

The roles of RecBCD, Ssb and RecA proteins in the formation of heteroduplexes from linear-duplex DNA in vitro

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Summary. The formation of heteroduplexes from linear duplex DNA, where one molecule possesses a DNA double-strand break, was assayed by agarose gel electrophoresis. Using unlabeled whole-length linear duplex DNA and ³H-labeled half-length linear duplex DNA (obtained from plasmid pACYC184), the appearance of ³H-labeled DNA that migrated as whole-length linear DNA was taken as evidence for formation of heteroduplex DNA. When the DNA mixtures were incubated with RecA, RecBCD, or Ssb proteins, or any double or triple combination of these proteins under a variety of reaction conditions, no heteroduplex DNA was detected. However, heteroduplex DNA was detected when the DNA mixtures were first incubated briefly with the RecBCD and Ssb proteins under reaction conditions that allow unwinding to proceed, and then the MgCl₂ concentration was raised such that renaturation could proceed. The inclusion of the RecBCD and Ssb proteins was sufficient to catalyze the slow formation of heteroduplex DNA, but the presence of RecA protein greatly increased the kinetics. The roles of the RecBCD, Ssb and RecA proteins in heteroduplex formation in vitro are discussed.

Key words: Recombination – DNA repair – RecBCD – Ssb – RecA

Introduction

The RecA and RecBCD proteins are the major components of the RecBCD pathway of recombination, and their enzymatic functions have been studied extensively in vitro. The RecA protein possesses protease and DNA-dependent ATPase activities, and is known to bind to single-stranded DNA and to promote homologous pairing and strand exchanges between several different types of DNA substrates, provided that one of the DNA substrates is at least partially single stranded (see Radding 1982; Cox and Lehman 1987 for reviews). However, *RecA-mediated pairing does not take place between homologous DNA duplexes that do not contain single-stranded regions* (West et al. 1981; Radding 1982; Cox and Lehman 1987). The RecBCD enzyme (Exo V) possesses ATP-dependent exonuclease, endonuclease and helicase activities (Taylor and Smith 1985; Amundsen et al. 1986). The helicase activity of the RecBCD enzyme has

been shown to unwind only linear duplex DNA with a nearly blunt-ended terminus whose 5' and 3' ends are offset by no more than about 25 nucleotides; molecules with longer single-stranded tails and circular duplex molecules with nicks or gaps are not detectably unwound (Taylor and Smith 1985). It has been suggested that the unwinding activity of the RecBCD enzyme can provide the single strandedness needed for RecA-mediated recombination between linear duplex DNA that does not contain single-stranded regions (Smith and Stahl 1985), and for the repair of DNA double-strand breaks (Wang and Smith 1985). In this work, we examine whether the inclusion of the RecBCD enzyme in a recombination assay can facilitate the formation of heteroduplex DNA from linear duplex DNA in vitro.

Materials and methods

Materials. RecA protein was purchased from United States Biochemical. Ssb protein was obtained from Worthington. The RecBCD enzyme (Fraction IV) (Taylor and Smith 1985) was kindly supplied by Dr. A. Taylor. The definition of RecBCD enzyme activity units follows that of Eichler and Lehman (1977). Nuclease S1 was purchased from Sigma; the restriction endonucleases *EcoRI* and *SalI* were purchased from Bethesda Research Laboratories, and were used according to the manufacturer's suggestions. Plasmid DNA was obtained from pACYC184 (Chang and Cohen 1978) grown in *Escherichia coli recA13 thyA* (AB2487), as described by West et al. (1983). ³H-labeled pACYC184 DNA was prepared as follows: cells were grown at 37° C in supplemented minimal medium (Wang and Smith 1982) containing 12.5 µg/ml chloramphenicol and 7.5 µg/ml tetracycline to an OD₆₅₀ of 0.4; the plasmid DNA was amplified by the addition of 300 µg/ml spectinomycin, and after 1 h [³H]thymidine (80 Ci/mmol) at 2.5 µCi/ml was added and the cells were incubated overnight. The cells were lysed with sodium dodecyl sulfate (SDS), and the plasmid DNA was purified by equilibrium banding in cesium chloride plus ethidium bromide (Maniatis et al. 1982). Whole-length linear DNA was prepared by digestion of the covalently closed circular pACYC184 DNA with the restriction enzyme *EcoRI*. Half-length linear DNA fragments were prepared by digestion of the whole-length linear DNA with *SalI* (Fig. 1). Heat-denatured ³H-labeled DNA was prepared by boiling linear duplex DNA in 10 mM Tris, pH 8, 1 mM EDTA for 5 min followed by quick chilling in an ice bath. The concentration of DNA was determined by measuring

the absorbance of DNA solutions at 260 nm, and was expressed as molarity of nucleotide equivalents.

Unwinding reactions. Agarose gel electrophoresis of DNA was used to detect the unwinding activity of the RecBCD enzyme. A typical reaction mixture contained in 15 μ l of reaction buffer [20 mM Tris, pH 7.5, 15 mM EDTA, 1 mM dithiothreitol, 7.5 mM MgCl₂, 10 mM ATP, 100 μ g/ml bovine serum albumin (BSA)]: 15 to 30 μ M linear duplex DNA, 1 to 2.5 μ M Ssb, and 3 to 12 units of the RecBCD enzyme. The mixture was incubated at 37° C, and the reaction was terminated by the addition of SDS to a final concentration of 1%. The DNA was fractionated by electrophoresis in a 1% agarose gel using Tris-borate buffer, pH 8 (Maniatis et al. 1982). The unwinding activity of the enzyme was demonstrated by the detection of single-stranded DNA on the agarose gel.

Renaturation assays. Two assays were employed in the reassociation studies: (1) agarose gel electrophoresis, and (2) S1 nuclease digestion (Bryant and Lehman 1985). Heat-denatured ³H-labeled DNA was incubated with 1 μ M Ssb or 5 μ M RecA protein in reaction buffer at 37° C. The reaction was terminated by adding SDS to a final concentration of 1%. The extent of renaturation was determined qualitatively from photographs of the agarose gels that had been stained with ethidium bromide.

For a quantitative determination, the S1 nuclease assay was used. The reaction mixture above plus SDS (12.5 μ l) was diluted with 300 μ l of digestion buffer (150 mM NaCl, 50 mM sodium acetate, pH 4.6, 1 mM zinc acetate) to which 6 μ g of heat-denatured calf thymus DNA and 40 units of S1 nuclease were added. After incubation at 37° C for 30 min, 30 μ g of heat-denatured calf thymus DNA was added and mixed well before adding 1 vol. of ice-cold 10% trichloroacetic acid. After sitting on ice for 45 min or longer, the mixtures were filtered on Whatman GF/C filters. The filters were washed with cold 10% trichloroacetic acid (three times with 1 ml) and 95% ethanol (twice with 1 ml) and then dried, and the radioactivity was measured. Under these conditions there was no detectable degradation of linear-duplex DNA, while greater than 95% of the heat-denatured DNA was degraded. Therefore, the amount of DNA that became double-stranded during reassociation was estimated from the amount of ³H-radioactivity that became resistant to S1 nuclease digestion.

Formation of heteroduplexes from linear duplex DNA. To detect whether the formation of heteroduplexes from linear duplex DNA takes place, and whether it can bypass a DNA double-strand break, unlabeled whole-length linear duplex DNA was mixed with ³H-labeled half-length linear duplex DNA fragments in the presence of RecA, RecBCD and Ssb proteins, and the appearance of ³H-labeled DNA that migrated as whole-length heteroduplex DNA (see Fig. 1) on agarose gels was examined. The details of particular experimental conditions are described in Results.

Autoradiography. The agarose gel was impregnated with fluor (En³Hance; New England Nuclear) according to the specifications of the manufacturer and dried. Kodak X-Omat AR Xar-2 film (Eastman Kodak) was exposed to the dried gel at -80° C and developed according to film specifications.

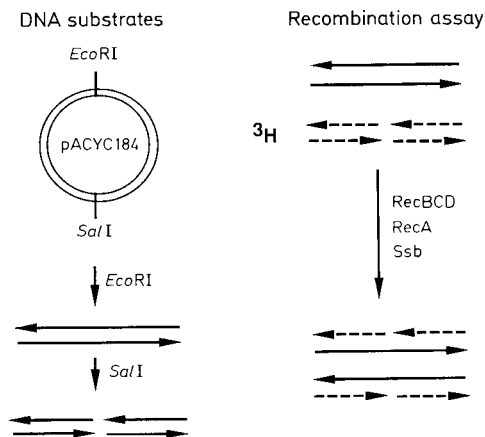


Fig. 1. DNA substrates and assay for in vitro recombination. Whole-length linear duplex DNA was produced by digesting plasmid pACYC184 DNA with *Eco*RI. Half-length linear duplex DNA was produced by digesting whole-length linear duplex DNA with *Sal*I. In the assay, unlabeled whole-length linear duplex DNA was reacted with ³H-labeled half-length linear duplex DNA in the presence of the RecBCD, RecA, and Ssb proteins. The formation of heteroduplexes from linear-duplex DNA resulted in the formation of ³H-labeled DNA that migrated as whole-length linear duplex DNA in agarose gel electrophoresis

Results

Preliminary experiments

The recent finding that the RecD polypeptide constitutes the essential third subunit of the RecBCD enzyme (Amundsen et al. 1986), and the fact that *recD* mutants are devoid of RecBCD nuclease activity, but are recombination proficient and UV radiation resistant (Chaudhury and Smith 1984; Amundsen et al. 1986), suggest that the helicase activity of the RecBCD enzyme is essential for recombination and repair. The reaction conditions used for the studies on the unwinding activity of the RecBCD enzyme by Rosamond et al. (1979) and by Taylor and Smith (1985) did not completely inhibit the endonucleolytic activity of RecBCD against linear duplex DNA. Under these conditions, the unwinding of linear duplex DNA to single-stranded DNA in the presence of Ssb protein was observed, but there was a smear of DNA bands that migrated faster than intact single-stranded DNA on an agarose gel (data not shown). Furthermore, when the MgCl₂ concentration was increased to 5 mM or higher, which is necessary for the several activities of RecA protein to proceed, considerable degradation of DNA was observed, which rendered our assay for heteroduplex formation impossible. When we employed EDTA as an inhibitor of the nuclease activities of the RecBCD enzyme, we observed that the presence of EDTA at 15 mM inhibited both the exonucleolytic and endonucleolytic activities of the RecBCD enzyme over a wide range of MgCl₂ concentrations (0–40 mM) (data not shown). Therefore, we examined whether the unwinding activity of the RecBCD enzyme and the annealing activity of the RecA protein could still proceed in the presence of 15 mM EDTA (see below).

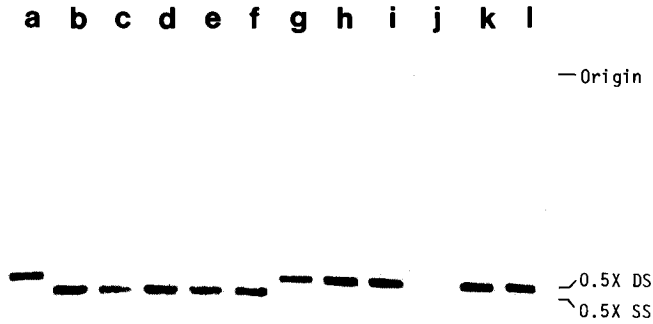


Fig. 2. Unwinding of linear duplex DNA by the RecBCD enzyme in the presence of Ssb protein. Autoradiograph of agarose gel showing products of the unwinding reaction in the reaction buffer with $30 \mu\text{M}$ ^3H -labeled half-length linear duplex DNA, $1.5 \mu\text{M}$ Ssb, and 12 units of RecBCD enzyme. Lanes a–c, 0, 5 and 10 min incubation at 37°C ; lanes d–l, 30 min; lane d, standard reaction mix; lanes e, f, 6 and 3 units of RecBCD enzyme; lanes g–k omission of Ssb protein (lane g), RecBCD enzyme (lane h), MgCl_2 (lane i), EDTA (lane j), ATP (lane k), lane l, MgCl_2 was present at 37.5 mM . The position of half-length double-stranded ($0.5 \times \text{DS}$) and half-length single-stranded ($0.5 \times \text{SS}$) DNA is indicated

Unwinding of linear duplex DNA by the RecBCD enzyme in the presence of EDTA

In a typical reaction mixture that contained 20 mM Tris, $\text{pH } 7.5$, 7.5 mM MgCl_2 , 15 mM EDTA, 10 mM ATP, 1 mM dithiothreitol, $100 \mu\text{g/ml}$ BSA and Ssb protein, linear duplex DNA was readily unwound by the RecBCD enzyme (Fig. 2). Denaturation of linear duplex DNA to form single-stranded DNA was not observed if one omitted MgCl_2 , or the RecBCD enzyme, or ATP, or Ssb protein from the reaction mixture. Omitting EDTA from the reaction mixture resulted in DNA degradation, and no DNA was detectable after incubation (Fig. 2, lane j). Interestingly, the unwinding of linear duplex DNA to yield single-stranded DNA was not observed when the MgCl_2 was present at higher concentrations (e.g., 37.5 mM) (Fig. 2, lane l).

Effect of RecA and Ssb proteins on the reassociation of DNA

Under our reaction conditions for unwinding, neither the RecA protein nor the Ssb protein could promote the reassociation of complementary DNA above the slow spontaneous reassociation rate (Fig. 3A). In fact, the Ssb protein bound tightly to single-stranded DNA and inhibited the spontaneous reassociation of DNA. However, when the MgCl_2 concentration was raised to 20 mM or higher, the RecA protein was capable of promoting renaturation (Fig. 3B). The Ssb protein only slightly enhanced the rate of reassociation above that of spontaneous reassociation (Fig. 3B).

The products of Ssb-promoted renaturation migrated to the same position as linear duplex DNA on an agarose gel (Fig. 4, lanes c and f), while in the RecA-promoted renaturation only a small portion of the DNA migrated to a position that corresponds to linear duplex DNA, and the majority of the DNA stayed at the top of the gel after electrophoresis (Fig. 5, lanes b and e). In the absence of ATP, both the RecA and Ssb proteins could promote renaturation in the presence of 40 mM MgCl_2 (Fig. 5A). One

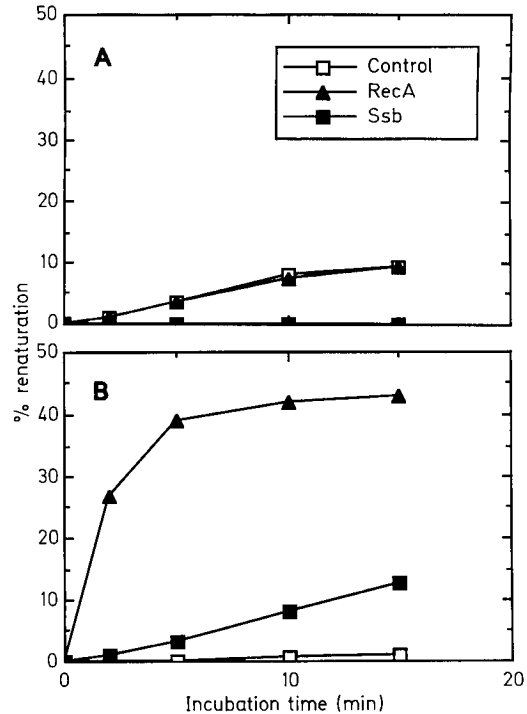


Fig. 3A and B. Renaturation of heat-denatured DNA by the RecA and Ssb proteins. ^3H -labeled whole-length linear duplex DNA was heat denatured and incubated with $5 \mu\text{M}$ RecA protein or $1 \mu\text{M}$ Ssb protein at 37°C . The extent of renaturation was determined from the amount of the ^3H -radioactivity that became resistant to S1 nuclease digestion, as described in Materials and methods. The reaction buffer contained **A** 7.5 mM MgCl_2 and **B** 40 mM MgCl_2 . Symbols: □, no enzyme added; ■, with Ssb protein; and ▲, with RecA protein

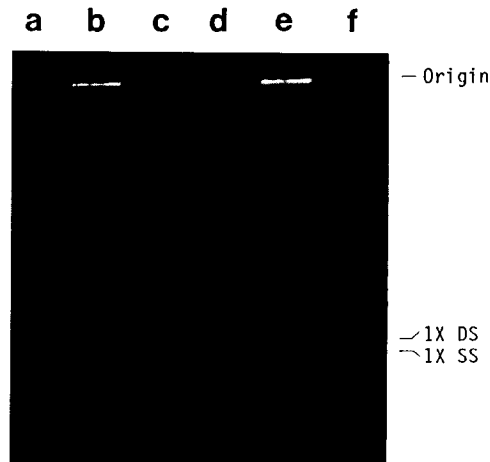


Fig. 4. Renaturation products promoted by the RecA and Ssb proteins. Whole-length linear duplex DNA was heat natured and incubated with $5 \mu\text{M}$ RecA protein or $1 \mu\text{M}$ Ssb protein for 15 min at 37°C . The reactions were terminated by the addition of SDS, and the DNA was separated by electrophoresis in an agarose gel. After staining with ethidium bromide, the gel was photographed with a Polaroid MP-4 camera. Lanes a–c, the reaction buffer contained 30 mM MgCl_2 ; lanes d–f, 40 mM MgCl_2 . Lanes a and d, no enzyme added; lanes b and e, with RecA protein; lanes c and f, with Ssb protein. Whole-length double-stranded ($1 \times \text{DS}$) and whole-length single-stranded ($1 \times \text{SS}$) bands are labeled

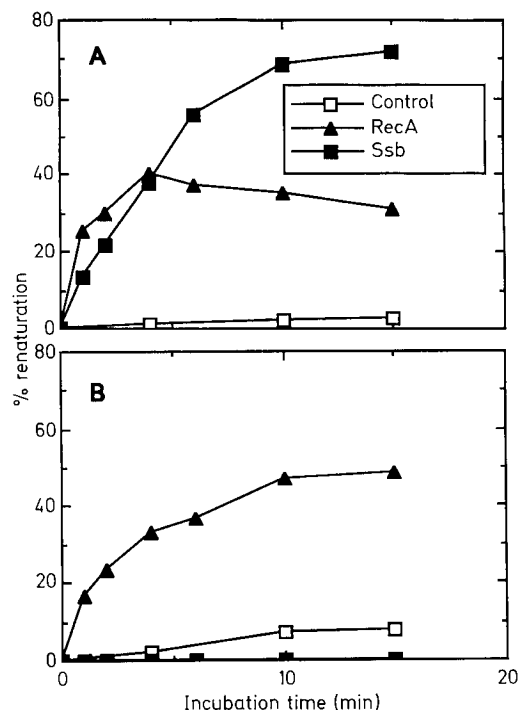


Fig. 5A and B. ATP-independent renaturation of heat-denatured DNA by the RecA and Ssb proteins. ³H-labeled whole-length linear duplex DNA was heat denatured and incubated with 5 μM RecA protein or 1 μM Ssb protein at 37° C. The extent of renaturation was determined from the amount of the ³H-radioactivity that became resistant to S1-nuclease digestion, as described in Materials and methods. **A** Reaction buffer with 40 mM MgCl₂ but lacking ATP; **B** as **A** except that EDTA was also omitted. Symbols: □, no enzyme added; ■, with Ssb protein; and ▲, with RecA protein

major difference between the ATP-independent renaturation promoted by the RecA protein and by the Ssb protein is the requirement for EDTA in the Ssb-promoted renaturation. In the presence of 40 mM MgCl₂ alone (i.e., no EDTA), the Ssb protein could not promote the renaturation of complementary single-stranded DNA, while the RecA protein could (Fig. 5B, and Bryant and Lehman 1985).

The formation of heteroduplexes from linear duplex DNA

In a 15 μl reaction mixture with 7.5 mM MgCl₂ that contained unlabeled whole-length linear duplex DNA and ³H-labeled half-length linear duplex DNA fragments, the addition of 6 units RecBCD, 1 μM Ssb and 5 μM RecA produced single-stranded DNA after incubation at 37° C (Fig. 6, lanes a–e); but no heteroduplex DNA was detected. In a reaction mixture that contained 40 mM MgCl₂ instead, no single-stranded DNA and no heteroduplex DNA was detected (data not shown). When the reaction mixture that contained 7.5 mM MgCl₂ was incubated at 37° C for 10 min in the presence of Ssb and RecBCD, and then the MgCl₂ concentration was raised to 27.5 or 37.5 mM together with the addition of RecA protein and incubated for an additional 10 min, ³H-labeled DNA that migrated as whole-length linear duplex DNA, and DNA aggregates that did not migrate into the gel were observed (Fig. 6, lanes h and i).

The kinetics of the appearance of heteroduplex DNA in the absence and presence of RecA protein are shown in Fig. 7. In the presence of RecA protein, the appearance of heteroduplex DNA that migrated as whole-length linear duplex DNA could be detected shortly after the addition of RecA protein and MgCl₂, but the major end products after longer incubation times were DNA aggregates (Fig. 7,

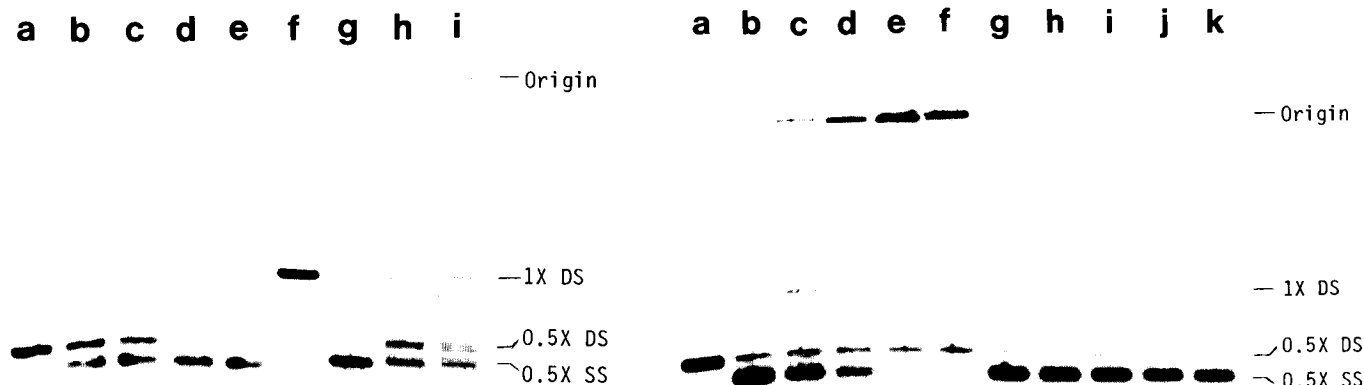


Fig. 6. The formation of heteroduplex DNA from linear duplex DNA. Reaction buffer with 7.5 mM MgCl₂, 15 μM unlabeled whole-length linear duplex DNA (1 × DS), and 15 μM ³H-labeled half-length linear duplex DNA (0.5 × DS). Lanes a–e, incubation at 37° C in the presence of the RecBCD, RecA and Ssb proteins for 0, 5, 10, 30 and 60 min, respectively; Lane f, ³H-labeled whole-length linear duplex DNA marker; lane g, incubation at 37° C in the presence of RecBCD and Ssb proteins for 10 min; lanes h and i, same as lane g except that after 10 min of incubation the MgCl₂ concentration was raised to 27.5 mM (lane h) and 37.5 mM (lane i) together with the addition of RecA protein, and the incubation was continued for an additional 10 min at 37° C

Fig. 7. Kinetics of formation of heteroduplex DNA. Reaction mixture as for Fig. 6. The reaction mixtures were first incubated at 37° C in the presence of the RecBCD and Ssb proteins for 0 min (lane a) or 10 min (lanes b–k). After incubation for 10 min, the MgCl₂ concentration was raised to 37.5 mM (lanes b–j), and RecA protein was added to the samples in lanes b–f. Incubation was continued for 0 min (lanes b, g), 5 min (lanes c, h), 10 min (lanes d, i), 30 min (lanes e, j) and 60 min (lane f). In lane k, only the RecA protein was added (i.e., the MgCl₂ concentration was not raised to 37.5 mM) and the reaction mixture was incubated for an additional 30 min. 1 × DS, 0.5 × DS, and 0.5 × SS as in Figs. 2 and 4

lanes c–f). A much slower appearance of heteroduplex DNA was observed if the RecA protein was absent (Fig. 7, lanes g–j). In this latter case, the heteroduplex DNA that was detected migrated as whole-length linear duplex DNA (Fig. 7, lane j), and no DNA aggregates were detected. Elimination of RecBCD or Ssb protein from the reaction mixture abolished the formation of unwound single-stranded DNA, and no heteroduplex DNA was detected (data not shown).

Discussion

Although earlier genetic data have indicated a major role of the *recA* (Clark and Margulies, 1965), *recB* (Howard-Flanders and Theriot 1966) and *recC* (Emmerson and Howard-Flanders 1967) gene products in recombination, to our knowledge no author has yet reported the inclusion of the RecBCD enzyme in RecA-mediated recombination studies in vitro. The major obstacle to including the RecBCD enzyme in an in vitro recombination assay is its nuclease activities, which can degrade DNA substrates in the reaction mixture. The inhibition of the nuclease activities of the RecBCD enzyme in our in vitro studies is warranted since it is known that in vivo if the nuclease activity of the RecBCD enzyme is not controlled, as in a *recA* strain, then “reckless” DNA degradation occurs after cells are irradiated (Howard-Flanders and Boyce 1966). This degradation is largely *recBC* dependent (Youngs and Bernstein 1973).

In this work, we have employed EDTA at 15 mM to inhibit both the exonucleolytic and endonucleolytic activities of the RecBCD enzyme over a wide range of MgCl₂ concentrations (0–40 mM) (data not shown). The absence of nuclease activity at higher MgCl₂ concentrations (e.g., 20–40 mM) suggests that the inhibitory effect of EDTA cannot simply be due to its chelation of divalent Mg⁺⁺ cations, rather it may chelate other rare metal ions that are needed for the nuclease activity of the RecBCD enzyme, or it may bind to and modify the active sites of the nucleases.

Three major differences were noted between RecA-promoted and Ssb-promoted renaturation: one is the requirement for EDTA in Ssb-promoted renaturation (Fig. 5); the second is the inhibitory effect of ATP on Ssb-promoted renaturation (compare Fig. 3B versus Fig. 5A) in contrast to the stimulating effect on RecA-promoted renaturation (Bryant and Lehman 1985); and the third difference is the nature of the products of the renaturation reaction (i.e., the presence or absence of DNA aggregates; Fig. 4). The observation that the Ssb protein can catalyze the renaturation of complementary single-stranded DNA was unexpected. However, this is consistent with the early work by Christiansen and Baldwin (1977) on the “helix destabilizing protein”, now known as Ssb. These authors found that the presence of polyamines, such as spermidine or spermine, were required for the reannealing activity of Ssb. Our observation that EDTA was required for this reaction suggests that the diamine structure of EDTA may be important in catalyzing this renaturation reaction.

The products of the Ssb-promoted renaturation migrated to the same position as linear duplex DNA on an agarose gel, but only a small portion of the products of the RecA-promoted reaction migrated to this position. The majority of the DNA stayed at the top of the agarose gel after electrophoresis (Fig. 5, lanes b and e), and may be

in the form of complex aggregates similar to those observed in the ATP-dependent renaturation catalyzed by RecA (Weinstock et al. 1979).

It is not known if the reannealing activity of the Ssb protein functions in vivo. However, it is worth noting that an *ssb-113* mutation produces a big deficiency in the post-replication repair of DNA daughter-strand gaps in UV-irradiated cells, and that the DNA double-strand breaks that are formed in UV-irradiated *uvrB ssb-113* cells are not repaired (Wang and Smith 1982). An increased affinity of Ssb-113 protein for single-stranded DNA has been noted (see Chase and Williams 1986), but no defect was detected in the activity of the Ssb-113 protein in facilitating the RecA-promoted pairing and strand exchange reactions in vitro (Egner et al. 1987). It would be of interest to test if the Ssb-113 protein is deficient in promoting the renaturation of DNA in vitro, which might account for its biological effect in vivo.

When the formation of heteroduplexes from linear duplex DNA was assayed at a low MgCl₂ concentration (7.5 mM), where the unwinding of duplex DNA could be detected but the Ssb-promoted and RecA-promoted reannealing was absent, no heteroduplex DNA was formed (Fig. 6, lanes a–e). Similarly, when the assay for heteroduplex formation was performed at a high concentration of MgCl₂ (e.g., 37.5 mM) where the RecA-promoted renaturation could proceed, no heteroduplex DNA was detected (data not shown). Under these latter reaction conditions, the linear duplex DNA did not appear to be unwound at all. Presumably, either the helicase activity of the RecBCD enzyme did not function under these conditions or the unwound single-stranded DNA simply renatured. However, when the reaction mixture that contained 7.5 mM MgCl₂ was first incubated in the presence of RecBCD and Ssb proteins at 37° C for 10 min and then the MgCl₂ concentration was raised to 37.5 mM together with the addition of RecA protein, ³H-DNA was detected that migrated both to the positions of whole-length linear duplex DNA and of DNA aggregates (Fig. 6, lanes h and i; Fig. 7, lanes c–f). Elimination of RecBCD or Ssb from the reaction mixture abolished the formation of heteroduplex DNA (data not shown), but the omission of RecA only slowed down the formation of heteroduplex DNA (Fig. 7, lanes g–j). The detection of heteroduplex DNA in the absence of RecA protein (Fig. 7, lane j) suggests that under these conditions the Ssb protein either promoted slow homologous pairing, or it no longer prevented spontaneous reassociation.

In summary, the formation of heteroduplex DNA from linear duplex DNA in vitro can be achieved enzymatically. In the present work, the RecBCD enzyme was provided to unwind the duplex DNA, and in the presence of Ssb protein, the unwound single-stranded DNA was prevented from reassociation. When the reaction conditions were then changed to favor reassociation, heteroduplex DNA was formed slowly in the absence of RecA protein, but the presence of RecA protein greatly increased the kinetics of heteroduplex formation.

Acknowledgements. We are grateful to Dr. Andrew Taylor for supplying the RecBCD enzyme, to Dr. Aziz Sancar for supplying the RecA protein that was used in our pilot experiments, and to Drs. Neil J. Sargentini and Gerald R. Smith for their helpful comments on the manuscript. This work was supported by Public Health Service research grant CA-06437, awarded by the National Cancer Institute, Department of Health and Human Services.

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Communicated by R. Devoret

Received April 5, 1988 / November 8, 1988