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## I. Properties of the Mutant RecA142 Protein

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We have characterized the biochemical properties of *Escherichia coli* RecA142 protein, the product of a *recA* allele that is phenotypically defective in genetic recombination. *In vitro*, this mutant RecA protein is totally defective in DNA heteroduplex formation. Despite this defect, RecA142 protein is not deficient in all other biochemical activities. RecA142 protein is proficient in single-strand (ss) DNA binding ability, ssDNA-dependent ATPase activity, and DNA-free self-association (although the first 2 properties show a greater sensitivity to NaCl concentration than does the wild-type protein). However, RecA142 protein is deficient in four properties: (1) its ssDNA-dependent ATPase activity is completely inhibited by ssDNA binding (SSB) protein, demonstrating that RecA142 protein is unable to compete effectively with SSB protein for ssDNA binding sites; (2) it is unable to promote the coaggregation of ssDNA and double-strand (ds) DNA; (3) its M13 dsDNA-dependent ATPase activity is attenuated to approximately 5% of the level of the wild-type protein; (4) it is unable fully to develop characteristics of the high-affinity ssDNA-binding state that is normally induced by ATP. The first three deficiencies correspond to defects in the presynaptic, synaptic and postsynaptic steps of the *in vitro* DNA strand exchange reaction, respectively; the fourth is the likely fundamental basis for defects 1 and 3. Therefore, one or more of these properties must be important to both the *in vitro* and *in vivo* processes.

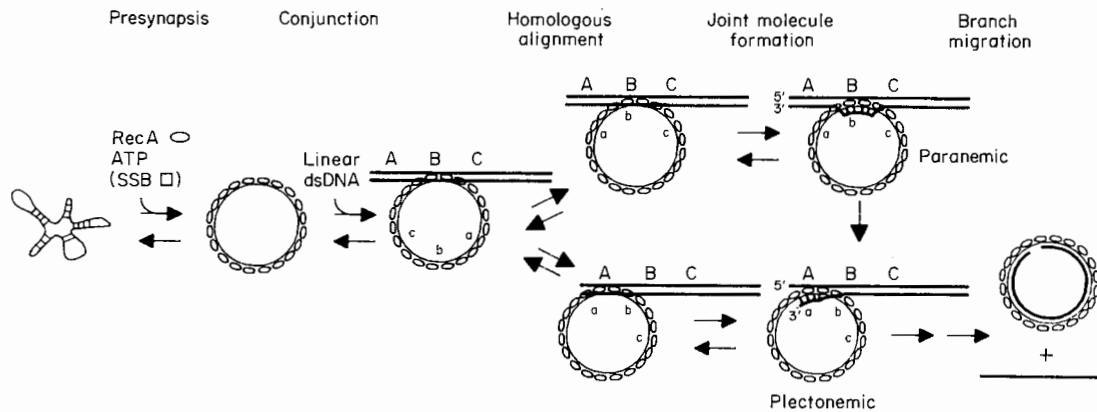
### 1. Introduction

Genetic studies have demonstrated that the *recA* gene product is essential for homologous recombination in *Escherichia coli* (Clark, 1973). Biochemical studies of purified RecA protein have shown that it possesses the unique ability to promote homologous pairing and subsequent exchange of strands between two DNA molecules, reactions which are thought to play an important role in the biological recombination process (McEntee *et al.*, 1979; Shibata *et al.*, 1979; Cassuto *et al.*, 1980; Cunningham *et al.*, 1980; West *et al.*, 1981a). In a reaction that serves as a prototype for *in vitro* studies, RecA protein can catalyze the complete reciprocal exchange of a single-strand (ss<sup>†</sup>) DNA molecule for

its homologue within a duplex DNA molecule to produce a new heteroduplex double-strand (ds) DNA molecule and a displaced ssDNA molecule (Cox & Lehman, 1981a; Kahn *et al.*, 1981; West *et al.*, 1981b).

Homologous pairing and exchange of DNA strands can be detected by several different functional assays, each of which measures a different aspect of the reaction: joint molecule (or D-loop) formation is detected by the nitrocellulose filter assay (Beattie *et al.*, 1977); total DNA heteroduplex formation can be determined using the S<sub>1</sub> nuclease assay, which measures the extent of labeled nucleotide exchange between DNA molecules (Cox & Lehman, 1981a); and complete DNA strand exchange can be shown using an agarose gel assay that measures the amount of nicked (or gapped) dsDNA formed as the result of complete exchange of homologous DNA strands (Cox & Lehman, 1981a). The mechanism of the complete DNA strand exchange reaction is quite complex and detailed biochemical studies have established that it proceeds *via* a number of intermediate steps (for

<sup>†</sup> Abbreviations used: ss, single-strand; ds, double-strand; SSB protein, *E. coli* single-strand DNA binding protein; etheno M13 DNA, M13 single-strand DNA containing 1,N<sup>6</sup>-etheno-adenosine and 3,N<sup>4</sup>-etheno-cytidine residues; ATP- $\gamma$ -S, adenosine-5'-O'-(3-thiophosphate); RFI, relative fluorescence increase.



**Figure 1.** Schematic illustration of the biochemical steps comprising the complete DNA strand exchange reaction (for details, see Kowalczykowski, 1987).

reviews, see Radding, 1982; Kowalczykowski, 1987; Cox & Lehman, 1987). These kinetically distinct steps include (see Fig. 1): *presynapsis*, the binding and saturation of ssDNA by RecA protein; *synapsis*, consisting of both conjunction and homologous alignment of the DNA molecules; *joint molecule formation*, the local denaturation of dsDNA and nascent strand exchange; and *branch migration*, the polar ATP-dependent exchange of the incoming DNA strand for the strand resident in the dsDNA molecule.

*In vitro* studies have also established that RecA protein possesses many biochemical activities, some of which must be involved mechanistically in the strand exchange process. These include binding to both ssDNA and dsDNA (West *et al.*, 1980; McEntee *et al.*, 1981; Dunn *et al.*, 1982; Chrysogelos *et al.*, 1983; Stasiak *et al.*, 1981; Silver & Fersht, 1982, 1983; Bryant *et al.*, 1985; Menetski & Kowalczykowski, 1985; Menetski *et al.*, 1988), ssDNA- and dsDNA-dependent ATPase activities (Weinstock *et al.*, 1981a,b,c; Roman & Kowalczykowski, 1986; Pugh & Cox, 1987; Kowalczykowski *et al.*, 1987a; Schutte & Cox, 1987), stimulation of ssDNA-dependent ATPase activity by the *E. coli* SSB protein (Roman & Kowalczykowski, 1986; Morrical *et al.*, 1986; Kowalczykowski & Krupp, 1987), coaggregation of non-homologous ssDNA and dsDNA (Chow & Radding, 1985; Tsang *et al.*, 1985a), and both DNA-dependent (Tsang *et al.*, 1985a) and DNA-independent protein aggregation (Cotterill & Fersht, 1983; Griffith & Shores, 1985; Morrical & Cox, 1985; Roman & Kowalczykowski, 1986).

Many of these activities have been associated with a function in a step of the strand exchange reaction. For example, the binding of RecA protein to ssDNA is a fundamental component of presynapsis. The affinity of RecA protein for ssDNA is affected by the nucleotide cofactors, ATP and ADP (Menetski & Kowalczykowski, 1985). This affinity modulation is thought to be a determinant in deciding the outcome of the competitive binding of RecA and SSB proteins for ssDNA (Kowalczykowski *et al.*, 1987b; Kowalczykowski & Krupp, 1987). Under experimental conditions that support

the *in vitro* strand exchange reaction, RecA protein can displace SSB protein from ssDNA (Roman & Kowalczykowski, 1986; Kowalczykowski *et al.*, 1987b). Under these same conditions, SSB protein will also stimulate the ssDNA-dependent ATPase activity of RecA protein (Roman & Kowalczykowski, 1986; Morrical *et al.*, 1986; Kowalczykowski & Krupp, 1987). The molecular basis for stimulation of this reaction, as well as the DNA strand exchange reaction, results primarily from the elimination of DNA secondary structure by SSB protein; DNA secondary structure prevents formation of a fully saturated presynaptic complex (Muniyappa *et al.*, 1984; Kahn & Radding, 1984; Kowalczykowski & Krupp, 1987). Presumably, since SSB mutations result in defects in recombinational repair (Glassberg *et al.*, 1979; Lieberman & Witkin, 1981; Whittier & Chase, 1983), SSB protein may serve a similar function *in vivo*. Mutations in either RecA protein or SSB protein that alter their relative ssDNA binding affinities should affect the outcome of ssDNA binding competition (Kowalczykowski & Krupp, 1987).

Another biochemical characteristic of RecA protein that may serve an important function in the DNA strand exchange process is its coaggregation activity (Chow & Radding, 1985; Tsang *et al.*, 1985a). In the coaggregation reaction, RecA protein forms a ternary complex with non-homologous ssDNA and dsDNA. Generally, inhibition of nucleoprotein network formation by an increase in either salt or ADP concentration prevents joint molecule formation. These, and similar observations, led to the hypothesis that coaggregation is the molecular event responsible for the conjunction phase of synapsis (Chow & Radding, 1985; Tsang *et al.*, 1985a). Coaggregate formation precedes joint molecule formation and is thought to be instrumental in the DNA sequence homology search (Tsang *et al.*, 1985a).

Finally, RecA protein has a dsDNA-dependent ATPase activity (Weinstock *et al.*, 1979, 1981a; Roman & Kowalczykowski, 1986; Kowalczykowski *et al.*, 1987a; Pugh & Cox, 1987). The earlier studies showed that dsDNA was a poor substrate for the

ATP hydrolysis reaction at pH 7.5, but the later studies showed that this was the result of an unusual lag in the ATP hydrolysis time-course. Although the reaction is apparently dependent on dsDNA, ATP hydrolysis actually occurs *via* a RecA protein-dsDNA intermediate that may be locally denatured and may be transiently single stranded (Kowalczykowski *et al.*, 1987a). As a consequence, this reaction may reflect molecular events important to steps of the DNA strand exchange reaction that require dsDNA denaturation, such as joint molecule formation and extension of DNA heteroduplex regions by branch migration (Kowalczykowski, 1987). In agreement with this expectation, a correlation is observed between the extent of ATP hydrolyzed in dsDNA-dependent reaction and the extent of branch migration; no such correlation is obtained for the ssDNA-dependent reaction (Roman & Kowalczykowski, 1986). This result implies that events associated with the dsDNA-dependent ATPase activity may be more directly related to steps involved in DNA heteroduplex formation (such as branch migration) than are those associated with the ssDNA-dependent ATPase activity. However, a limiting view proposed for the mechanistic role of the dsDNA-dependent ATPase in DNA heteroduplex formation does not require ATP hydrolysis for exchange of DNA strands (Kowalczykowski, 1987). ATP binding (and the induction of the high-affinity DNA-binding form of RecA protein), but not ATP hydrolysis, is necessary to promote binding and polymerization of RecA protein on dsDNA as the essential step in DNA heteroduplex formation; dsDNA-dependent ATP hydrolysis is an event that follows exchange of DNA. Recent studies suggest that this latter view may be more appropriate and that, in fact, ATP hydrolysis is needed for dissociation of RecA protein from the DNA (Menetski, 1988).

Despite the biochemical studies of RecA protein function, it is not yet certain how many of the activities described above play a role in the genetic recombination process *in vivo*. To establish such functionality, ideally, requires a mutant RecA protein defective in genetic recombination *in vivo* but defective in only a limited number of biochemical activities *in vitro*. Studies on RecA1 protein have shown that it is unable to catalyze DNA strand exchange (Rusche *et al.*, 1985) and that it is also defective in ssDNA-dependent ATPase activity (Ogawa *et al.*, 1978) and ssDNA binding (Rusche *et al.*, 1985; Bryant & Lehman, 1986). However, since this mutant protein is also defective in nearly every other RecA protein function (Kowalczykowski *et al.*, unpublished results), it is not possible to ascertain the relative importance of each biochemical property to biological function.

Here we describe the *in vitro* properties of a mutant RecA protein, RecA142 protein. Strains containing the *recA142* allele are defective in genetic recombination (Clark, 1973) and display a +/- phenotype for lambda prophage induction (Dutreix *et al.*, 1985). Previously, RecA142 protein had been

shown to be defective in lambda repressor cleavage activity and to display a ssDNA-dependent ATPase activity that is more salt-sensitive than that of wild-type RecA protein (Roberts & Roberts, 1981). We have examined the biochemical activity of RecA142 protein thought to be important in the recombination process. Consistent with its recombination phenotype, we show that this mutant RecA protein is totally defective in DNA heteroduplex formation *in vitro*. However, in contrast to the mutant RecA1 protein, RecA142 protein is defective in only a limited number of biochemical properties. Thus, the mutant RecA142 protein serves to define biochemical properties that must be important to the biological function of RecA protein. The relative importance and physiological significance of each of these activities is discussed.

## 2. Materials and Methods

### (a) Chemicals and buffers

All chemicals were reagent grade and solutions were made using glass-distilled water. ATP and ADP were purchased from Pharmacia PL Biochemicals and were dissolved as concentrated stocks at approximately pH 7.5. NADH and phosphoenolpyruvate were purchased from Sigma. Unless otherwise noted, the standard buffer used in all experiments consisted of 20 mM-Tris-acetate (pH 7.5), 10 mM-magnesium acetate, 0.1 mM-dithiothreitol; when ATP was present, its concentration was 1 mM and an ATP-regenerating system consisting of 7.5 mM-phosphoenolpyruvate, 2 units pyruvate kinase/ml was added. All reactions were carried out at 37 °C.

### (b) DNA and protein

Single- and double-stranded DNA were isolated from bacteriophage M13mp7, using the procedure of Messing (Messing, 1983). M13 dsDNA was labeled by infecting with phage in the presence of [<sup>3</sup>H]thymidine. The dsDNA was linearized by digestion with *EcoRI* restriction endonuclease. Plasmid DNA (pBEU41), which is non-identical to the M13 DNA, was prepared from strain BEU293 (kindly provided by A. John Clark of University of California-Berkeley); pBEU41 is plasmid pBEU2 (Uhlen *et al.*, 1983) containing the cloned *recA56* gene. Concentrations were determined using molar extinction coefficients of 8780 and 6500 M<sup>-1</sup> cm<sup>-1</sup> at 260 nm for ssDNA and dsDNA, respectively. Etheno M13 DNA was prepared from the phage DNA as described (Menetski & Kowalczykowski, 1985); its concentration was determined using an extinction coefficient of 7000 M<sup>-1</sup> cm<sup>-1</sup> (Menetski & Kowalczykowski, 1987a).

RecA142 protein was purified from strain JWR259 (kindly provided by Jeffrey Roberts of Cornell University) using a preparative protocol (Kowalczykowski *et al.*, unpublished results) based on spermidine precipitation (Griffith & Shores, 1985). Wild-type RecA protein was purified from strain JC12772 (Uhlen & Clark, 1981) using the same procedure. RecA protein concentrations were determined using an extinction coefficient of 2.7 × 10<sup>4</sup> cm<sup>-1</sup> at 280 nm. SSB protein was isolated from strain RLM727 using a preparative procedure provided by Roger McMacken of Johns Hopkins University. The SSB protein concentration was determined using an extinction coefficient of 3.0 × 10<sup>4</sup> at 280 nm (Ruyechan & Wetmur, 1975).

(c) *DNA strand exchange assay*

DNA strand exchange activity was measured using an agarose gel assay (Cox & Lehman, 1981a). Reaction conditions were modified to use acetate-based buffers rather than chloride, and product formation was quantified by densitometric scanning of a photographic negative of the gel as described (Roman & Kowalczykowski, 1986). The percentage of product formed in this assay was defined as the percentage of the total dsDNA that was converted to gapped circular dsDNA from linear dsDNA. Standard buffer containing ATP was used, except that the Tris-acetate concentration was 25 mM, the dithiothreitol concentration was 1 mM, and 3.7% (v/v) glycerol was present. The concentrations employed were 9.9  $\mu$ M-ssDNA, 16.8  $\mu$ M-linear dsDNA, 6  $\mu$ M-RecA protein, and 0.9  $\mu$ M-SSB protein, unless otherwise noted. RecA protein and the ssDNA were preincubated together for 1 to 2 min prior to the addition of SSB protein. Reactions were stopped by the addition of 50 mM-EDTA and 1% (w/v) SDS and samples were run on a 0.8% agarose gel containing 10  $\mu$ g ethidium bromide/ml in TAE buffer (40 mM-Tris-acetate, 1 mM-EDTA, pH 8).

(d) *DNA heteroduplex formation assay*

Formation of heteroduplex dsDNA was monitored using the  $S_1$  nuclease assay as described (Cox & Lehman, 1981a), but with the following modifications.  $^3$ H-labeled M13mp7 dsDNA that was linearized with *Eco*RI restriction endonuclease was employed rather than labeled ssDNA. Component concentrations and order of addition were identical with those described for the above strand exchange assay. Samples were removed from the strand exchange reaction mixture at various times, and SDS was added to a final concentration of 1% and a 6 $\times$  volume of  $S_1$  nuclease buffer was added.  $S_1$  nuclease was added to a final concentration of 100 units/ml and the mixture was incubated at 37°C for 30 min. Cold trichloroacetic acid and calf thymus DNA were added to final concentrations of 10% (v/v) and 0.25 mg/ml, respectively, and the mixture was centrifuged in an Eppendorf microfuge for 15 min at 4°C. A portion of the supernatant was withdrawn and counted in liquid scintillation cocktail. Complete strand exchange results in the susceptibility of one strand of the  $^3$ H-labeled dsDNA to digestion by  $S_1$  nuclease and corresponds to the appearance in the supernatant fraction of 50% of the total input tritiated DNA.

(e) *Joint molecule assay*

Joint molecule formation was detected using the nitrocellulose filter assay described (Beattie *et al.*, 1977). Component concentrations and the order of addition were identical with those described above for the strand exchange assays. Samples (50  $\mu$ l) were removed from the reaction mixture and stopped by the addition of SDS and EDTA to final concentrations of 0.5% and 12.5 mM, respectively. One ml of cold D-loop buffer was added, then filtered through a nitrocellulose filter (Schleicher & Schuell BA85, 45  $\mu$ m pore size) that had been soaking in D-loop buffer. The filters were then washed with 2 ml of D-loop buffer, dried, and counted in liquid scintillation cocktail. (D-loop buffer is: 2 M-NaCl, 0.15 M-sodium citrate).

(f) *ssDNA binding assay*

The binding of RecA protein to ssDNA was monitored using a fluorescent derivative of single-stranded M13

DNA, referred to as etheno M13 DNA. Experiments were carried out as described (Menetski & Kowalczykowski, 1985). Standard buffer was employed, except that the anion present was chloride rather than acetate and the phosphoenolpyruvate concentration was 0.6 mM when the ATP concentration was less than 250  $\mu$ M or 1.5 mM when the ATP concentration was greater than 250  $\mu$ M (Menetski *et al.*, 1988). Titrations of the etheno M13 DNA by RecA protein were carried out by adding portions of a concentrated RecA protein stock to 3  $\mu$ M-etheno M13 DNA. Salt titrations were carried out by adding portions of 5 M-NaCl to complexes of 1.2  $\mu$ M-RecA protein and 3  $\mu$ M-etheno M13 DNA. The salt titration midpoint is defined as the salt concentration required to dissociate one-half of the protein-DNA complexes present (Menetski & Kowalczykowski, 1985).

(g) *ATPase assay*

ATPase assays were carried out using a spectrophotometric assay that couples the production of ADP to the oxidation of NADH (Kreuzer & Jongeneel, 1983). The ssDNA-dependent ATPase activity was measured in standard buffer containing ATP, as described (Kowalczykowski & Krupp, 1987) but with the following modifications: the NADH concentration was 0.2 mg/ml, the phosphoenolpyruvate concentration was 1.5 mM, and the pyruvate kinase and lactate dehydrogenase were present at 25 units/ml. Unless otherwise indicated, the ssDNA concentration was 3  $\mu$ M. When SSB protein was present, it was added 1 to 2 min after formation of the RecA protein-ssDNA complex to a final concentration of 0.6  $\mu$ M.

The dsDNA-dependent ATPase activity was also measured in standard buffer containing ATP, except that both the pyruvate kinase and the lactate dehydrogenase were present at 100 units/ml (Roman & Kowalczykowski, 1986; Kowalczykowski *et al.*, 1987a). The concentration of linear double-stranded M13 DNA was 8.4  $\mu$ M and the RecA protein concentration was 3  $\mu$ M; these concentrations are one-half the concentrations employed in the DNA strand exchange experiments.

(h) *Coaggregation assay*

Coaggregation was measured as described (Tsang *et al.*, 1985a) except that, instead of using labeled DNA, agarose gel electrophoresis was used to identify the DNA. Assays (100  $\mu$ l) were carried out in a buffer consisting of 25 mM-Tris-acetate (pH 7.5), 1 mM-dithiothreitol, 1 mM-ATP, 2.25 mM-phosphoenolpyruvate, 10 units pyruvate kinase/ml, unless otherwise noted. Magnesium acetate was present in the preincubation and final incubation steps at the concentration indicated. Component concentrations were: 2.0  $\mu$ M-RecA protein, 0.28  $\mu$ M-SSB protein (when present), 2.8  $\mu$ M-single-stranded M13 DNA, and 10  $\mu$ M-duplex pBEU41 DNA. The ssDNA, RecA protein and SSB protein (added last, when present) were preincubated for 10 min in either 1 mM or 10 mM-Mg acetate, as indicated. Following the preincubation step, additional magnesium acetate, if indicated, was added to a final concentration of 10 mM, and then followed by the dsDNA. After a further 10 min of incubation, the mixture was centrifuged in a Fisher microfuge for 4 min. Supernatant fraction (30  $\mu$ l) was removed and SDS was added to 1%. The remainder of the supernatant fraction was removed and the pellet fraction was suspended by vortexing in 100  $\mu$ l of 1% SDS. The supernatant and pellet fractions (33  $\mu$ l) were then loaded onto a 0.8%

agarose gel and run in TAE buffer. To quantify the amount of ssDNA and dsDNA in each fraction, a photographic negative of the ethidium bromide-stained gel was scanned using a Bio-Med Instruments laser densitometer interfaced to an HP 3390A integrator.

(i) *Aggregation assay*

RecA protein aggregation was determined by following the increase in light-scattering that accompanies self-association (Cotterill & Fersht, 1983). Assays were carried out in standard buffer containing 6  $\mu\text{M}$ -RecA protein (Roman & Kowalczykowski, 1986).

### 3. Results

(a) *RecA142 protein is defective in both DNA strand exchange activity and DNA heteroduplex formation*

Because the *recA142* mutation shows a defect in genetic recombination *in vivo*, it was of interest to determine whether the *in vitro* DNA strand exchange reaction was affected. Figure 2 displays the results obtained from the agarose gel assay†. When SSB protein is present, wild-type protein is capable of converting more than 90% of the linear dsDNA substrate into the gapped circular dsDNA product after 30 min (Roman & Kowalczykowski, 1986). In contrast, RecA142 protein shows no trace of DNA strand exchange activity (Fig. 2). This is true whether or not SSB protein is present. In

† We have observed that analysis of DNA strand exchange reaction products on agarose gels containing ethidium bromide does not permit the separation of reaction intermediates (i.e. joint molecules) from final product (i.e. gapped circular dsDNA; Menetski, 1988). Consequently, the term product, as shown in Fig. 2, represents the sum of both intermediates and final product. When reactions are analyzed on ethidium bromide-free agarose gels, final product begins to appear after about 10 min (see Fig. 7 in the accompanying paper (Kowalczykowski & Krupp, 1989)).

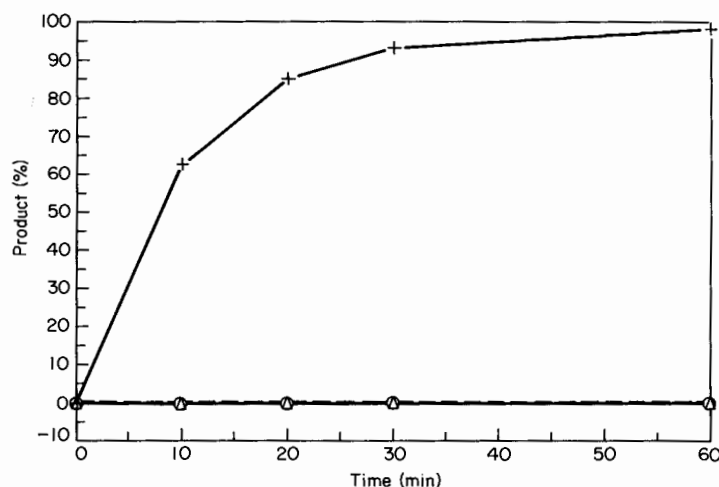
addition, there was no evidence of DNA intermediates that may have undergone only partial exchange. Hence, the absence of DNA strand exchange activity for RecA142 protein is consistent with its defect in genetic recombination *in vivo*.

To ensure that the results obtained from the agarose gel-based DNA strand exchange experiments were an accurate measure of heteroduplex DNA formation, parallel experiments were carried out using the  $S_1$  nuclease assay (Cox & Lehman, 1981a,b). This assay detects DNA heteroduplex formation as an increased sensitivity of  $^3\text{H}$ -labeled dsDNA to  $S_1$  nuclease due to strand displacement by the invading ssDNA. Wild-type RecA protein can catalyze nearly complete (80 to 90%) heteroduplex DNA formation after approximately 30 minutes (not shown; for typical data, see Kowalczykowski & Krupp, 1989). In comparison, RecA142 protein is unable to form any heteroduplex DNA ( $\pm 4\%$ ) as detected by this assay. Thus, RecA142 protein appears to be defective in even limited DNA heteroduplex formation.

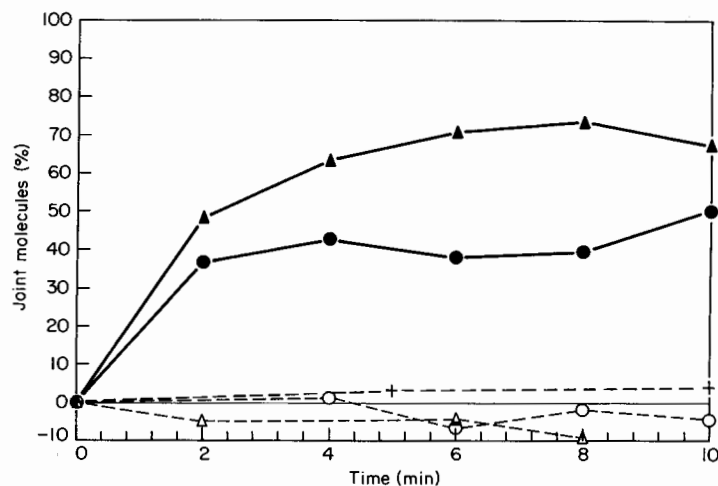
(b) *RecA142 protein is defective in joint molecule formation*

The assays employed in the preceding section are most useful for detection of extensive DNA heteroduplex formation. However, RecA142 protein may be capable of only very limited DNA heteroduplex formation, which escaped detection in those assays. To examine this possibility, joint molecule formation was measured using the nitrocellulose filter assay.

Figure 3 shows that, in the presence of SSB protein, wild-type RecA protein incorporates approximately 70% ( $\pm 15\%$ ) of the dsDNA into joint heteroduplex DNA molecules and that, without SSB protein, the yield is approximately 30% (not shown). In contrast, there is no detectable joint molecule formation by RecA142 protein either in the presence of SSB protein or in its absence.



**Figure 2.** DNA strand exchange activities of wild-type and RecA142 proteins. Agarose gel assays were carried out and analyzed as described in Materials and Methods: (+) wild-type RecA protein; (O) RecA142 protein; (Δ) RecA142 protein but without SSB protein.



**Figure 3.** Joint molecule formation catalyzed by wild-type and RecA142 proteins. Nitrocellulose filter assays were carried out as described in Materials and Methods: (▲) wild-type RecA protein in the presence of SSB protein; (△) RecA142 protein in the presence of SSB protein; (●) wild-type RecA protein in the absence of SSB protein, using a preincubation step in 1 mM-magnesium acetate; (○) RecA142 protein in the absence of SSB protein, using a preincubation step in 1 mM-magnesium acetate; (+) RecA142 protein in the absence of SSB protein.

The stimulation of joint molecule formation by SSB protein can be somewhat mimicked by preincubation of the RecA protein-ssDNA complex in 1 mM-Mg<sup>2+</sup> prior to dsDNA addition (Muniyappa *et al.*, 1984; Kahn & Radding, 1984). This is shown in Figure 3. However, this same preincubation procedure has no effect on the ability of the mutant protein to form joint molecules; RecA142 protein is still fully defective in joint molecule formation (Fig. 3).

The inability of RecA142 protein to form incipient heteroduplex DNA structures implies that it is deficient in either one or a combination of the following steps: formation of a RecA protein-ssDNA presynaptic complex, synapsis of DNA molecules, or local denaturation of dsDNA during initial strand invasion. The proficiency of RecA142 protein in each of these individual steps was examined.

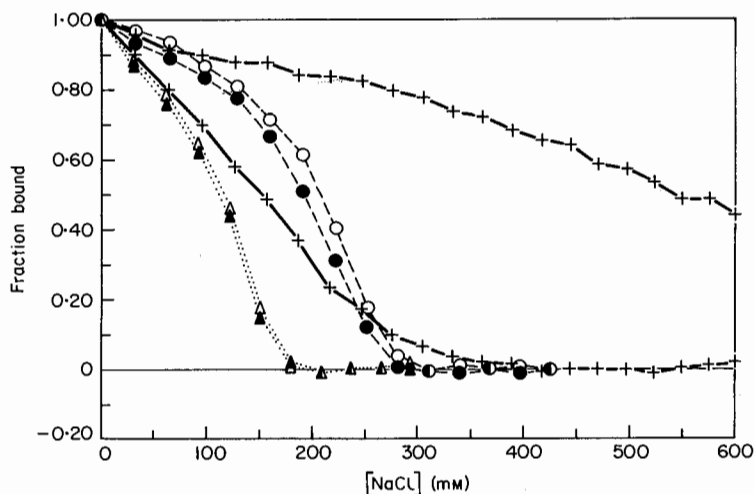
#### (c) Presynaptic complex formation by RecA142 protein

The formation of an active presynaptic complex by RecA protein requires, at the minimum, the binding of RecA protein to ssDNA. This binding step can be readily assayed using the properties of the fluorescently modified single-stranded M13 DNA, referred to as etheno M13 DNA (Menetski & Kowalczykowski, 1985). When RecA protein binds to etheno M13 DNA, the DNA fluorescence increases in proportion to the amount of protein-DNA complex formed, until saturation of the DNA is achieved. For the wild-type protein, saturation occurs at a stoichiometry of 8(±1) nucleotides/RecA protein monomer (Menetski & Kowalczykowski, 1985, 1987a). RecA142 protein binds to ssDNA with an identical stoichiometry of 8(±1) nucleotides/protein monomer (not shown).

Although RecA142 and wild-type proteins are both able to bind ssDNA, quantitative differences in binding affinity may exist that would go undetected in a stoichiometric titration. Such differences in affinity are readily detected by measuring the sensitivity of the RecA protein-etheno DNA complexes to dissociation by NaCl (Menetski & Kowalczykowski, 1985). Figure 4 (filled circles) shows that the salt titration midpoint for wild-type RecA protein is approximately 200 mM-NaCl in the absence of any nucleotide cofactor. For RecA142 protein (open circles), the salt titration midpoint is identical, within experimental error. Since both protein-etheno M13 DNA complexes dissociate at an identical NaCl concentration, this demonstrates that both the ssDNA binding affinity and salt dependence of binding are identical in the absence of nucleotide cofactor.

The effect of ADP on the stability of the RecA142 protein-etheno M13 DNA complex is also shown in Figure 4 (open triangles). ADP destabilizes the wild-type RecA protein-DNA complex, resulting in a lower salt titration midpoint (Menetski & Kowalczykowski, 1985). It is evident that ADP has an identical destabilizing effect on the affinity of the RecA142 protein-etheno M13 DNA complex. Thus, both in the presence and absence of ADP, the wild-type and mutant proteins have identical ssDNA binding properties.

Similar salt titration experiments can be carried out in the presence of ATP and an ATP-regenerating system; however, because of ATP hydrolysis, such experiments do not reflect simple equilibrium properties but rather some steady-state average of the kinetic and equilibrium properties of the system (Menetski & Kowalczykowski, 1985). Nevertheless, these experiments are useful for discerning differences in apparent DNA binding affinities. Figure 4 shows that, in the presence of 0.5 mM-ATP (crosses),

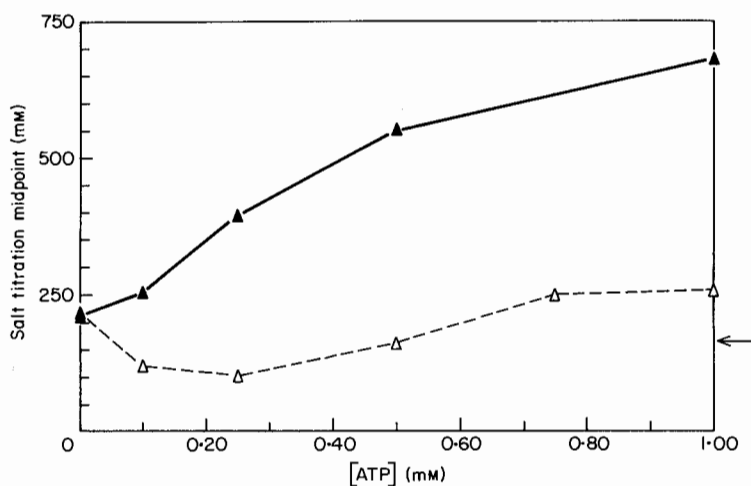


**Figure 4.** Stability of RecA protein-etheno M13 DNA complexes to dissociation by NaCl. Salt titration experiments were carried out as described in Materials and Methods; filled symbols represent wild-type protein and open symbols represent RecA142 protein: ( $\blacktriangle$ ,  $\triangle$ ) presence of 1.0 mM-ADP; ( $\circ$ ,  $\bullet$ ) absence of nucleotide cofactor; (-+-+-, -+-+--), presence of 0.5 mM-ATP and an ATP-regenerating system, for wild type and RecA142 protein, respectively).

there is a distinct difference in the salt titration midpoint for the wild-type and mutant proteins. The midpoint is approximately 550 mM-NaCl for RecA protein but only approximately 160 mM-NaCl for RecA142 protein; this difference is in distinct contrast to the results obtained without ATP.

Since the affinity of RecA protein for ssDNA is thermodynamically coupled to its affinity for ATP, the effect of ATP concentration on the salt titration midpoint for each of the two proteins was compared. Figure 5 shows that increasing concentrations of ATP result in an increased salt titration midpoint for the wild-type protein, indicative of a progressive increase in steady-state affinity. For RecA142 protein, however, low concentrations of ATP actually *decrease* the salt titration midpoint to a level that is normally observed only with ADP (arrow). A similar effect is observed for wild-type

protein, but only at ATP concentrations less than 100  $\mu$ M (Menetski *et al.*, 1988). The salt titration midpoint for RecA142 protein increases at higher ATP concentrations but, at 1 mM-ATP, it is only at a level normally observed without ATP. At higher ATP concentrations (e.g. 8 mM), the affinity increases above the no cofactor state (not shown), but a quantitative difference between the wild-type and mutant proteins remains; the observed salt titration midpoints are 1.25 M-NaCl and 0.66 M-NaCl, respectively. The non-hydrolyzable ATP analogue, ATP- $\gamma$ -S, induces a very high affinity form of RecA142 protein that is not dissociable by 1.0 M-NaCl (not shown); this behavior is identical with that of the wild-type protein (Menetski & Kowalczykowski, 1985). However, in contrast to the wild-type protein, the ATP- $\gamma$ -S form of RecA142 protein can be dissociated in 0.7 M-NaCl by EDTA, demon-



**Figure 5.** Effect of ATP concentration on the stability of RecA protein-etheno M13 DNA complexes to dissociation by NaCl. Salt titration midpoint values were obtained as described in Materials and Methods: ( $\blacktriangle$ ) wild-type RecA protein; ( $\triangle$ ) RecA142 protein. The arrow indicates the salt titration midpoint obtained in the presence of 1.0 mM-ADP.



strating that this complex is also less stable than the equivalent wild-type protein complex.

An additional parameter obtained from the etheno M13 DNA binding experiments is the relative fluorescence increase (RFI) upon complex formation (Menetski & Kowalczykowski, 1985); this parameter is simply the ratio of the fluorescence of the saturated protein-etheno DNA complex relative to the dissociated state. For wild-type protein, the RFI is approximately 1.6 in the absence of nucleotide cofactor, and increases to  $2.4 \pm 0.1$  and  $2.6$  in the presence of ATP and ATP- $\gamma$ -S, respectively. The additional fluorescence increase in the presence of ATP or ATP- $\gamma$ -S is indicative of an ATP-induced conformation change in the RecA protein-DNA complex (Menetski & Kowalczykowski, 1987b; Menetski et al., 1988). RecA142 protein shows an identical RFI in the absence of cofactor but, in the presence of ATP, the RFI is lower ( $2.1 \pm 0.1$ ) than for wild-type protein. The RFI obtained for RecA142 protein does not increase with increasing ATP concentration, despite the induction of a higher salt titration midpoint. In the presence of ATP- $\gamma$ -S, however, the RFI values for both RecA proteins are identical.

Taken together, these results suggest that RecA142 protein is quantitatively defective in an ATP-induced conformation change that is manifest as both a decreased stability to dissociation by NaCl and a lower characteristic fluorescence increase upon complex formation with etheno M13 DNA. However ATP- $\gamma$ -S, a potent inducer of this transition, is capable of inducing this state.

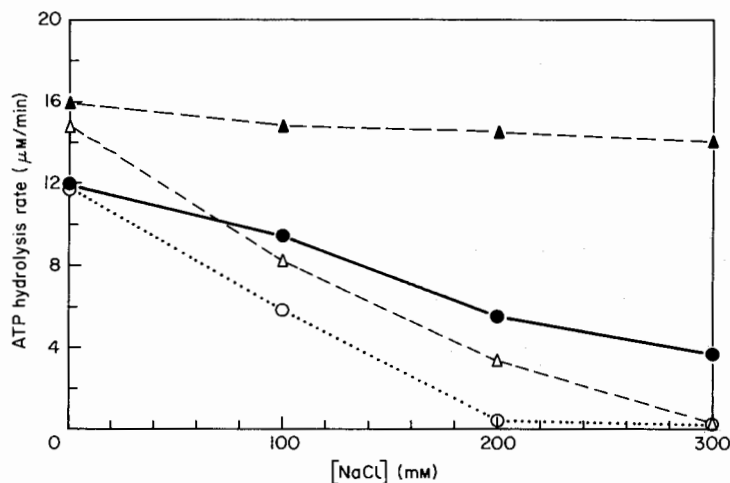
(d) *Properties of the single-stranded DNA-dependent ATPase activity of RecA142 protein*

RecA protein has a ssDNA-dependent ATPase activity that may play a role in DNA strand exchange. This activity is also useful for assaying presynaptic complex formation, since the rate of

ATP hydrolysis is proportional to the amount of RecA protein-ssDNA complex formed (Kowalczykowski & Krupp, 1987). Figure 6 shows that RecA142 protein possesses ssDNA-dependent ATPase activity and that, under strand exchange conditions (i.e. at 0 mM-NaCl), the ATPase activity of RecA142 protein is nearly identical with that of the wild-type protein. This equivalence is true at two different RecA protein concentrations. Thus the defect in strand exchange activity exhibited by RecA142 protein cannot be attributed to an absence of ssDNA-dependent ATPase activity *per se*.

However, compared with wild-type protein, the ATPase activity of RecA142 protein is more sensitive to increasing NaCl concentrations. The activity of  $1.5 \mu\text{M}$ -wild-type protein is inhibited by only 12% at 300 mM-NaCl, whereas the activity of RecA142 protein is inhibited by 99% relative to the levels measured without added NaCl. A similar differential salt sensitivity is also observed when the protein and ssDNA concentrations are identical with those employed in the strand exchange experiments (not shown). These results agree with the original observations made by Roberts & Roberts (1981).

The ATPase activity of RecA142 protein also displays salt sensitivity when etheno M13 DNA is used as the DNA substrate (not shown). Under conditions identical with those used in Figure 6 ( $1.5 \mu\text{M}$ -protein), the ATPase activity of the wild-type protein is reduced by only 10% at 300 mM-NaCl, whereas the mutant protein shows a 42% reduction. This inhibition is less than that observed with native single-stranded M13 DNA because the affinity of RecA protein for the modified DNA is greater than for the unmodified DNA (Silver & Fersht, 1983; Menetski & Kowalczykowski, 1985). The increased salt sensitivity for the ssDNA-dependent ATPase activity of RecA142 protein relative to wild-type protein is consistent with the etheno M13 DNA binding studies described above, which also



**Figure 6.** Effect of NaCl on the ssDNA-dependent ATPase activity of RecA protein. ATPase assays were carried out as described in Materials and Methods; filled symbols represent wild-type RecA protein and open symbols represent RecA142 protein: (▲, △)  $1.5 \mu\text{M}$ -protein; (○, ●)  $0.5 \mu\text{M}$ -protein.

showed an increased sensitivity of the mutant protein-DNA complexes to disruption by NaCl in the presence of ATP.

Since induction of a higher affinity ssDNA-binding form of RecA142 protein required greater concentrations of ATP compared with wild-type protein, this suggested that the affinity of RecA142 protein for ATP was reduced. Therefore, the apparent  $K_m$  for ATP was determined from ATPase assays using etheno M13 DNA as a cofactor. The ATP concentration-dependence curve for RecA142 protein was sigmoid (not shown) as observed for wild-type protein (Weinstock *et al.*, 1981b; Kowalczykowski, 1986; Menetski & Kowalczykowski, 1987b); however, the apparent  $K_m$  (measured at the midpoint of the sigmoid curve) was 400  $\mu\text{M}$  in contrast to the value of approximately 50  $\mu\text{M}$  obtained with wild-type protein. Thus, the ATPase assays yield results that are in qualitative agreement with the etheno M13 DNA binding experiments described above.

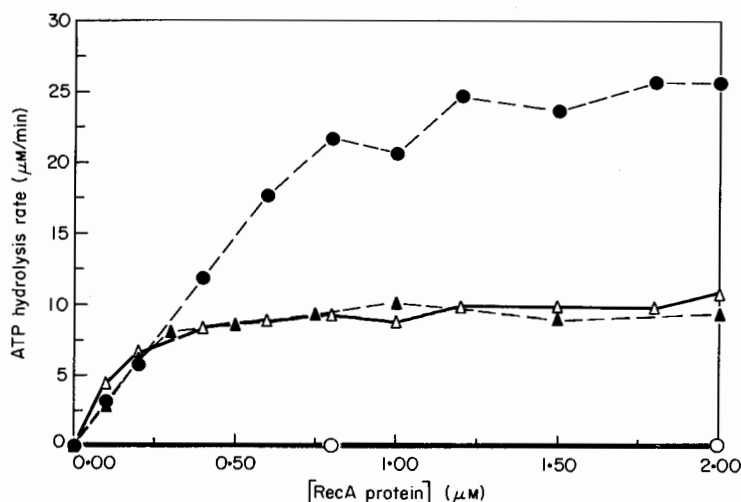
(e) *Effect of SSB protein on presynaptic complex formation*

Under DNA strand exchange conditions, SSB protein has been shown to facilitate presynaptic complex formation by removing ssDNA secondary structure which is inhibitory to complex formation (Kahn & Radding, 1984; Muniyappa *et al.*, 1984; Tsang *et al.*, 1985b; Kowalczykowski & Krupp, 1987). This stimulatory effect of SSB protein is manifest as an approximately 2 to 2.5-fold increase in the ssDNA-dependent ATP hydrolysis rate when the concentration of RecA protein is in stoichiometric excess relative to the amount required to saturate the M13 ssDNA (3 to 4 nucleotides/RecA protein monomer) (Roman & Kowalczykowski, 1986; Kowalczykowski & Krupp, 1987). In the

absence of SSB protein, the apparent stoichiometry of RecA protein binding to M13 ssDNA is six to nine nucleotides per RecA protein monomer, depending on conditions and the method used to determine the stoichiometric point (Lavery & Kowalczykowski, 1988). Figure 7 shows that, in the absence of SSB protein, both RecA proteins display similar protein titration curves for ATP hydrolysis and saturate at a similar  $V_{\text{max}}$  value. This demonstrates that both the apparent binding stoichiometry to a natural ssDNA substrate and the turnover of ATP are nearly identical for these two proteins.

In contrast to the wild-type protein, SSB protein completely inhibits the ssDNA-dependent ATPase activity of RecA142 protein (Fig. 7). This inhibition is not overcome by increasing the RecA142 protein concentration to as high as 6  $\mu\text{M}$ , increasing the ATP concentration to 8 mM, or substituting 1 mM-dATP for ATP (all not shown). Inhibition by SSB protein is observed at SSB concentrations as low as 0.15  $\mu\text{M}$  (at 1.5  $\mu\text{M}$ -RecA142 protein) and also at 100 mM-NaCl (not shown). At protein and DNA concentrations that are one-half of the concentrations employed in the DNA strand exchange experiments inhibition of RecA142 protein activity by SSB protein is still obtained (not shown). Elsewhere, we have shown that such an inhibition of ATPase activity is due to a displacement of RecA protein from the ssDNA by SSB protein (Kowalczykowski *et al.*, 1987b). Therefore, these data demonstrate that SSB protein prevents, rather than enhances, presynaptic complex formation by RecA142 protein under all conditions examined.

The presynaptic defect of RecA142 protein observed in the presence of SSB protein is, by itself, sufficient to explain both the inability of RecA142 protein to catalyze DNA heteroduplex formation (in the presence of SSB protein) *in vitro* and the defect in recombination *in vivo*. However, it does not



**Figure 7.** Effect of SSB protein on the ssDNA-dependent ATPase activity of RecA protein. ATPase assays were conducted as described in Materials and Methods, using M13 ssDNA; filled symbols represent wild-type protein and open symbols represent RecA142 protein: ( $\blacktriangle$ ,  $\triangle$ ) in the absence of SSB protein; ( $\circ$ ,  $\bullet$ ), in the presence of 0.6  $\mu\text{M}$ -SSB protein.

**Table 1**  
*DNA coaggregation and aggregation properties*

Conditions‡	% in pellet†			
	Wild-type		RecA142 protein	
	ssDNA	dsDNA	ssDNA	dsDNA
1/10	91	89	<1	10
1/10 with SSB	80	88	1	7
10/10	98	29	15	2
10/10 with SSB	99	99	13	5
1/10§	21	—	<1	—
10/10§	70	—	38	—

Coaggregation assays were carried out as described in Materials and Methods.

† The uncertainty in these values is approximately 10 percentage units.

‡ The coaggregation reaction conditions are described in terms of the  $Mg^{2+}$  concentration present during the preincubation step to the left of the slash, and the  $Mg^{2+}$  concentration during the incubation step to the right of the slash.

§ Aggregation of ssDNA was measured using the coaggregation protocol, except that dsDNA was omitted from the reaction.

explain why RecA142 protein is unable to promote both DNA strand exchange (Fig. 2) and joint molecule formation (Fig. 3) in the *absence* of SSB protein *in vitro*. Even without SSB protein, RecA142 protein (but not wild-type protein) fails to produce joint molecules, using either the elevated  $Mg^{2+}$  concentration typically employed or preincubation in 1 mM-magnesium acetate (a step that eliminates the need for SSB protein in presynapsis *in vitro*). Yet, under both of these conditions, RecA142 protein can bind to ssDNA and hydrolyze ATP, demonstrating the ability to form a functional (in ATP hydrolysis) ATP–RecA142 protein–ssDNA complex. This implies that ssDNA-dependent ATPase activity is not sufficient for DNA heteroduplex formation and that RecA142 protein must be defective in a step following presynapsis as well.

(f) *Coaggregation properties of RecA142 protein*

Two events that follow presynapsis are conjunction and homologous alignment. A biochemical property of RecA protein that is thought to play a

key role in these steps is its ability to form coaggregates, complexes of RecA protein and DNA that can be pelleted in a centrifuge (Chow & Radding, 1985; Tsang *et al.*, 1985a). Table 1 compares the DNA coaggregation and aggregation properties of the mutant and wild-type proteins. As expected, the wild-type protein can form coaggregates of non-homologous ssDNA and dsDNA under conditions that have been defined (Chow & Radding, 1985; Tsang *et al.*, 1985a); identical results are obtained if chloride anion is substituted for acetate in the buffer (not shown). RecA142 protein is striking in that it is totally defective in coaggregation. This is true regardless of whether or not SSB protein is present, and is independent of the preincubation protocol used. Using protein and DNA component concentrations identical with those in the DNA strand exchange studies yields similar results (not shown). Table 1 also shows the RecA142 protein is partially defective in aggregation of ssDNA.

The inability of RecA142 protein to promote coaggregation implies that, besides its defect in presynaptic complex formation in the presence of SSB protein, RecA142 protein also is defective in the synaptic phase of strand exchange (even in the absence of SSB protein).

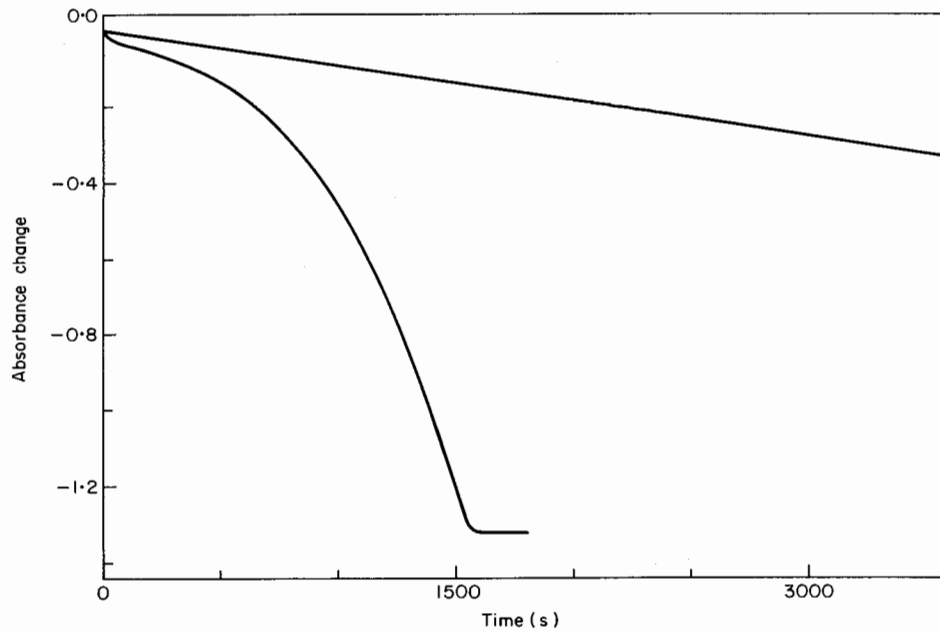
(g) *Self-association properties of RecA142 protein*

Since RecA142 protein is defective in coaggregation, its DNA-independent aggregation (i.e. self-association) properties (Cotterill & Fersht, 1983; Register & Griffith, 1985; Roman & Kowalczykowski, 1986) were also examined to see if these two different aggregation processes are related. This self-association was assayed by measuring the increase in light-scattering that accompanies association. Table 2 shows that there are no differences in the protein aggregation properties of the mutant and wild-type proteins. The  $Mg^{2+}$  concentration required for formation of protein aggregates is the same for both proteins, either in the presence or absence of ATP; the NaCl concentration required to disrupt these aggregates is also the same. In addition, the magnitude of the light-scattering increase for both proteins is identical (not shown). Thus the

**Table 2**  
*Self-association properties*

[ATP] (mM)	[Mg acetate] (mM) required for formation		[NaCl] (mM) required for disruption	
	RecA protein	RecA142 protein	RecA protein	RecA142 protein
0	9	9	30	30
1.0	3	3	—	—

From light-scattering experiments as described in Materials and Methods. Magnesium acetate was used to induce self-association and the shape of the curves in the absence of ATP were sigmoid (Cotterill & Fersht, 1983; Roman & Kowalczykowski, 1986), whereas the curves in the presence of ATP were hyperbolic with an approximately 10-fold reduced intensity (Roman & Kowalczykowski, 1986). NaCl was added to disrupt the magnesium-induced aggregates; the magnesium acetate concentration was 16 mM and the midpoint of the transition is reported. The complexes formed in the presence of ATP are stable to at least 250 mM-NaCl (Roman & Kowalczykowski, 1986).



**Figure 8.** Double-stranded DNA-dependent ATP hydrolysis activity of RecA protein. ATPase assays were carried out as described in Materials and Methods, using linear M13 dsDNA; the lower and upper traces represent  $3\ \mu\text{M}$ -wild-type RecA protein and  $3\ \mu\text{M}$ -RecA142 protein, respectively. A decrease of 1 absorbance unit corresponds to the hydrolysis of 0.16 mM-ATP.

defect of RecA142 protein in coaggregation is distinct from any effect on its DNA-independent protein self-association properties.

(h) *Double-stranded DNA-dependent ATPase activity of RecA142 protein*

In addition to its ssDNA-dependent ATPase activity, RecA protein has a dsDNA activity (Roman & Kowalczykowski, 1986; Kowalczykowski *et al.*, 1987a). Figure 8 displays the time-course of ATP hydrolysis stimulated by linear M13 dsDNA, for both wild-type and mutant proteins under DNA strand exchange conditions. The wild-type protein displays a "lag" in ATP hydrolysis that precedes attainment of the final steady-state ATP hydrolysis rate. (The lag phase is the period of time during which the ATP hydrolysis rate is lower than, and is approaching, the final steady-state value; this lag phase is less distinct for the compositionally heterogeneous M13 dsDNA than it is for the homogeneous synthetic dsDNA molecules (Kowalczykowski *et al.*, 1987).) In comparison, RecA142 protein shows a greatly reduced rate of dsDNA-dependent ATP hydrolysis, which is almost linear in time (even for as long as 2 h). Because of the non-linear profile of the wild-type reaction, the magnitude of the RecA142 protein defect is dependent on the definition employed for activity. Comparison of the ATP hydrolysis rate attained at the terminal portion of each curve shows that RecA142 protein has only 5% of the activity of the wild-type protein. Regardless of definition, the M13 dsDNA-dependent ATPase activity of the mutant protein is severely attenuated relative to that of the wild-type protein.

Since RecA142 protein is defective in an enzymatic property shown to correlate with the extent of DNA heteroduplex formation (Roman & Kowalczykowski, 1986), we expect that RecA142 protein would be defective in steps requiring opening of M13 dsDNA.

However, RecA142 protein does show a significant rate of dsDNA-dependent ATPase activity using poly(dA-dT) as a substrate rather than M13 dsDNA (not shown). The lag observed for RecA142 protein with poly(dA-dT) is similar to that obtained for the wild-type protein (Kowalczykowski *et al.*, 1987a), but the terminal rate of ATP hydrolysis is only two-thirds of the rate obtained with wild-type protein. Since RecA142 protein displays 5% of the activity of wild-type protein on M13 dsDNA, it is possible that RecA142 protein may be invading A+T-rich regions in the native DNA substrates. However, the extent of this invasion must not be sufficient to permit stable joint molecule formation (Fig. 3). Thus, there seems to exist a more quantitative relationship between the dsDNA-dependent ATPase activity and heteroduplex DNA formation; i.e. the presence of a reduced amount of the ATPase activity does not mean that DNA strand exchange will occur if given sufficient time.

#### 4. Discussion

We have characterized the biochemical properties of RecA142 protein, the product of a *recA* allele that displays a defect in genetic recombination (Clark, 1973). The RecA142 protein is unable to catalyze the exchange of DNA strands between circular single-strand and linear double-strand M13 DNA

molecules as measured by either an agarose gel assay or by an  $S_1$  nuclease assay. Further dissection of the strand exchange deficiency showed that RecA142 protein is defective in joint molecule formation. This indicates that, at the minimum, either presynaptic or synaptic complex formation is impaired. These results confirm the expectation that these *in vitro* activities of RecA protein are important to the genetic recombination process *in vivo*.

One or more of the following four biochemical properties must be responsible for the defect in the recombination activities of RecA142 protein *in vitro*. First, the ssDNA-dependent ATPase activity of RecA142 protein is completely inhibited by SSB protein, indicating that SSB protein is able to displace the mutant protein from ssDNA. Second, RecA142 protein is unable to form coaggregates of ssDNA and dsDNA. Third, the dsDNA-dependent ATPase activity of RecA142 protein is substantially attenuated relative to the activity of the wild-type protein. Fourth, the mutant protein is defective in the formation of the ATP-induced high-affinity ssDNA-binding state. The first three deficiencies correspond to defects in the presynaptic, synaptic and postsynaptic steps of the overall DNA strand exchange reaction as defined by biochemical studies *in vitro*; the fourth defect is likely to be the fundamental cause of the first and third deficiencies and, perhaps, of the second as well. Since the relevance of each of these reactions to the DNA strand exchange and heteroduplex formation activities of RecA protein is based solely on correlations between *in vitro* properties, these results also furnish biochemical evidence that any one or all of these partial reactions are important to *in vivo* function as well.

Just as significant as the finding that RecA142 protein is aberrant in the four properties listed above is the observation that RecA142 protein is *not* defective in the following properties, under *in vitro* strand exchange reaction conditions. First, RecA142 protein retains the ability to bind to ssDNA in the presence or absence of ATP or ADP (though the stability of the ATP-RecA142 protein-ssDNA complex is also more salt sensitive than the equivalent wild-type protein complex). Second, it is not defective in its ssDNA-dependent ATPase activity (though this activity is more salt sensitive than wild-type protein activity). Third, RecA142 protein has the same DNA-independent self-association properties as the wild-type protein. The first and second observations establish that, in the absence of SSB protein, RecA protein is capable of forming the protein-ssDNA complexes necessary for presynapsis. Also, since RecA142 protein can bind to ssDNA and hydrolyze ATP under the typical *in vitro* DNA strand exchange conditions, this implies that these properties, by themselves, are not responsible for the defective DNA strand exchange activity. Instead, the increased salt sensitivity of these two properties reflects an underlying *quantitative* fault in binding properties. Finally, the observation that RecA142 protein is proficient in self-association yet

defective in coaggregation implies that these properties are unrelated. Thus the above mentioned biochemical properties cannot be responsible for the *in vivo* deficiency in genetic recombination of RecA142 protein.

The relative importance of the essential biochemical properties of RecA protein (defined by the defects in the RecA142 mutant protein) to the biological mechanism of genetic recombination can be assessed by assuming that the *in vivo* process follows a mechanism similar to that determined for the *in vitro* reaction. The first step in the DNA strand exchange reaction is presynapsis. Since RecA142 protein is unable to form a presynaptic complex with ssDNA in the presence of SSB protein, this observation alone is sufficient to explain the recombination phenotype of strains carrying the *recA142* allele. Assuming that the concentration of ssDNA in the cell is limiting compared with the concentrations of RecA and SSB proteins, presynaptic complex formation will be dependent on the ability of RecA protein to compete successfully for the ssDNA binding sites. In the case of the mutant RecA142 protein, the *in vitro* studies suggest that SSB protein will displace nearly all of it from the ssDNA, inhibiting presynaptic complex formation and, hence, homologous recombination.

This observation regarding the competition between RecA142 and SSB proteins for ssDNA-binding sites also has implications for explaining the defect in SOS induction reported for this mutant allele (Roberts & Roberts, 1981; Dutreix *et al.*, 1985). RecA142 protein has less than 10% of the lambda repressor cleavage activity of wild-type RecA protein in the presence of ATP (Roberts & Roberts, 1981). We have found that RecA142 protein retains approximately 20% of the LexA repressor cleavage activity, when compared with wild-type protein in the presence of ATP (Kowalczykowski *et al.* unpublished results). However, in the presence of SSB protein, LexA repressor cleavage activity is totally abolished (Kowalczykowski *et al.*, unpublished results), whereas wild-type RecA protein activity is stimulated. Thus, under *in vivo* conditions where the concentration of ssDNA is limiting, RecA142 protein is expected to display little protease activity; but upon production of ssDNA (e.g. upon ultraviolet irradiation), the limited protease activity of RecA142 protein could be activated. These expectations are consistent with the observed physiological property associated with the *recA142* allele (Dutreix *et al.*, 1985); i.e. *recA142* shows a tenfold reduced level of spontaneous lambda prophage induction and an induced, but delayed, response in ultraviolet-damaged bacteria.

The importance of the competition between RecA and SSB proteins for limited ssDNA sites was first established using biochemical arguments (Tsang *et al.*, 1985b; Roman & Kowalczykowski, 1986; Kowalczykowski *et al.*, 1987b; Kowalczykowski & Krupp, 1987). Those studies established that a correlation exists between conditions that favor the binding of RecA protein to ssDNA over SSB protein

and conditions that allow DNA strand exchange to occur (Roman & Kowalczykowski, 1986). The observation that RecA142 protein is unable to compete successfully with SSB protein further strengthens this interpretation and also provides strong support for the presumption that this competition is important to the proper *in vivo* functioning of both genetic recombination and SOS induction.

Although the inability of RecA142 protein to compete with SSB protein is sufficient to explain its defect in genetic recombination, it is possible that additional biochemical faults could further contribute to the biological defect. *In vitro*, the requirement for SSB protein in presynapsis can be circumvented, thus making it possible to assess the relative importance of steps that occur later in DNA strand exchange. This can be accomplished either by eliminating SSB protein from the reaction or by preincubating the RecA protein-ssDNA complex in 1 mM-Mg<sup>2+</sup> prior to carrying out the reaction in 10 mM-Mg<sup>2+</sup> (Muniyappa *et al.*, 1984; Kahn & Radding, 1984). Studies with the wild-type protein have shown that this latter protocol eliminates the requirement for SSB protein by allowing RecA protein to bind to regions of secondary structure in the DNA (Muniyappa *et al.*, 1984; Kowalczykowski & Krupp, 1987). However, using either procedure with RecA142 protein failed to produce any joint DNA molecules (Fig. 3). One possible explanation is that, although RecA142 protein can bind to ssDNA and ATP, forming an active ATP hydrolytic complex, the structure of this nucleoprotein complex is incorrect and precludes subsequent pairing events. However, the other defects of RecA142 protein may also impede joint molecule formation.

Another possible reason for this inability to produce joint molecules is that RecA142 protein is defective in coaggregation as well. Coaggregation precedes joint molecule formation, and this process has been proposed to play a critical role in the DNA sequence homology search (Gonda & Radding, 1986). As pointed out (Gonda & Radding, 1986), coaggregation may be less critically important *in vivo*, where the DNA concentrations are higher, than *in vitro*. The coaggregation defect of RecA142 protein lends credence to the conclusion that coaggregation, or some unrecognized property that is manifest in the coaggregation reaction, is important to RecA protein function. However, there are conditions *in vitro* where no detectable coaggregation by wild-type RecA protein occurs, yet the rate of DNA heteroduplex formation is relatively unaffected (Kowalczykowski & Krupp, 1989). Thus, while visible coaggregate formation may reflect an important underlying property of RecA protein, it does not appear to be essential to the DNA strand exchange reaction.

The failure of RecA142 protein to form joint molecules might also be ascribed to the defect in its dsDNA-dependent ATPase activity. Unfortunately, since DNA heteroduplex formation follows both the presynaptic and synaptic steps of the DNA strand

exchange reaction, the defects in competition with SSB protein and coaggregation preclude a direct determination of the relative importance of the dsDNA-dependent ATPase activity to loss of DNA strand exchange activity in RecA142 protein.

The final significant distinction found between mutant and wild-type proteins is a quantitative difference in ssDNA binding affinity (Fig. 5). At equivalent ATP concentrations, RecA142 protein displays a lower affinity for ssDNA-binding than does the wild-type protein. In addition, the characteristic relative fluorescence increase of the mutant protein-etheno M13 DNA complex is lower than for the wild-type protein, suggesting that the complex formed has an altered conformation. Thus, RecA142 protein is unable to form the ATP binding-induced high-affinity ssDNA-binding state with the characteristics observed for the wild-type protein. This result is probably the fundamental basis for the inability of RecA142 protein to compete with SSB protein and the defect in dsDNA-dependent ATPase activity. Both of these RecA protein functions require a protein with a high affinity for ssDNA and the ATP-induced form was strongly implicated in both functions (Kowalczykowski *et al.*, 1987*a,b*; Kowalczykowski & Krupp, 1987).

In addition, the binding studies described here demonstrate that a high affinity for ssDNA may be a necessary, though not sufficient, condition for these biochemical activities. This statement is based on the fact that higher concentrations of ATP (e.g. 8 mM) induce a RecA142 protein-ssDNA complex that has the same salt titration midpoint as the wild-type complex at 1 mM-ATP, yet the effect of SSB protein is still inhibitory. However, the RFI of the mutant complex is still reduced at the higher ATP concentration. This implies either that a unique RecA protein-ssDNA complex is required to resist inhibition by SSB protein or that the steady-state kinetic properties of the mutant complex are altered sufficiently to be manifest as a lower steady-state fluorescence level in the complex. Elsewhere, we presented further phenomenological evidence for the significance of both the high ssDNA binding affinity state and the high RFI state (Menetski *et al.*, 1988).

These studies also contribute information to the mapping of structure-function relationships within the RecA protein polypeptide. The increased sensitivity to increasing salt concentration for ssDNA binding in the presence of ATP and for ssDNA-dependent ATPase activity might imply that RecA142 protein is a ssDNA binding mutant. However, in the absence of ATP or in the presence of ADP, its affinity for ssDNA is identical with that of wild-type protein. Therefore, RecA142 protein cannot be categorized simply as a ssDNA-binding mutant *per se*. Since the  $K_m$  for ATP determined from a ssDNA-dependent ATPase assay is greater than the  $K_m$  for ATP for wild-type protein, RecA142 protein might also be viewed as an ATP-binding mutant; however, this view is inconsistent with the failure to obtain wild-type levels of enzy-

matic activity at ATP concentrations high enough to obtain RecA142 protein-ssDNA complexes with a stability comparable to that of wild-type protein. These apparently incompatible ideas can be explained by the fact that recA protein exists in at least two different ssDNA binding affinity states (Menetski *et al.*, 1988): the low-affinity form found in the presence of ADP and the high-affinity form found in the presence of ATP. Both of these forms are characterized by representative affinities for ssDNA and nucleotide cofactors, and by RFI values. Interconversion between these forms is regulated by ATP (or ATP- $\gamma$ -S) binding and ATP hydrolysis (Menetski & Kowalczykowski, 1985). Therefore, RecA142 protein is actually defective in the ATP-dependent induction of the high-affinity ssDNA binding state that is normally attained by the wild-type protein. Since the affinities for ATP and ssDNA are thermodynamically linked, a reduction in the DNA binding affinity of the high-affinity state of RecA protein results in a reduction in ATP affinity as well; this would explain the higher  $K_m$  for ATP observed with RecA142 protein. Also the RFI value for RecA142 protein is always lower than that of wild-type protein, even though a high affinity for etheno M13 ssDNA is achieved. Thus, the most likely physical categorization of the RecA142 protein is that the structure of the high-affinity form of RecA142 protein is somehow altered and/or the steady-state rate of interconversion between the low- and high-affinity forms is adversely affected. The site of the mutation lies at residue 225 and is a substitution of a valine for an isoleucine residue (Dutreix *et al.*, unpublished results). Unfortunately, straightforward assignment of RecA protein functional domains will suffer from the complexity mentioned above and points out some of the subtleties involved in proper assignment of structure-function relationships for RecA protein. Nevertheless, future detailed studies of this and other mutant RecA proteins will be useful for discerning aspects of biochemical mechanism, biological function, and structure-function relationships.

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### References

- Beattie, K. L., Wiegand, R. C. & Radding, C. M. (1977). *J. Mol. Biol.* **116**, 783-803.
- Bryant, F. R. & Lehman, I. R. (1986). *J. Biol. Chem.* **261**, 12988-12993.
- Bryant, F. R., Taylor, A. R. & Lehman, I. R. (1985). *J. Biol. Chem.* **260**, 1196-1202.
- Cassuto, E., West, S. C., Mursalim, J., Conlon, S. & Howard-Flanders, P. (1980). *Proc. Nat. Acad. Sci., U.S.A.* **77**, 3962-3966.
- Chow, S. A. & Radding, C. M. (1985). *Proc. Nat. Acad. Sci., U.S.A.* **82**, 5646-5650.
- Chrysogelos, S., Register II, J. C. & Griffith, J. (1983). *J. Biol. Chem.* **258**, 12624-12631.
- Clark, A. J. (1973). *Annu. Rev. Genet.* **7**, 67-86.
- Cotterill, S. M. & Fersht, A. R. (1983). *Biochemistry*, **22**, 3525-3531.
- Cox, M. M. & Lehman, I. R. (1981a). *Proc. Nat. Acad. Sci., U.S.A.* **78**, 3433-3437.
- Cox, M. M. & Lehman, I. R. (1981b). *Proc. Nat. Acad. Sci., U.S.A.* **78**, 6018-6022.
- Cox, M. M. & Lehman, I. R. (1987). *Annu. Rev. Biochem.* **56**, 229-262.
- Cunningham, R. P., DasGupta, C., Shibata, T. & Radding, C. M. (1980). *Cell*, **20**, 223-235.
- Dunn, K., Chrysogelos, S. & Griffith, J. (1982). *Cell*, **28**, 757-765.
- Dutreix, M., Bailone, A. & Devoret, R. (1985). *J. Bacteriol.* **161**, 1080-1085.
- Glassberg, J., Meyer, R. R. & Kornberg, A. (1979). *J. Bacteriol.* **140**, 14-19.
- Gonda, D. K. & Radding, C. M. (1986). *J. Biol. Chem.* **261**, 13087-13096.
- Griffith, J. & Shores, C. G. (1985). *Biochemistry*, **24**, 158-162.
- Kahn, R. & Radding, C. M. (1984). *J. Biol. Chem.* **259**, 7495-7503.
- Kahn, R., Cunningham, R. P., DasGupta, C. & Radding, C. M. (1981). *Proc. Nat. Acad. Sci., U.S.A.* **78**, 4786-4790.
- Kowalczykowski, S. C. (1986). *Biochemistry*, **25**, 5872-5881.
- Kowalczykowski, S. C. (1987). *Trends Biochem. Sci.* **12**, 141-145.
- Kowalczykowski, S. C. & Krupp, R. A. (1987). *J. Mol. Biol.* **193**, 97-113.
- Kowalczykowski, S. C. & Krupp, R. A. (1989). *J. Mol. Biol.* **207**, 735-747.
- Kowalczykowski, S. C., Clow, J. & Krupp, R. A. (1987a). *Proc. Nat. Acad. Sci., U.S.A.* **84**, 3127-3131.
- Kowalczykowski, S. C., Clow, J. C., Somani, R. & Varghese, A. (1987b). *J. Mol. Biol.* **193**, 81-95.
- Kreuzer, K. N. & Jongeneel, C. V. (1983). *Methods Enzymol.* **100**, 144-160.
- Lavery, P. & Kowalczykowski, S. C. (1988). *J. Mol. Biol.* **203**, 861-874.
- Lieberman, H. B. & Witkin, E. M. (1981). *Mol. Gen. Genet.* **183**, 348-355.
- McEntee, K., Weinstock, G. M. & Lehman, I. R. (1979). *Proc. Nat. Acad. Sci., U.S.A.* **76**, 2615-2619.
- McEntee, K., Weinstock, G. M. & Lehman, I. R. (1981). *J. Biol. Chem.* **256**, 8835-8844.
- Menetski, J. P. (1988). Ph.D. thesis, Northwestern University.
- Menetski, J. P. & Kowalczykowski, S. C. (1985). *J. Mol. Biol.* **181**, 281-295.
- Menetski, J. P. & Kowalczykowski, S. C. (1987a). *J. Biol. Chem.* **262**, 2085-2092.
- Menetski, J. P. & Kowalczykowski, S. C. (1987b). *J. Biol. Chem.* **262**, 2093-2100.
- Menetski, J. P., Varghese, A. & Kowalczykowski, S. C. (1988). *Biochemistry*, **27**, 1205-1212.
- Messing, J. (1983). *Methods Enzymol.* **101**, 20-78.
- Morriscal, S. W. & Cox, M. M. (1985). *Biochemistry*, **24**, 760-767.
- Morriscal, S. W., Lee, J. & Cox, M. M. (1986). *Biochemistry*, **25**, 1482-1494.

- Muniyappa, K., Shaner, S. L., Tsang, S. S. & Radding, C. M. (1984). *Proc. Nat. Acad. Sci., U.S.A.* **81**, 2757-2761.
- Ogawa, T., Wabiko, H., Tsurimoto, T., Horii, T., Masukata, H. & Ogawa, H. (1978). *Cold Spring Harbor Symp. Quant. Biol.* **43**, 909-916.
- Pugh, B. F. & Cox, M. M. (1987). *J. Biol. Chem.* **262**, 1326-1336.
- Radding, C. M. (1982). *Annu. Rev. Genet.* **16**, 405-437.
- Register, J. C. & Griffith, J. (1985). *Mol. Gen. Genet.* **199**, 415-420.
- Roberts, J. W. & Roberts, C. W. (1981). *Nature (London)*, **290**, 422-423.
- Roman, L. J. & Kowalczykowski, S. C. (1986). *Biochemistry*, **25**, 7375-7385.
- Rusche, J. R., Konigsberg, W. & Howard-Flanders, P. (1985). *J. Biol. Chem.* **260**, 949-955.
- Ruyechan, W. T. & Wetmur, J. G. (1975). *Biochemistry*, **14**, 5529-5533.
- Schutte, B. C. & Cox, M. M. (1987). *Biochemistry*, **26**, 5616-5625.
- Shibata, T., DasGupta, C., Cunningham, R. P. & Radding, C. M. (1979). *Proc. Nat. Acad. Sci., U.S.A.* **76**, 1638-1642.
- Silver, M. S. & Fersht, A. R. (1982). *Biochemistry*, **21**, 6066-6072.
- Silver, M. S. & Fersht, A. R. (1983). *Biochemistry*, **22**, 2860-2866.
- Stasiak, A., DiCapua, E. & Koller, T. (1981). *J. Mol. Biol.* **151**, 557-564.
- Tsang, S. S., Chow, S. A. & Radding, C. M. (1985a). *Biochemistry*, **24**, 3226-3232.
- Tsang, S. S., Muniyappa, K., Azhderian, E., Gonda, D. K., Radding, C. M., Flory, J. & Chase, J. W. (1985b). *J. Mol. Biol.* **185**, 295-309.
- Uhlin, B. E. & Clark, A. J. (1981). *J. Bacteriol.* **148**, 386-390.
- Uhlin, B. E., Schweikart, V. & Clark, A. J. (1983). *Gene*, **22**, 255-265.
- Weinstock, G. M., McEntee, K. & Lehman, I. R. (1979). *Proc. Nat. Acad. Sci., U.S.A.* **76**, 126-130.
- Weinstock, G. M., McEntee, K. & Lehman, I. R. (1981a). *J. Biol. Chem.* **256**, 8829-8834.
- Weinstock, G. M., McEntee, K. & Lehman, I. R. (1981b). *J. Biol. Chem.* **256**, 8845-8849.
- Weinstock, G. M., McEntee, K. & Lehman, I. R. (1981c). *J. Biol. Chem.* **256**, 8856-8858.
- West, S. C., Cassuto, E., Mursalim, J. & Howard-Flanders, P. (1980). *Proc. Nat. Acad. Sci., U.S.A.* **77**, 2569-2573.
- West, S. C., Cassuto, E. & Howard-Flanders, P. (1981a). *Proc. Nat. Acad. Sci., U.S.A.* **78**, 2100-2104.
- West, S. C., Cassuto, E. & Howard-Flanders, P. (1981b). *Proc. Nat. Acad. Sci., U.S.A.* **78**, 6149-6153.
- Whittier, R. F. & Chase, J. W. (1983). *Mol. Gen. Genet.* **190**, 101-111.

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