSB protein on the binding of RecA protein to etheno M13 DNA*

The usefulness of the fluorescent derivative of natural M13 single-stranded DNA, referred to as etheno M13 DNA, is that it can be used directly to monitor the binding of RecA protein to this DNA (Silver & Fersht, 1982; Menetski & Kowalczykowski, 1985). Consequently, etheno M13 DNA can be used to study the effect of SSB protein on both the equilibrium and kinetic properties of RecA protein–single-stranded DNA complexes (see below). However, it is first necessary to determine whether the competitive effects that have been characterized here using the natural M13 DNA substrate are observed using the etheno M13 DNA substrate.

Table 1 summarizes the results of SSB protein titration experiments that were obtained using etheno M13 single-stranded DNA in the presence of RecA protein and no cofactor, ATP-$\gamma$-S or ATP (the SSB protein fluorescence is still being measured). SSB protein binds to etheno M13 DNA in the absence of RecA protein with an observed site size ($14(\pm 1)$ nucleotides/SSB protein monomer) and an extent of fluorescence quenching ($\approx 70\%$) that are comparable to values obtained with the natural single-stranded DNA substrate (not shown). The data in Table 1 demonstrate that SSB protein is able to bind to etheno M13 when RecA protein and no cofactor are present but is unable to bind when RecA protein and ATP-$\gamma$-S are present. Thus, as with the natural M13 DNA substrate, the same relative binding affinities of these two proteins for etheno M13 DNA are observed in the presence and absence of ATP-$\gamma$-S. Identical competition results are obtained at either 25°C or 37°C and either 4 mM or 10 mM-$MgCl_2$.

In the presence of ATP and RecA protein at 25°C, the SSB protein titration results obtained using etheno M13 DNA are again identical with those obtained using the unmodified DNA substrate, i.e. complete displacement of RecA protein is observed (Table 1). However, in contrast to the binding data obtained using the natural DNA substrate at 37°C and 10 mM-$MgCl_2$ (see Fig. 5), equivalent data obtained using etheno M13 DNA shows that SSB protein will bind to the etheno DNA in the presence of RecA protein and ATP (Table 1). Complete displacement of the RecA protein from the etheno DNA substrate by SSB protein is always observed, regardless of temperature and magnesium concentration. Although the binding results obtained at 37°C in the presence of ATP with each of the etheno and natural DNA substrates have opposite binding outcomes, they are completely consistent with the effects of SSB protein on the ATP hydrolytic properties of RecA protein described in the accompanying paper (Kowalczykowski & Krupp, 1987); namely that under all conditions, SSB protein inhibits the ATPase activity of RecA protein when etheno M13 DNA is used as a substrate, whereas SSB protein inhibits this activity only at lower temperature and magnesium concentration conditions when the natural DNA substrate is used. Thus, although contrasting results are obtained depending on the DNA substrate used, the binding studies yield results that closely parallel the results obtained from the ATPase studies. The self-consistence of these two different experimental methods serves to confirm our interpretation of the DNA binding results

further. Although the difference in the properties of these two DNA substrates could be easily attributed to the chemical modification present in the etheno M13 DNA, we show that it is actually due to the absence of secondary structure in the etheno M13 DNA (see Kowalczykowski & Krupp, 1986). This conclusion is also consistent with the observation that under similar experimental conditions, the synthetic homopolymer poly(dT) yields SSB protein-binding competition results identical with those obtained with etheno M13 DNA (not shown).

### (f) *Effect of RecA protein on the salt stability of SSB protein–single-stranded M13 DNA complexes*

If an interaction existed between RecA and SSB proteins that resulted in an increased affinity of RecA protein for single-stranded DNA, then from thermodynamic considerations it would be expected that the converse would also be true, i.e. that RecA protein would increase the affinity of SSB protein for single-stranded DNA. Since the stability of most protein–DNA complexes is decreased by increasing salt concentration (Record *et al.*, 1978), a simple way to determine the relative affinity of a protein–nucleic acid complex under a given set of experimental conditions is to measure the salt concentration required to dissociate half the amount of complex present; this value is referred to as the salt titration midpoint (Kowalczykowski *et al.*, 1981; Menetski & Kowalczykowski, 1985). Thus, if RecA protein increases the stability of the SSB protein–DNA complex, then an increase in the salt titration midpoint of the complex would be expected.

To determine whether such an effect could be observed, complexes of SSB protein and single-stranded M13 DNA were formed in the presence of various amounts of RecA protein. These complexes were then titrated with increasing concentrations of NaCl, while monitoring the change in intrinsic protein fluorescence; upon complex dissociation an increase in SSB protein fluorescence will occur. Such titrations are shown in Figure 6, and it is clear that the presence of RecA protein has no effect on the salt titration midpoint value of the SSB protein–single-stranded M13 DNA complex.

However, on the basis of data in Figure 1, the concentrations of proteins and DNA employed in the experiments presented in Figure 6 were such that the SSB protein should have displaced all of the RecA protein. Perhaps to have detected an effect of the RecA protein, it was necessary to have some RecA protein also bound to the DNA. Therefore a similar experiment was carried out, except that the concentration of DNA-binding sites was in excess of both the SSB and RecA protein concentrations so that both proteins were bound to the DNA. Again, no effect of increasing concentrations of RecA protein on SSB protein–single-stranded M13 DNA complex stability was detected (not shown).

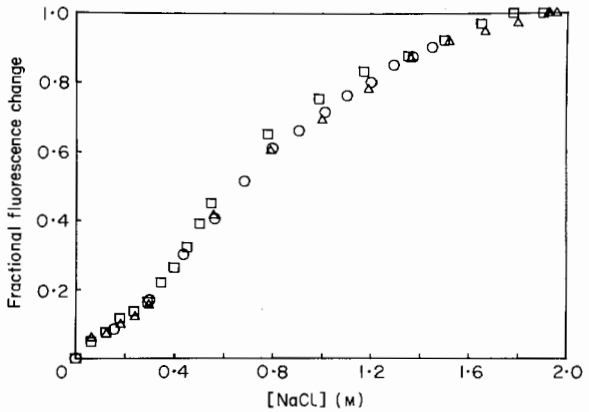


**Figure 6.** Effect of salt concentration on the stability of SSB protein–single-stranded M13 DNA complexes in the presence of RecA protein. Complexes were formed by adding RecA protein, at the concentrations indicated, to 6 $\mu$M-single-stranded DNA followed by the addition of 1·2 $\mu$M-SSB protein. Standard buffer containing 4 mM-$MgCl_2$ at 25°C was used. The complexes were titrated with NaCl while monitoring the SSB protein intrinsic fluorescence: (○) no RecA protein; (□) 0·3 $\mu$M-RecA protein; (△) 0·6 $\mu$M-RecA protein.

Finally, to ensure that RecA protein remains bound to the single-stranded M13 DNA during the course of the salt titration experiments, salt titrations of the SSB protein–single-stranded M13 DNA complex in the presence of RecA protein and ATP-$\gamma$-S were carried out (not shown). Once again, the result was negative; no effect on the stability of the SSB protein–DNA complexes was observed.

Thus, under all these conditions, there is no evidence for the formation of a complex of SSB and RecA proteins that has an effect on SSB protein–single-stranded DNA-binding affinity. However, if this hypothetical protein–protein complex is unstable at salt concentrations approaching 0·4 to 0·6 M-NaCl, then little or no effect would be observed. This question will be more fully addressed in the kinetic experiments described below.

### (g) *Effect of SSB protein on the salt stability of Rec A protein–etheno M13 DNA complexes*

In order to address more directly the question of whether SSB protein has a stabilizing effect on RecA protein–single-stranded DNA complexes, the effect of SSB protein on the salt stability of RecA protein–etheno M13 DNA complexes was investigated. As mentioned above, an advantage of the etheno M13 DNA substrate in these binding studies is that the binding of RecA protein to this DNA substrate can be monitored directly by means of the increase in etheno M13 DNA fluorescence that occurs upon formation of the RecA protein–etheno M13 DNA complex. Although the binding of SSB protein induces a fluorescence increase (not shown) of a magnitude similar to that induced by

RecA protein, salt titration experiments such as those described in section (f), above, can readily differentiate the RecA protein–etheno M13 DNA complexes from the SSB protein–DNA complexes. This is because the salt titration midpoint for the former complexes is less than 400 mM-NaCl (Menetski & Kowalczykowski, 1985), whereas it is greater than 2·0 M-NaCl for the latter complexes (not shown). In addition, the salt-induced dissociation of the SSB protein–etheno M13 DNA complexes can also be followed, independent of any fluorescence signal from RecA protein, by monitoring the change in intrinsic SSB protein fluorescence that accompanies dissociation of the SSB protein–etheno M13 DNA complexes (see Fig. 6); both experimental procedures yield similar results for the SSB protein–etheno M13 DNA complex stability. Therefore, using the etheno M13 DNA substrate, the effect of DNA-bound SSB protein on the salt stability of RecA protein–single-stranded DNA complexes can be determined directly.

The results of a salt titration experiment using etheno M13 DNA are demonstrated in Figure 7. In this Figure, the NaCl concentration range shown corresponds to a region of salt concentration where the decrease in etheno M13 DNA fluorescence observed is due only to dissociation of the RecA protein–etheno M13 DNA complexes. In this experiment, complexes of RecA protein and etheno M13 DNA were formed at concentrations such that less than 30% of the etheno M13 DNA was complexed with RecA protein. SSB protein was then added at a concentration so that the DNA concentration remained in excess of the total protein concentration to prevent displacement of the RecA protein. If SSB protein stabilized the RecA protein–single-stranded DNA complex, then a shift of the salt titration midpoint to higher salt concentration would be observed. Figure 7 shows clearly that this is not the case and that, in fact, there is no effect of SSB protein on the stability of the RecA protein–etheno M13 DNA complex (the slight decrease in fluorescence at low salt concentration is due to a decrease in light scattering, resulting from a salt-induced disaggregation of the RecA protein). Similar results are obtained when such salt titration experiments are carried out under strand assimilation conditions (i.e. 37°C and 10 mM-$MgCl_2$), even when RecA protein is present at a concentration sufficient to saturate the DNA and concentration of SSB protein is varied from 0 to 100% saturation of the etheno M13 DNA (not shown). At the higher SSB protein concentrations, the magnitude of the salt titration transition for the RecA protein–etheno M13 complex is decreased due to expected displacement of the RecA protein by the increasing amounts of SSB protein.

Although no effect of SSB protein on the stability of the RecA protein–DNA complex was observed in the absence of any nucleotide cofactors, the possibility existed that perhaps SSB protein somehow moderated the destabilizing effect of ADP

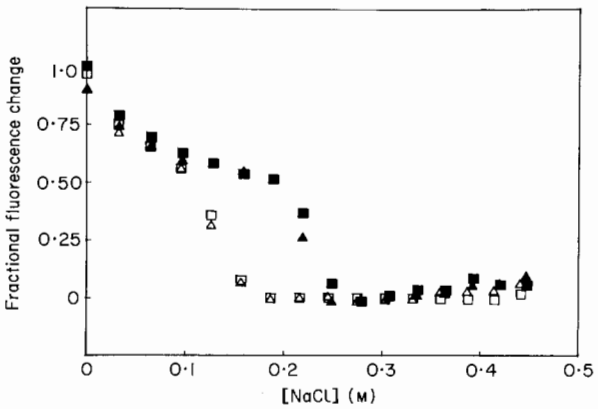


**Figure 7.** Effect of SSB protein on the stability of RecA protein–etheno M13 DNA complexes in the presence and absence of ADP. The etheno M13 DNA fluorescence was monitored. Cuvettes contained 4 mM-$MgCl_2$, 10·7 μM-etheno M13 DNA, and 0·4 μM-RecA protein: (■) no ADP or SSB protein; (▲) no ADP plus 0·27 μM-SSB protein; (□) 100 μM-ADP and no SSB protein; (△) 100 μM-ADP plus 0·27 μM-SSB protein.

on this complex (Menetski & Kowalczykowski, 1985). Thus, an experiment identical with that described above was performed, except in the presence of 100 μM-ADP. The results of this experiment are also shown in Figure 7 and they demonstrate that addition of ADP results in an identical destabilization of the RecA protein–etheno M13 DNA complex both in the presence of SSB protein and in its absence. Again, experiments conducted at 37°C and 10 mM-$MgCl_2$ revealed no effect of SSB protein on the salt-induced dissociation of RecA protein–etheno M13 DNA complexes in the presence of ADP (not shown).

Finally, the effect of various concentrations of SSB protein on the stability of the RecA protein–etheno M13 DNA complex in the presence of ATP (and an ATP-regenerating system) was determined using the salt titration procedure. Under all conditions examined, whether the concentration of RecA protein was in excess of the etheno M13 DNA concentration, or whether the DNA concentration was in excess of the total protein concentration (RecA plus SSB proteins), no effect of SSB protein was observed on the salt titration midpoint (not shown). In addition, identical results were obtained when salt titration experiments were carried out at 25°C and 4 mM-$MgCl_2$ (not shown). Thus, on the basis of all of the experiments, there seems to be no effect of SSB protein on the equilibrium stability of the RecA protein–etheno M13 single-stranded DNA complex either in the presence or the absence of ADP or ATP, despite the fact that in all cases SSB protein is bound to single-stranded DNA.

As mentioned above, one possible limitation of these salt titration studies is that, although both RecA protein and SSB protein are bound to the DNA, the putative interaction between them may be sensitive to increasing salt concentration. Thus,

it is possible that, at salt concentrations greater than approximately 150 mM-NaCl (the lowest value of the salt titration midpoint obtained in the presence of ADP), it is still too weak to be detected by these equilibrium procedures. Although this is a possible explanation, the studies of the effect of SSB protein on the kinetic stability of the RecA protein–single-stranded DNA complex in the absence of added NaCl (see below) also fail to demonstrate any specific interaction.

### (h) *Effect of SSB protein on the kinetic lifetime of RecA protein–etheno M13 DNA complexes*

In order to determine whether SSB protein has a detectable effect on the stability of the RecA protein–single-stranded DNA complex at low salt conditions, the kinetic stability of the RecA protein–DNA complex in the presence and the absence of SSB protein was determined. Since studies of the effect of competing single-stranded DNA on the initiation of strand assimilation suggested that SSB protein increased the half-life of the RecA protein–single-stranded DNA complex from 17 seconds to greater than 90 minutes (Cox & Lehman, 1982), it was of interest to determine whether a similar stabilization could be detected through direct measurement of the lifetime of RecA protein complexes formed with etheno M13 DNA.

These kinetic lifetime experiments were performed by forming complexes of RecA protein and etheno M13 DNA at molar ratios such that only one-half of the DNA was saturated. The complexes were incubated for several minutes in the presence of ATP, an ATP-regenerating system and various amounts of SSB protein. Following this incubation step, the kinetic transfer experiments were initiated by the addition of a sixfold molar excess of poly(dT) as a competing DNA. Since RecA protein binds more tightly to poly(dT), transfer of RecA protein from the ethano M13 DNA to the poly-(dT) occurs, resulting in a decrease in etheno M13 DNA fluorescence (detailed aspects of this kinetic process will be described elsewhere: Menetski & Kowalczykowski, unpublished results). The results of such transfer experiments performed under strand assimilation buffer conditions are shown in Figure 8. In the absence of SSB protein, RecA protein transfers from the etheno M13 DNA to poly(dT) with an apparent rate constant of $4{\cdot}8(\pm 0{\cdot}2)\times 10^{-3}$/s, corresponding to a RecA protein–DNA complex half-life of 2·4 minutes. If SSB protein increased the lifetime of the RecA protein–DNA complex, then the observed half-time would be expected to increase. The results in Figure 8 shows that this is not the case; the effect of increasing the SSB protein concentration is to *increase* the rate of dissociation by a factor of 2 at the maximum SSB protein concentration of 0·45 μM. (Control experiments demonstrate that essentially no dissociation of the SSB protein occurs during the RecA protein dissociation time course (not shown).) Similar experiments carried out at

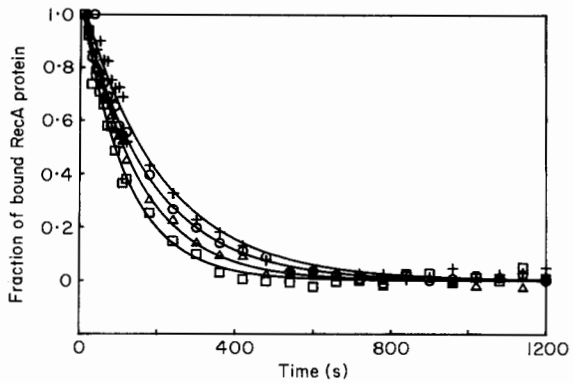


**Figure 8.** Time course of RecA protein transfer from etheno M13 DNA to poly(dT) in the presence of SSB protein. The etheno M13 DNA fluorescence was monitored. Cuvettes contained standard buffer plus 10 mM-$MgCl_2$, 10·2 μM-etheno M13 DNA, 0·51 μM-RecA protein, 1 mM-ATP, 3 mM-phosphoenol pyruvate, and 24 units pyruvate kinase/ml. Mixtures were pre-incubated at 37°C for 2 min prior to the addition of SSB protein. Reactions were initiated at time zero by the addition of 37 μM-poly(dT) and contained the following amounts of SSB protein: (+) no SSB protein; (○) 0·15 μM; (△) 0·3 μM; (□) 0·45 μM. The continuous lines represent least-squares fits of the data to single-exponential functions; the apparent rate constants are (+) $4{\cdot}8\times 10^{-3}$/s; (○) $5{\cdot}5\times 10^{-3}$/s; (△) $6{\cdot}9\times 10^{-3}$/s; (□) $8{\cdot}5\times 10^{-3}$/s.

25°C and 4 mM-$MgCl_2$ yielded results identical with those in Figure 8; i.e. SSB protein does not stabilize the RecA protein–etheno M13 DNA complex but, instead, increases the rate of transfer to poly(dT) twofold (not shown). Under these conditions, the transfer rate constant is $5{\cdot}0\times 10^{-2}$/s in the absence of SSB protein and increases to $1{\cdot}1\times 10^{-1}$/s in the presence of 0·3 μM-SSB protein. Thus, these kinetic data demonstrate that SSB protein has no stabilizing effect on the RecA protein–etheno M13 DNA complex. Although the chemical modification of the etheno M13 DNA can be suspected as being the reason for the lack of an effect of SSB protein, it should be realized that, as a consequence of this chemical modification, etheno M13 DNA is also devoid of secondary structure and that the absence of secondary structure may play a role. But, regardless of the molecular interpretation, the fact that no increased kinetic stability of the RecA protein–etheno M13 DNA complex by SSB protein is observed implies that the stabilization of RecA protein–single-stranded DNA complexes is not a general property of SSB protein. As shown below, stabilization of a RecA protein–DNA complex is a property related to the DNA substrate.

### (i) *Effect of SSB protein on the kinetic lifetime of RecA protein–single-stranded M13 DNA complexes*

To ensure that the observed lack of stabilization of the RecA protein–single-stranded DNA complex by SSB protein is not an artifact of the etheno M13

DNA substrate, a transfer experiment similar to that shown in Figure 8 was performed, except using native single-stranded M13 DNA to form the initial RecA protein–DNA complex, and with etheno M13 DNA as the competing DNA. In this case, the transfer of RecA protein from single-stranded M13 DNA to etheno M13 DNA could be monitored as an increase in fluorescence. Since SSB protein does not displace RecA protein totally from the native single-stranded M13 DNA substrate under these conditions (see Fig. 5), it was possible to perform these experiments using either saturating or subsaturating amounts of RecA protein. Figure 9 shows the effect of increasing amounts of SSB protein on such a transfer reaction when an excess concentration of RecA protein (relative to saturation of the single-stranded M13 DNA) is present. In the absence of SSB protein, the rate of transfer of RecA protein from single-stranded M13 DNA to etheno M13 DNA is $2{\cdot}0\times10^{-2}$/s, corresponding to a half-life of 35 seconds. However, in contrast to the results obtained with the etheno M13 DNA-to-poly(dT) transfer experiments, the effect of increasing SSB protein concentrations on these experiments is to *decrease* the transfer rate (Table 2); at 0·75 μM-SSB protein, a transfer rate constant of $1{\cdot}8\times10^{-3}$/s is obtained. This represents a nearly tenfold apparent stabilization of the RecA protein–single-stranded DNA complex by SSB protein. The effect appears to saturate at approximately 0·3 μM-SSB protein and data obtained at higher SSB protein concentrations yield identical rate constants within experimental reproducibility.

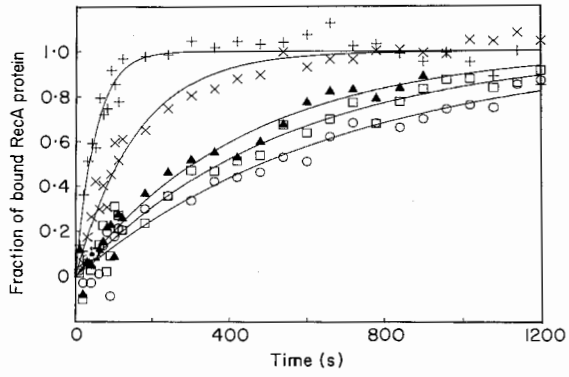


**Figure 9.** Time course of RecA protein transfer from single-stranded M13 DNA to etheno M13 DNA. The etheno M13 DNA fluorescence was monitored. Cuvettes contained standard buffer plus 10 mM-$MgCl_2$, 6 μM-single-stranded M13 DNA, 1·5 μM-RecA protein, 1 mM-ATP, 3 mM-phosphoenol pyruvate and 24 units pyruvate kinase/ml. The same procedure as that described for Fig. 8 was used except that the reactions were initiated by the addition of 37 μM-etheno M13 DNA, and contained the following amounts of SSB protein: (+) none; (×) 0·15 μM; (○) 0·30 μM; (□) 0·75 μM. The filled triangles represent a transfer experiment performed in the absence of SSB protein and instead, all of the components in the cuvette were first incubated in 1 mM-$MgCl_2$ for 5 min, followed by the addition of $MgCl_2$ to a final concentration of 10 mM, and then followed by the addition of the etheno M13 DNA at zero time. The continuous lines represent least-squares fits of the data to single-exponential functions; the apparent rate constants are: (+) $2{\cdot}0\times10^{-2}$/s; (×) $6{\cdot}4\times10^{-3}$/s; (○) $1{\cdot}4\times10^{-3}$/s; (□) $1{\cdot}8\times10^{-3}$/s; (▲) $2{\cdot}3\times10^{-3}$/s.

**Table 2**

*Rate of RecA protein transfer from single-stranded M13 DNA to etheno M13 DNA*

| [SSB protein] (μM) | Apparent rate constant ($s^{-1}$) |
|---|---|
| A. *At excess RecA protein* | |
| 0 | $2{\cdot}0(\pm0{\cdot}2)\times10^{-2}$ |
| 0·15 | $6{\cdot}4(\pm0{\cdot}7)\times10^{-3}$ |
| 0·30 | $1{\cdot}4(\pm0{\cdot}3)\times10^{-3}$ |
| 0·60 | $1{\cdot}2(\pm0{\cdot}3)\times10^{-3}$ |
| 0·75 | $1{\cdot}8(\pm0{\cdot}3)\times10^{-3}$ |
| 0; pre-incubate at 1 mM-$MgCl_2$ | $2{\cdot}3(\pm0{\cdot}4)\times10^{-3}$ |
| B. *At subsaturating RecA protein* | |
| 0 | $3{\cdot}7(\pm0{\cdot}4)\times10^{-3}$ |
| 0·15 | $4{\cdot}4(\pm0{\cdot}3)\times10^{-3}$ |
| 0·45 | $2{\cdot}5(\pm0{\cdot}4)\times10^{-3}$ |
| 0·60 | $1{\cdot}7(\pm0{\cdot}2)\times10^{-3}$ |
| 0·75 | $2{\cdot}2(\pm0{\cdot}3)\times10^{-3}$ |
| 0; pre-incubate at 1 mM-$MgCl_2$ | $2{\cdot}2(\pm0{\cdot}8)\times10^{-3}$ |

Experimental conditions are identical with those described in the legend to Fig. 9, except that for B, the concentration of RecA protein is 0·5 μM. The experimental error listed is the standard deviation obtained from the least-squares fit of the data set.

Although these data can be interpreted as the result of a direct effect (i.e. protein–protein) of SSB protein on the RecA protein–DNA complex, the data in Figure 9 also reveal that a nearly identical stabilization of the RecA protein–single-stranded M13 DNA complex can be obtained by simply incubating the complex in the presence of ATP in buffer containing 1 mM-$MgCl_2$ for five minutes, and then adding $MgCl_2$ to a final concentration of 10 mM, prior to the start of the transfer experiment. If such a sequence of events is followed, the observed rate constant changes to $2{\cdot}2\times10^{-3}$/s. Within experimental error, this result is indistinguishable from those obtained at SSB protein concentrations greater than 0·3 μM. Thus, pre-incubation of a RecA protein–DNA complex in a low $MgCl_2$ concentration mimics the effect of SSB protein. Results from experiments identical with those shown in Figure 9, except using a subsaturating amount of RecA protein (0·5 μM), are also shown in Table 2. SSB protein is observed also to have an apparent stabilizing effect on the RecA protein–single-stranded M13 DNA complex, although under these conditions it is only twofold. A twofold effect is observed primarily because the transer rate in the absence of SSB protein at 0·5 μM-RecA protein is approximately fivefold slower than at 1·5 μM-RecA protein; the transfer rate observed in the presence of SSB protein is similar in both cases. In agreement with the results obtained at higher RecA protein concentrations, pre-incubation of the RecA protein–single-stranded M13 DNA complexes in 1 mM-$MgCl_2$ followed by

addition of $MgCl_2$ to a final concentration of 10 mM results in the formation of RecA protein–single-stranded M13 DNA complexes that have kinetic properties identical with those formed with SSB protein.

Although initially it may seem unusual that pre-incubation of RecA protein–single-stranded M13 DNA complexes in 1 mM-$MgCl_2$ results in the formation of complexes that behave kinetically in a way identical with that of complexes formed in the presence of SSB protein, Muniyappa *et al.* (1984) demonstrated that such a pre-incubation step facilitated the formation of joint DNA molecules and, on the basis of this and other experimental data, concluded that both SSB protein and low $MgCl_2$ concentrations were responsible for removing secondary structure that impedes the formation of joint molecules. Consistent with this interpretation, in the accompanying paper (Kowalczykowski & Krupp, 1987) it is demonstrated that both SSB protein and low $MgCl_2$ concentrations eliminate secondary structure that prevents the binding of RecA protein to single-stranded DNA. In addition, those ATPase studies demonstrate that, in the presence of secondary structure, RecA protein can form, with DNA, complexes that have a unique stability. Thus, while the result that SSB protein increases the kinetic stability of the RecA protein–M13 DNA complex might be ascribed to a directed effect of SSB protein, it is almost certainly an indirect effect mediated through the DNA secondary structure. In any event, the magnitude of the SSB protein stabilization of the RecA protein–M13 DNA complex is not nearly as great as the >300-fold effect that would be expected from the strand assimilation studies (Cox & Lehman, 1982; Cox *et al.*, 1983*a*).

## 4. Discussion

In this paper, we have attempted to determine whether SSB protein has any effect on the equilibrium or kinetic stability of complexes of RecA protein and single-stranded DNA. Three complementary approaches were used to attempt to detect directly the existence of a stabilizing interaction between these two proteins and these included: (1) the effect of RecA protein on the stability of SSB protein–single-stranded M13 DNA complexes; (2) the effect of SSB protein on the stability of RecA protein–etheno M13 single-stranded DNA; and (3) the effect of SSB protein on the transfer rate of RecA protein–single-stranded DNA complexes. In each case, little or no direct effect of SSB protein on the stability of the RecA protein–single-stranded DNA complex was detected.

The simplest interpretation of the binding experiments is that the binding of SSB and RecA protein to single-stranded DNA is competitive, with SSB protein displacing RecA protein from the DNA when no cofactor is present, but with RecA protein displacing SSB protein when ATP-$\gamma$-S is present. Similar competition experiments performed in the presence of ATP are very interesting in that the results obtained depend on the DNA substrate employed and on the temperature and magnesium concentration. The data show clearly that, in the presence of ATP, SSB protein is able to displace RecA protein completely from etheno M13 DNA under all conditions examined (see Table 1). When unmodified single-stranded M13 DNA is used as the substrate, complete displacement by SSB protein is also observed, but only at 25°C and 4 mM-$MgCl_2$. However, at 37°C and either 4 mM or 10 mM-$MgCl_2$, RecA protein (in the presence of ATP) is able partially to prevent binding of SSB protein to the natural DNA substrate, and the relative amounts of RecA and SSB protein bound to the DNA depends on their input concentrations (see Fig. 5). These data are also consistent with the interpretation that the binding of RecA and SSB proteins to single-stranded DNA in the presence of ATP is competitive, and that the outcome of this competition reflects the steady-state properties (i.e. affinities and association and dissociation rates of the various components involved).

Our conclusion that the binding of these two proteins is competitive, and therefore mutually exclusive, is in agreement with similar conclusions based on gel filtration and electron microscopic data that also demonstrate the displacement of RecA protein by SSB protein (Cohen *et al.*, 1983; Tsang *et al.*, 1985; Register & Griffith, 1985). In addition, Tsang *et al.* (1985) demonstrated that, in the presence of ATP, RecA protein was more effective in displacing SSB protein from single-stranded DNA when the $MgCl_2$ concentration was 13 mM rather than 1 mM; this result is in qualitative agreement with our fluorescent binding data obtained at 4 mM *versus* 10 mM-$MgCl_2$.

The results from our binding studies are also in good agreement with the effects of SSB protein on the single-stranded DNA-dependent ATPase activity of RecA protein described in the accompanying paper (Kowalczykowski & Krupp, 1986). There it is shown that SSB protein will inhibit the ATPase activity of RecA protein under all conditions examined if etheno M13 DNA (or any other DNA devoid of secondary structure) is used as a substrate; this result is in quantitative agreement with our binding studies, which demonstrate complete displacement of RecA protein by SSB protein. The ATPase activity study also shows that SSB protein will inhibit the activity of RecA protein when single-stranded M13 DNA is used, but only at 25°C and 4 mM-$MgCl_2$; at 37°C and either 4 mM or 10 mM-$MgCl_2$ a stimulation of activity is observed. This result also is in agreement with the results of our binding studies as summarized above. Thus, our conclusion that the binding of these two proteins is competitive is in accordance with results obtained using experimental procedures unrelated to the fluorescence methods employed here.

In addition, the DNA binding studies presented

here demonstrate that SSB protein has no effect on the thermodynamic stability of a RecA protein–etheno M13 RNA complex, as measured by salt titration experiments. No effect of SSB protein on the stability of this RecA protein–DNA complex is observed even if the conditions are such that both RecA protein and SSB protein are bound to DNA (i.e. the DNA concentration is in excess) or if either ATP or ADP is present. Also, consistent with thermodynamic principles, these results show that RecA protein has no effect on the stability of the SSB protein–single-stranded M13 DNA complex. Thus, there appears to be no interaction between these two proteins that is manifest as an increased salt stability of either protein–DNA complex.

Other than the absence of a specific interaction between RecA protein and SSB protein, an alternative explanation for the lack of any observed equilibrium effects is that the putative protein–protein interactions are very sensitive to salt concentration, and that these interactions could be disrupted at the salt concentrations required to dissociate the protein–DNA complexes. For the complexes formed with etheno M13 DNA, this would correspond to salt concentrations greater than 150 mM-NaCl. Although in theory a slight stabilization would still be expected, experimental detection of this effect may not be possible. However, this potential explanation is inconsistent with the kinetic studies described here. The kinetic transfer experiments carried out in the absence of added salt under strand assimilation conditions (37°C and 10 mM-$MgCl_2$) demonstrated clearly that SSB protein *decreased* twofold the lifetime of the RecA protein–etheno M13 DNA complex. Thus, as stated previously, stabilization of RecA protein–DNA complexes is not a general property of SSB protein, implying that there is no specific direct interaction between these two proteins.

We have also shown that when single-stranded M13 DNA is substituted for the etheno M13 DNA in the kinetic transfer studies, an apparent tenfold stabilization of this RecA protein–DNA complex is observed when SSB protein is present. However, the magnitude of this effect is much less than the >300-fold effect of SSB protein on RecA protein–DNA complex lifetime that was inferred from strand-assimilation studies (Cox & Lehman; 1982; Cox *et al.*, 1983*a*). In addition, we have demonstrated that this stabilizing effect of SSB protein can be mimicked by simply pre-incubating RecA protein with the single-stranded M13 DNA at 1 mM-$MgCl_2$ prior to adding the competitor DNA. These two observations suggest that this tenfold kinetic effect is mediated through changes in DNA structure rather than through protein–protein interactions. Consistent with this conclusion, Muniyappa *et al.* (1984) found that RecA protein can overcome an inhibitory effect of secondary structure on joint molecule formation at 1 mM-$MgCl_2$ and Kowalczykowski & Krupp (1987) demonstrated that, at 1 mM-$MgCl_2$, all of the single-stranded M13 DNA was available to stimulate RecA protein ATPase activity, whereas at 10 mM-$MgCl_2$ less than half of that DNA was available unless SSB protein was present. Thus, we would conclude that stabilization of RecA protein–DNA complexes is not a general property of SSB protein, but rather is dependent on the single-stranded DNA employed and is not observed on DNA molecules devoid of secondary structure. SSB protein is required only when secondary structure is present, as with single-stranded M13 DNA, and, as will be demonstrated further (Kowalczykowski & Krupp, 1987), is necessary to remove secondary structure in the DNA and facilitate the formation of a unique RecA protein–DNA complex that appears to have greater kinetic stability.

The one possibility that still remains is that only a small fraction of the RecA protein molecules interact with SSB protein and that this fraction represents the critical "active" complex that is stably bound to the DNA. Since the limits of detection in both the equilibrium and kinetic experiments are such that at least 90% of the protein–DNA complexes can be accounted for through measurements of the magnitudes of the fluorescence changes, it is unlikely that, if there is a special class of RecA protein–SSB complexes, it constitutes more than 10% of the proteins present and, in some experiments less than 5%.

The suggestion that SSB protein stabilizes the RecA protein–single-stranded DNA was made by Cox & Lehman (1982) to explain the fact that SSB protein stimulates the RecA protein-catalyzed strand assimilation reaction. Their DNA challenge studies showed that SSB protein appeared to increase the lifetime of the RecA protein–single-stranded DNA complex by greater than 300-fold as measured by its ability to participate in the strand assimilation reaction. However, our direct DNA transfer studies presented in this paper have failed to detect any kinetic stabilization of a comparable magnitude of the RecA protein–single-stranded DNA complex. The data we have presented in Figure 9 may provide a plausible explanation for the results obtained by Cox & Lehman (1982), who showed that in the presence of SSB protein, RecA protein would not transfer from single-stranded DNA to which it is initially bound when challenged with identical single-stranded DNA. Since we showed that the transfer rate decreased tenfold, due to the addition of SSB protein (or by pre-incubation in 1 mM-$MgCl_2$), it is reasonable to assume that this change is also reflected in a tenfold increase in the affinity of this new RecA protein–DNA complex compared to that formed in the absence of SSB protein. Thus, if this higher affinity complex is challenged with seemingly identical DNA, little or no transfer would occur to the lower affinity challenge DNA molecule. In addition, as described by Kowalczykowski & Krupp (1987), if SSB protein is allowed to bind to the challenge DNA first (because the RecA protein concentration is limiting, as is the case in the strand assimilation studies), then RecA protein binding to

the challenge DNA will be inhibited. Therefore, both effects will contribute to the inhibition of RecA protein transfer between two apparently identical DNA molecules in the presence of SSB protein.

Since we see essentially no effect of SSB protein on the equilibrium or kinetic properties of the complexes of RecA protein formed with etheno M13 DNA (a single-stranded DNA molecule devoid of secondary structure), and since the tenfold stabilizing effect of SSB protein on the kinetic lifetime of the RecA protein–single-stranded M13 DNA complexes can be mimicked by pre-incubation at 1 mM-$MgCl_2$, we would conclude that the effects of SSB protein on RecA protein–single-stranded DNA interactions are mediated through changes in DNA secondary structure and that these effects are at the level of the *association* of RecA protein with single-stranded DNA. Such a conclusion is consistent with the interpretation put forward by Muniyappa *et al.* (1984), who demonstrated that the role of SSB protein is to remove secondary structure from single-stranded DNA and thereby facilitate the formation of a proper RecA protein–single-stranded DNA complex. If the rate of formation of a "kinetically competent" RecA protein–single-stranded DNA complex is a rate-limiting step in the strand assimilation process, and since this rate is accelerated by the ability of SSB protein to remove stable secondary structure in the single-stranded DNA (Kahn & Radding, 1984), then the effects of SSB protein on RecA protein-catalyzed strand assimilation are consistent with our data. In the accompanying paper (Kowalczykowski & Krupp, 1987) it is shown (on the basis of ATPase activity studies) that the role of SSB protein is to allow RecA protein access to regions of secondary structure within the single-stranded DNA, consistent with its function in facilitating the association of RecA protein with DNA, and with the conclusion of Muniyappa *et al.* (1984). Further, it is demonstrated that the complexes formed in the presence of SSB protein are resistant to displacement by additional SSB protein, and that this effect is due to DNA secondary structure and not to SSB protein directly consistent with the DNA binding results presented here. The ATPase studies also provide insight into the effect of SSB protein in the strand assimilation reaction and further discussion is deferred to the accompanying paper.

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