Characterization of the Interaction of *Saccharomyces cerevisiae* Strand Exchange Protein 1 with DNA*

(Received for publication, May 12, 1993, and in revised form, September 28, 1993)

Arlen W. Johnson† and Richard D. Kolodner‡

From the Division of Cellular and Molecular Biology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115 and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

We have analyzed in greater detail the interaction of strand exchange protein 1 (Sep1) from *Saccharomyces cerevisiae* with DNA. The binding site size of Sep1 on single-stranded DNA (ssDNA) was determined to be 70 nucleotides per protein monomer using a fluorescence assay and 100 nucleotides using an exonuclease titration technique. The amount of Sep1 required for maximum aggregation of ssDNA was the amount needed to saturate the DNA. When double-stranded DNA (dsDNA) and ssDNA were both present, the duplex DNA was efficiently aggregated only at protein concentrations above that required for saturation of ssDNA. Strand exchange reactions with blunt-ended linear dsDNA and homologous ssDNA substrates required saturation of the ssDNA with Sep1 since free Sep1 is needed for exonuclease activity to initiate pairing with the dsDNA substrate. Precubation of Sep1 with resected duplex DNA before adding ssDNA allowed joint molecule formation to occur at protein concentrations at least 10-fold below that required for saturation of the ssDNA. However, precubation of Sep1 with ssDNA before the addition of resected duplex DNA required saturating amounts of Sep1 for joint molecule formation to occur. These results suggest that pairing requires Sep1 on both the ssDNA and the resected ends of the dsDNA.

Our knowledge of how DNA molecules pair during genetic recombination comes primarily from studies of *Escherichia coli* RecA protein and related proteins (Kowalczykowski, 1991; West, 1992; Radding, 1991). The formation of heteroduplex DNA between single-stranded circular and homologous linear duplex DNA substrates (Cox and Lehman, 1981) has been widely used to study the RecA protein since this reaction is thought to reflect some of the salient elements of recombination reactions *in vivo*. RecA promoted strand exchange is an ordered process with at least three distinct phases: 1) presynapsis, the assembly of a RecA filament on single-stranded DNA, a reaction that is facilitated by the removal of secondary structure, such as by the addition of single-stranded DNA binding protein; 2) synopsis, the homologous pairing of the single-stranded DNA (ssDNA) in the protein-DNA filament with double-stranded DNA. This homology search and pairing process is thought to occur in aggregates of ssDNA and dsDNA that initially form independent of homology; and 3) branch migration and concomitant strand exchange. In addition to promoting homologous pairing, RecA is an ATP binding protein and a DNA-dependent ATPase. However, the ability of RecA to promote the formation of extensive heteroduplex DNA does not require ATP hydrolysis (Menetski et al., 1990; Rehrauer and Kowalczykowski, 1993). ATP hydrolysis by RecA during strand exchange may promote the cycling of RecA on and off of the presynaptic filament to allow the formation of a continuous protein-DNA filament (Menetski et al., 1990). It may also allow the recycling of RecA protein by promoting the dissociation of RecA from the product heteroduplex (Menetski et al., 1990; Roselli and Stasiak, 1990).

Strand exchange protein 1 (Sep1) is the major protein in whole cell extracts of mitotic yeast that promotes pairing and strand exchange between single-stranded and homologous duplex linear DNA substrates *in vitro* (Kolodner et al., 1987). Recent genetic studies have suggested that Sep1 plays a role in meiotic recombination and the progression through meiosis in *Saccharomyces cerevisiae* (Tishkoff et al., 1991), consistent with this homologous pairing activity. Sep1 is a large protein of 175,000 Da that contains 5'-3' exonuclease, DNA renaturation, aggregation, and strand exchange activities (Kolodner et al., 1987; Heyer et al., 1988; Dykstra et al., 1990; Johnson and Kolodner, 1991). The requirement for exonuclease activity during synapsis (Johnson and Kolodner, 1991) suggests that the Sep1-mediated reaction is mechanistically different from the RecA reaction in which a single-stranded DNA molecule can be paired directly with homologous duplex molecules (Bianchi et al., 1983; Riddles and Lehman, 1985). Indeed, in a companion study (Johnson and Kolodner, 1994) we present evidence indicating that the formation of stable joint molecules by Sep1 involves the pairing of two single strands. An additional feature that distinguishes the two proteins is their polarity of strand transfer. Whereas RecA promotes strand exchange with a defined polarity (Cox and Lehman, 1981), Sep1 appears to promote branch migration in either direction, the polarity being determined by the polarity of the initial exonuclease digestion (Johnson and Kolodner, 1991). Other aspects of Sep1-mediated strand exchange appear similar to that of RecA. For instance, Sep1 is required in stoichiometric amounts relative to the ssDNA present (Kolodner et al., 1987). Sep1 renatures and aggregates DNA (Heyer et al., 1988) and strand exchange promoted by Sep1 is ATP independent (Kolodner et al., 1987). In this report we have more thoroughly characterized the interaction of Sep1 with ssDNA and dsDNA to develop a more comprehensive understanding of how Sep1 promotes strand exchange.

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by National Institutes of Health Postdoctoral Fellowship GM13594.

‡ Supported by National Institutes of Health Grant GM29383. To whom correspondence should be addressed: Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115. Tel.: 617-632-3806; Fax: 617-632-4326.

The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; Sep1, strand exchange protein 1; Sub, single-stranded DNA-binding protein; BSA, bovine serum albumin; ε-DNA, etheno DNA; RF, replicative form.

D. Tishkoff and R. Kolodner, manuscript in preparation.
**Materials and Methods**

**Enzymes and Proteins**—All restriction endonucleases and T4 polynucleotide kinase were from New England Biolabs Inc. T7 gene 6 exonuclease and Escherichia coli single-stranded DNA binding protein (Ssb), were from U. S. Biochemical Corp. Sep 1 fraction V was as described a companion study (Johnson and Kolodner, 1994). Protein standards for gel filtration chromatography and scrosu density gradient centrifugation were from Pharmacia LKB Biotechnology Inc.

DNA Substrates—Single-stranded M13mp19 viral DNA, M13mp19 viral [*H*DNA] (7.040 cpm/nmol), double-stranded M13mp19 RF DNA, and M13mp19 RF [*H*DNA] (5280 cpm/nmol) were purified as described elsewhere (Norriss and Kolodner, 1990). Linear double-stranded M13 DNA was prepared by digesting with Smal. Fluorescence-derivatized etheno-DNA (e-DNA) was as described and was generously provided by F. Alani (Alani et al., 1989), and the DNA concentration was quantitated by assaying for total phosphate (Ames, 1966) using native viral M13mp19 ssDNA as a control. 5'-Resected double-stranded linear M13mp19 RF DNA, with single-stranded tails having an average length of 50 nucleotides, was prepared as previously described (Johnson and Kolodner, 1991). T7 [*H*DNA] was purified by a published procedure (Richardson, 1986) and had a specific activity of 11.5 cpm/μmol. Sonication of DNA solutions was performed on ice for 2 min with a Heat Systems sonicator using a microtip at a setting of 2. For oligonucleotide binding reactions a 51-base long oligonucleotide: 5'-Al'TACCGGTCC-

**Enzyme Assays—Standard strand exchange reactions (30 μl) contained 33 μM Tris-HCl, pH 7.5, 1.5 μg MspCI, 1.8 μg dithiothreitol, 68 μg/ml BSA, 20 μM Smal-digested linear duplex M13mp19 DNA, 10 μM single-stranded viral M13mp19 DNA, and Sep1. Reactions were incubated at 30 °C for 20 min, stopped by the addition of 0.5 μL EDTA, 10 mg/ml of proteinase K, and 10% SDS to final concentrations of 50 μM, 600 μM, and 0.1%, respectively, and incubated at 37 °C for an additional 10 min. Where indicated, 5 μg CaCl2 was used in place of MgCl2, and resected linear duplex molecules were used as substrate in place of Smal-digested duplex DNA. For agarose gel analysis of strand exchange reactions, 6 μl of a solution containing 15% Ficoll 400, 0.25% bromophenol blue, 0.25% xylene cyanol, and 120 μM EDTA were added, and samples were electrophoresed through 1% agarose gels at 250 V for 1 h, stained with 0.5 μg/ml ethidium bromide for 1 h at 5 V/cm. For quantitation, the gels were photographed with a Polaroid type 665 positive/negative film, and the negatives were scanned using an LKB Ultrascan XL laser densitometer.

**Oligonucleotide Binding Reactions for Scatchard Analysis** contained 33 μM Tris-HCl, pH 7.5, 5 μM McIP1, 1.8 μg dithiothreitol, 88 μg/ml BSA, 0.3 nmoi of single-stranded M13 viral DNA or 0.6 nmol of double-stranded M13 DNA, or 0.3 nmol of ssSDNA and 0.6 nmol of double-stranded M13 linear DNA, and varying amounts of Sep1 protein in a total volume of 30 μl. Where indicated 5'-resected Smal-digested DNA with single-stranded tails of 50 nucleotides was used as substrate. The reactions were incubated at 30 °C for 10 min followed by centrifugation for 3 min at 12,000 x g. The supernatant fraction (27 μl) was removed, and 24 μl of T2 (10 mm Tris buffer, pH 8.0, 1 mm EDTA) were added to the remaining pellets. The pellet and supernatant fractions were then deproteinized and analyzed by agarose gel electrophoresis as described for strand exchange (Johnson and Kolodner, 1991). The ethidium bromide-stained gels were photographed using Polaroid type 665 film, and the negatives were quantitated by densiometry using an LKB Ultrascan densitometer. Alternatively, gels were photographed digitally using a CoHU CCD camera, and the digital images were quantitated using Image Pro Plus software (National Institutes of Health). The amount of DNA in the supernatants was corrected for the total reaction volume, and 10% of this value was subtracted from the amount of DNA in the pellet fraction to account for the 10% of the supernatant remaining in these samples.

**Hydrodynamic Properties of Sep1—** Gel filtration chromatography of Sep1 was performed using Superose 6 resin (Pharmacia). The column (0.34 cm x 20.3 cm) was equilibrated in 20 μM Tris-HCl, pH 7.5, 240 μM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 10% (w/v) glycerol, and samples were chromatographed at a flow rate of 0.25 ml/min using a Bio-Rad gel chromatograph. The column was equilibrated in 20 μM Tris-HCl, pH 7.5, 5 mM CeCl3, 1.8 μg dithiothreitol, 88 μg/ml BSA, 0.3 nmol of single-stranded M13 viral DNA or 0.6 nmol of double-stranded M13 DNA, or 0.3 nmol of ssSDNA and 0.6 nmol of double-stranded M13 linear DNA, and varying amounts of Sep1 protein in a total volume of 30 μl. Where indicated 5'-resected Smal-digested DNA with single-stranded tails of 50 nucleotides was used as substrate. The reactions were incubated at 30 °C for 10 min followed by centrifugation for 3 min. The supernatant fraction (27 μl) was removed, and 24 μl of T2 (10 mm Tris buffer, pH 8.0, 1 mm EDTA) were added to the remaining pellets. The pellet and supernatant fractions were then deproteinized and analyzed by agarose gel electrophoresis as described for strand exchange. The position observed for the protein eluted in 240 fraction is indicated by a vertical line. The pellets and supernatant fractions were then deproteinized and analyzed by agarose gel electrophoresis as described for strand exchange (Johnson and Kolodner, 1991). The ethidium bromide-stained gels were photographed using Polaroid type 665 film, and the negatives were quantitated by densiometry using an LKB Ultrascan densitometer. Alternatively, gels were photographed digitally using a CoHU CCD camera, and the digital images were quantitated using Image Pro Plus software (National Institutes of Health). The amount of DNA in the supernatants was corrected for the total reaction volume, and 10% of this value was subtracted from the amount of DNA in the pellet fraction to account for the 10% of the supernatant remaining in these samples.

**RESULTS**

**Hydrodynamic Properties of Sep1—** The Stokes’ radius of Sep1 was determined by gel filtration chromatography (Laurant and Killander, 1964). In the presence of 240 μM NaCl, Sep1 chromatographed as a single species with a Stokes’ radius of 58.8 Å (Fig. 1a). At 40 μM NaCl, Sep1 appeared to aggregate, eluting from the column in the void volume and trailing to the position observed for the protein eluted in 240 μM NaCl with no apparent discrete intermediate species (data not shown). The sedimentation coefficient of Sep1 was 5.9 S (Fig. 1b), determined by centrifugation through 5–20% (w/v) sucrose gradients in the presence of 240 μM NaCl (Martin and Ames, 1961).
Hydrodynamic properties of Sep1. a, gel filtration determination of Stokes' radius. Superose 6 gel filtration chromatography was performed in the presence of 20 mM Tris-HCl, pH 7.5, 240 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 10% (w/v) glycerol as described in the text. The elution positions of protein standards and Sep1 are indicated. At least two independent determinations were made for each point. b, sucrose density gradient determination of sedimentation coefficient. Gradients of 5–20% sucrose were prepared in the presence of 20 mM Tris-HCl, pH 7.5, 240 mM NaCl, and 0.1 mM EDTA as described in the text. The positions of protein standards and Sep1 are indicated.

Assuming a partial specific volume of 0.736 cm³/g calculated from the translated DNA sequence (Steensgaard et al., 1992), a molecular weight of 150,000 was determined for Sep1 using the method of Siegel and Monty (1966). This is in reasonable agreement with a calculated monomeric molecular weight of 175,000 calculated from a DNA sequence. In addition, the Perrin factor, F, was calculated from the molecular weight and Stokes' radius to be 1.7 (Siegel and Monty, 1966), indicating an elongated shape with an axial ratio of 11 to 14 (Cantor and Schimmel, 1980). These data indicate that Sep1 is an elongated monomer in solution at 240 mM NaCl. It should be noted that the exonuclease and strand exchange activities of Sep1 are inhibited at 240 mM NaCl, whereas Sep1 aggregates under the salt concentrations typically used for exonuclease and strand exchange assays.

Determination of Binding Site Size—The binding of proteins to nucleic acids can be monitored spectrofluorometrically (Kowalczykowski et al., 1981). Sep1 binding to native ssDNA did not result in any appreciable quenching of the intrinsic fluorescence of Sep1 protein when tryptophan or tyrosine fluorescence was monitored (data not shown). Consequently an extrinsic fluorescence technique of monitoring the change in fluorescence of the bound protein was used. The intrinsic fluorescence of Sep1 is quenched by binding to ssDNA. The change in fluorescence, DΔF, is related to the change in binding, Δn, by a linear relationship, DΔF = sΔn, where s is the slope of the curve. The change in binding, Δn, is a function of the change in the binding constant, K, which is related to the change in the binding site size, ΔS, by the equation ΔS = -2.303 Δn log K; hence DΔF = -2.303 sΔn log K.
cience of chemically modified, fluorescent DNA, e-DNA, as protein bound to the DNA was used to monitor the interaction of Sep1 with DNA. The addition of increasing amounts of Sep1 protein to the e-DNA resulted in a biphasic increase in fluorescence (Fig. 2). The initial slope of this curve was due to the combination of enhanced DNA fluorescence from protein binding and the intrinsic fluorescence of the protein itself. The final slope was due to the intrinsic fluorescence of the protein alone after the DNA had been saturated with protein and the maximal enhanced fluorescence from protein binding had been reached. The intercept of these two slopes gives the saturation point for protein binding. From this saturation point the apparent site size of Sep1 was determined to be approximately 70 nucleotides. Similar results were obtained from multiple determinations and a similar binding site size was observed in the presence of 5 mM CaCl₂ (data not shown). One potential complication of this analysis was the possible digestion of e-DNA by the ssDNA exonuclease activity of Sep1 leading to increased fluorescence independent of DNA binding. However, in the experiments reported here using circular viral DNA, no time dependent increase in Sep1-dependent fluorescence was observed indicating that the exonuclease activity of Sep1 did not contribute to the observed increase in fluorescence (data not shown). Fig. 2 shows a slight nonlinearity of the fluorescence response at low protein concentrations indicative of cooperative binding. Experiments in which higher salt concentrations were used to reduce the binding affinity to allow more accurate analysis of binding at low protein concentrations resulted in altered slopes for both phases of the binding curve without increasing the sigmoidal character of the curve. This suggested that increasing salt concentrations alter the nature of Sep1-DNA or Sep1-Sep1 interaction complicating any determination of cooperativity. Because of this difficulty in evaluating the cooperativity of binding it was not possible to determine a $K_d$ from these data (Kowalczewski et al., 1986).

Tritium Exonuclease Activity by ssDNA—Since e-DNA has been chemically modified and lacks secondary structure, the properties of Sep1 binding to e-DNA may be different from binding to native DNA. We had observed that Sep1 bound to ssDNA was inert as a dsDNA exonuclease (see below). This result was exploited in a novel approach to determine the apparent site size of Sep1 binding to native ssDNA. Fig. 3 shows the results of titrating the dsDNA exonuclease activity of Sep1 with various amounts of ssDNA. In the absence of ssDNA, there was a linear relationship between the concentration of Sep1 and the extent of dsDNA exonuclease activity. If Sep1 was preincubated with a fixed concentration of ssDNA, the dsDNA exonuclease versus protein concentration curve was displaced to higher concentrations of Sep1. As greater amounts of ssDNA competitor were added, the dsDNA exonuclease activity versus protein concentration curve was shifted to proportionally higher protein concentrations. Similar results were obtained when Sep1 was added directly to reactions containing both single-stranded and double-stranded substrate DNA (data not shown), reflecting the higher affinity of Sep1 for ssDNA.

These results demonstrate two points. First, Sep1 bound to ssDNA is not free to act as a nuclease on free duplex DNA molecules. Second, the displacement of these curves to higher Sep1 concentrations reflects the amount of Sep1 protein required to saturate the ssDNA before free Sep1 was available to act as a nuclease. Linear regression analysis of points of these curves surrounding their inflection points gives lines that intercept the abscissa at the titration points for each reaction. An apparent site size of 100 ± 5 nucleotides was obtained from this analysis. The apparent site size determined by this method is approximately 40% greater than that obtained with e-DNA. This difference could reflect the exclusion of Sep1 from regions of secondary structure present in the native ssDNA. Consistent with this idea, electron microscopic analysis of Sep1 binding to native ssDNA has indicated that it binds first to open regions and begins to disrupt secondary structure only at higher pro-
FIG. 4. a, Sep1 binding to ssDNA and dsDNA measured by nitrocellulose filter binding. Reactions contained Sep1 or E. coli Ssb and 1.67 μM of M13mp19 viral [3H]DNA or 1.67 μM of M13mp19 linear duplex [3H]DNA in 33 mM Tris-HCl, pH 7.5, 13 mM MgCl₂ or 5 mM CaCl₂, as indicated, 1.8 mM dithiothreitol, and 88 μg/ml BSA in a total volume of 300 μl. Reactions were incubated at 30 °C for 1 min and were then applied, using a Millipore filtration manifold, to KOH-treated nitrocellulose filters. The DNA bound by protein was measured as radioactivity retained on the filters. Symbols: △, Sep1 and ssDNA in Mg²⁺; ●, Sep1 and dsDNA in Mg²⁺; ○, Sep1 and ssDNA in Ca²⁺; ○, Sep1 and dsDNA in Ca²⁺; □, Ssb and ssDNA in Mg²⁺; and ■, Ssb and dsDNA in Mg²⁺. b, Scatchard analysis of oligonucleotide binding. Sep1 binding to a 5'-32P-labeled, 51-nucleotide long oligonucleotide was measured by retention of the complex on nitrocellulose filters as described under “Materials and Methods.” Reactions were done in duplicate and contained 3.8 nM Sep1 and 0.6 nM to 6.2 nM oligonucleotide in 33 mM Tris-HCl, pH 7.5, 5 mM CaCl₂, 1.8 mM dithiothreitol, 88 μg/ml BSA. The data were treated by Scatchard analysis in which the slope of the curve represents the negative inverse of the dissociation constant, [DNA]/([DNA]+[PROT]) is the concentration of bound DNA, [DNA]/([DNA]+[PROT]) the concentration of free DNA, and [PROT] the concentration of bound protein.

It seems likely that the actual site size is the same for e-DNA and native ssDNA and that the difference in apparent site sizes is due to the inaccessibility of Sep1 to regions of the native ssDNA containing secondary structure.

Nitrocellulose Filter Binding Measurements of the Interaction of Sep1 with DNA—The binding of Sep1 to ssDNA and dsDNA was first analyzed by filter binding using a proteolytic fragment of Sep1 (Heyer et al., 1988). Because intact protein was used in the present analysis, we believed it was important to determine the DNA binding properties of the native protein. The binding of Sep1 to ssDNA and dsDNA was measured by nitrocellulose filter binding under conditions in which DNA is retained by the filters only when bound by protein. For binding measurements with dsDNA, closed circular DNA was used as substrate to avoid the effects of binding ends. Sep1 was incubated with ³H-labeled single-stranded or ³H-labeled double-stranded circular M13mp19 DNA under strand exchange conditions in the presence of Mg²⁺ or Ca²⁺ and the amount of DNA bound by Sep1 was determined. Sep1 bound ssDNA preferentially over dsDNA (Fig. 4a). Approximately three times as much...
Sep1 was required to retain the same molar amount of dsDNA as ssDNA. Similar results were obtained in either 5 mM CaCl$_2$ or 13 mM MgCl$_2$. The binding of *E. coli* Ssb protein was measured as a control. Ssb showed a nearly absolute specificity for ssDNA and the DNA was efficiently bound at low levels of protein reflecting the high apparent affinity of Ssb for ssDNA. The lack of binding of the dsDNA by Ssb showed that the dsDNA did not contain significant single-stranded regions further supporting the idea that Sep1 has dsDNA binding activity.

Nitrocellulose filter binding was also used to quantitate the binding of Sep1 to a 5'-$^{32}$P-end-labeled, 51-nucleotide long, single-stranded oligonucleotide. Binding was performed in the presence of Ca$^{2+}$ in order to inhibit the nuclease activity of the protein reflecting the high apparent affinity of Ssb for ssDNA, $K_d$.

Binding of Sep1 to dsDNA was measured by incubation of protein and DNA together for 10 min at 30 °C. DNA aggregation was measured as material pelleted by centrifugation for 3 min at 12,000 × g. Symbols: ■, aggregation of ssDNA alone; ○, aggregation of dsDNA alone; □, aggregation of ssDNA in the presence of dsDNA; ○, aggregation of dsDNA in the presence of ssDNA; and ▲, aggregation of resected dsDNA alone.

**Sep1 Interaction with dsDNA**

**Fig. 5. Aggregation of ssDNA and dsDNA by Sep1.** DNA aggregation was measured under standard strand exchange reaction conditions in the presence of 5 mM CaCl$_2$. Reactions contained ssDNA (10 µM) alone, dsDNA (20 µM) alone, or ssDNA (10 µM) and dsDNA (30 µM). After incubation of protein and DNA together for 10 min at 30 °C, DNA aggregation was measured as material pelleted by centrifugation for 3 min at 12,000 × g. Symbols: ■, aggregation of ssDNA alone; ○, aggregation of dsDNA alone; □, aggregation of ssDNA in the presence of dsDNA; ○, aggregation of dsDNA in the presence of ssDNA; and ▲, aggregation of resected dsDNA alone.

Sep1 was titrated into strand exchange reactions at 30 °C and the concentration of DNA was varied. In control experiments, saturation of the oligonucleotide with protein resulted in retention of 100% of the labeled oligonucleotide (data not shown). Fig. 4B shows a representative Scatchard plot of such an experiment. From these data a dissociation constant, $K_d$, of $2.7 \times 10^{-8}$ M with respect to nucleotide concentration was obtained.

**DNA Aggregation.—**Although the aggregation of DNA by Sep1 was demonstrated previously (Heyer et al., 1988), the stoichiometry of protein needed for aggregation, the effect of single-stranded ends on the duplex DNA and coaggregation of ssDNA and dsDNA were not addressed or not addressed in detail. Aggregation of dsDNA and dsDNA by Sep1 under strand exchange conditions was examined. Reactions were carried out in the presence of Ca$^{2+}$ so that the Sep1 exonuclease was inactive. We have shown previously that strand exchange by Sep1 occurs efficiently in the presence of Ca$^{2+}$ instead of Mg$^{2+}$ (Johnson and Kolodner, 1991), and that the DNA binding properties of Sep1 are similar in the presence of either cation (see above). When Sep1 was incubated with ssDNA alone, 100% of the DNA was aggregated at a stoichiometry of 1 Sep1 to 1 ssDNA of approximately 1 protein monomer/100 nucleotides (0.095 µM Sep1, Fig. 5). The complete aggregation of dsDNA required approximately 1 protein monomer/70 base pairs (0.133 µM Sep1, Fig. 5). Evidently aggregation of ssDNA and dsDNA does not require the same stoichiometry of protein to nucleotides. When both DNAs were present, the aggregation of ssDNA occurred essentially as if only ssDNA was present. Most of the ssDNA could be aggregated when only minimal aggregation of the dsDNA was observed, reflecting the preference of Sep1 for ssDNA. Complete aggregation of the dsDNA in the presence of ssDNA required a protein concentration that was approximately the sum of the amounts required to aggregate the two DNAs alone. Hence, Sep1 bound to ssDNA did not appear to facilitate the aggregation of dsDNA. Similar results were obtained when the aggregation reactions contained Mg$^{2+}$ instead of Ca$^{2+}$ (data not shown). The presence of single-stranded tails on the linear dsDNA changed the aggregation pattern of dsDNA. In this case, the dsDNA alone was more efficiently aggregated than ssDNA alone in terms of nucleotides of DNA aggregated per amount of protein added. This difference in aggregation between ssDNA alone and resected dsDNA alone could be explained by the preferential binding of Sep1 to ssDNA and if Sep1 binds to dsDNA cooperatively and if aggregation of DNA requires that it is bound by protein. Under these conditions, the single-stranded tails of the resected dsDNA would be saturated with Sep1 at a lower protein concentration than that needed to saturate the circular ssDNA. Because of the cooperativity of binding, larger amounts of protein would be needed for all the circular ssDNA molecules to have bound protein and be aggregated. Resected dsDNA would also be aggregated more efficiently than blunt-ended dsDNA because Sep1 preferentially binds ssDNA, and at low protein concentrations a greater proportion of resected duplex molecules compared to blunt-ended duplex DNA molecules would be bound by Sep1 and found in aggregates. The presence of single-stranded ends on the dsDNA also reduced somewhat the amount of Sep1 required to aggregate the dsDNA in the presence of ssDNA. This result also suggests that aggregation of dsDNA does not require a specific stoichiometry of Sep1 in relation to DNA.

**Sep1 Preloaded onto Single-stranded Ends of dsDNA Promotes Joint Molecule Formation at Lower Sep1 Concentrations.—**The amount of Sep1 required for joint molecule formation in the presence of Mg$^{2+}$ using blunt-ended DNA is proportional to the amount of ssDNA in the reaction (Kolodner et al., 1987). As Sep1 was titrated into strand exchange reactions a sharp transition point was observed for the formation of joint molecules (Fig. 6). Under these conditions, joint molecules were observed when the stoichiometry of Sep1 to DNA was approximately 1 Sep1 monomer/100 nucleotides of ssDNA substrate. Different results were obtained when the dsDNA was resected to contain 3'-single-stranded ends that were approximately 50 nucleotides long. Sep1 was preincubated with resected dsDNA in the presence of Ca$^{2+}$ for 3 min at 30 °C, followed by the addition of homologous ssDNA. After an additional 20 min at 30 °C, the reactions were analyzed for the presence of joint molecules. Under these conditions, joint molecules were observed at Sep1 concentrations that were 10-30 times lower than in the case of blunt-ended dsDNA substrates (Fig. 6). In addition, under these conditions there was a more gradual increase in the proportion of joint molecules formed as a function of the concentration of Sep1 than was observed with blunt-ended dsDNA substrates. This experiment suggests that loading of Sep1 onto the ends of the duplex molecules is necessary for pairing.

Alternatively, Sep1 was preincubated with the circular ssDNA in the presence of Ca$^{2+}$, followed by the addition of resected linear dsDNA. Joint molecule formation was observed only at Sep1 concentrations at and above those required to saturate the circular ssDNA, similar to results observed in the presence of Mg$^{2+}$ with single-stranded circular and blunt-ended
Sepl-DNA Interaction

Fig. 6. Titration of Sepl in strand exchange reactions. Strand exchange reactions containing increasing amounts of Sepl were performed and quantitated as described in the text. When blunt-ended dsDNA was used, no protein-dependent pairing was observed. The small amount (≤7%) of protein-independent pairing observed with resected substrates was subtracted from the total amount of joint molecule product in those reactions. The products of protein-independent pairing were sigma molecules and did not show any strand exchange (Johnson and Kolodner, 1991). Symbols: ■, standard reaction containing ssDNA and complementary blunt-ended duplex DNA in the presence of Mg\textsuperscript{2+}; ○, Sepl was preincubated with duplex DNA containing single-stranded tails, followed by the addition homologous ssDNA in the presence of Ca\textsuperscript{2+}; and ●, Sepl was preincubated with single-stranded circular DNA, followed by the addition resected homologous duplex linear DNA in the presence of Ca\textsuperscript{2+}.

linear dsDNA substrates. In this reaction, the use of resected duplex DNA avoids any requirement for exonuclease activity. Consequently the presence of the same titration point in this experiment indicates that the pairing function of Sepl could be titrated by binding to ssDNA and is consistent with the idea that pairing requires binding of Sepl to both DNA molecules.

DISCUSSION

In this report we have characterized the interaction of Sepl with DNA in order to understand how Sepl promotes the pairing of circular ssDNA with homologous dsDNA followed by a strand exchange reaction. We have analyzed the binding of Sepl to ssDNA and dsDNA in terms of stoichiometry, affinity, and the ability of binding to aggregate DNA and to modulate the exonuclease activity of Sepl. In Fig. 7 we present a model for Sepl-mediated homologous pairing that represents our interpretation of the results presented in this and a companion study (Johnson and Kolodner, 1994). This model provides a description of what we envision happens when Sepl is titrated into a reaction containing circular ssDNA and homologous linear dsDNA substrates. In the following sections we will discuss specific aspects of Sepl-mediated strand exchange. For a complete description of the model, see Fig. 7.

In a standard strand exchange reaction containing circular ssDNA and homologous linear dsDNA, Sepl binds first to ssDNA due to its higher affinity for ssDNA. Sepl bound to ssDNA is inactive as an exonuclease on dsDNA. This finding was exploited to determine the binding site size of Sepl on native ssDNA. Since the nuclelease activity of Sepl is necessary for the formation of stable joint molecules (Johnson and Kolodner, 1991), Sepl must be present at concentrations in excess of that needed to saturate the ssDNA before free protein is available to act as a nuclease on the ends of the linear dsDNA and initiate pairing. This need to saturate the ssDNA to overcome the inhibition of exonuclease activity results in a sharp titration point in protein concentration required for the observation of joint molecules.

Two additional phenomena appear to contribute to producing the titration point observed for joint molecule formation. First, saturation of the ssDNA is necessary for the aggregation of the dsDNA with ssDNA. Second, stable pairing requires protein on both the ssDNA and the duplex ends. This requires saturation of the ssDNA because the preferential binding of Sepl to ssDNA prevents binding of Sepl to dsDNA until there is free Sepl available. The concentration of Sepl at which the ssDNA was completely saturated with Sepl was the concentration at which ssDNA was fully aggregated. It was also the lowest Sepl concentration at which joint molecule formation was observed when Sepl was added to reactions containing both ssDNA and dsDNA substrates. However, maximal joint molecule formation was observed at Sepl concentrations approximately twice that needed to saturate the ssDNA. At such Sepl concentrations, the dsDNA was fully aggregated, suggesting that the observed pairing reaction occurs within these aggregates. Possibly these aggregates are obligatory intermediates in the pairing process. However, since the formation of aggregates is independent of homology (data not shown), aggregation does not reflect the homologous pairing of DNA. That aggregation of the dsDNA occurs only after the ssDNA had been saturated and aggregated by Sepl and that aggregation of the ssDNA and dsDNA together requires the sum of the amount of Sepl required to aggregate the ssDNA and dsDNA separately suggest that Sepl aggregation of dsDNA is not facilitated by the binding of Sepl to ssDNA.

These results obtained with Sepl are in some respects similar to results from aggregation studies of RecA protein. Under conditions that favor joint molecule formation RecA aggregates ssDNA at protein concentrations lower than that needed to aggregate duplex DNA, much like the reaction observed with Sepl. The aggregation and renaturation of ssDNA by RecA is thought to result from the ability of a RecA-ssDNA filament to accept a second protein-free DNA molecule in a second binding site on RecA (Tsang et al., 1985). However, concentrations of RecA protein above that required to saturate ssDNA tend to inhibit ssDNA aggregation and ssDNA renaturation, but promote strand exchange. This inhibition apparently results from the inability of RecA protein on one ssDNA molecule to bind another DNA strand that is also coated with RecA (Tsang et al., 1985). Such inhibition of ssDNA aggregation by increasing amounts of Sepl was not observed. The aggregation of dsDNA with ssDNA by Sepl appears to occur by a mechanism different from that of RecA, possibly through the formation of protein-DNA complexes that further associate through protein-protein interactions.

Although stoichiometric amounts of Sepl were required for joint molecule formation when the protein was presented with ssDNA and homologous dsDNA together, a different result was obtained when Sepl was preloaded onto resected duplex DNA substrates. In this case, when ssDNA was added to reactions in which Sepl was allowed first to bind to resected duplex DNA in the absence of ssDNA, joint molecules were observed at concentrations of Sepl that were 10–30-fold below that required to saturate the circular ssDNA. Thus, the loading of Sepl onto the duplex ends appears to be critical for joint molecule formation. At these low protein concentrations, Sepl was still above the concentration required to saturate the duplex ends. Consequently, because joint molecule formation was observed only under conditions where there was free Sepl in solution that would bind to the ssDNA substrate, we cannot argue that Sepl is needed only on the resected duplex end for pairing. We have
The Sep1-DNA Interaction

FIG. 7. Model for Sep1 DNA binding and strand exchange. When Sep1 is titrated into reactions containing both ssDNA and dsDNA it binds first and preferentially to the single-stranded circle. The Sep1-ssDNA complex has been depicted in an ordered open arrangement only for simplicity of discussion and not to imply that Sep1 bound to circular ssDNA forms such ordered complexes. When the ssDNA is saturated with Sep1 there is free Sep1 to act as a double-strand exonuclease yielding short single-stranded tails on the ends of the duplex DNAs. The exonuclease digestion is the rate limiting step in stable joint molecule formation. When the single-stranded tail is approximately 20 nucleotides in length and Sep1 can bind to the end of the duplex substrate, Sep1 efficiently pairs the duplex and circular DNAs in an annealing-like reaction. The ssDNA is fully aggregated when saturated with Sep1. Higher concentrations of Sep1 lead to aggregation of blunt ended dsDNA with the ssDNA and exonuclease digestion of the duplex ends leads to more efficient aggregation of the dsDNA. These aggregates may be intermediates in the strand exchange reaction. Since Sep1 must be above saturating concentrations on the ssDNA before pairing with resected duplexes is observed, it is likely that both pairing and aggregation of the ssDNA and dsDNA substrates requires Sep1 present on both the circular ssDNA and the ends of the linear duplex molecules. Once pairing is achieved, strand displacement and branch migration proceed independently of the need for further nuclease activity. When the nuclease is active, however, branch migration leads to increased rates of exonucleolytic degradation on the displaced 5' single-stranded tail.

not determined the extent of strand exchange, if any, that occurs at these low Sep1 concentrations and it is possible that only renaturation of the single-stranded tail to the circular ssDNA had occurred under these conditions. In addition, the presence of single-stranded ends on the dsDNA allowed aggregation of linear dsDNA to occur at lower Sep1 concentrations both in the presence (data not shown) and absence of circular ssDNA. This effect appears to be due to the higher affinity of Sep1 for ssDNA compared to dsDNA leading to saturation of the single-stranded ends at relatively low protein concentrations. Thus, the need for Sep1 on the duplex ends may be to facilitate the aggregation of the duplex DNA.

The last phenomenon that appears to contribute to the titration point in joint molecule formation by Sep1 is the titration of the pairing function of Sep1, even when the duplex DNA contains single-stranded tails. When Sep1 was preloaded onto the circular ssDNA and then resected linear dsDNA was added to the reaction, joint molecule formation required concentrations of Sep1 at or above saturation for the ssDNA. Thus, in addition to inhibition of the exonuclease activity, binding to ssDNA also appeared to titrate the homologous pairing activity of Sep1. This is most simply understood if Sep1-mediated pairing requires protein on both the circular ssDNA and the tails of the linear dsDNA molecules, the latter complex only being formed after there is more Sep1 present than required to saturate the ssDNA and hence there is free Sep1 in solution. These observations are consistent with the idea that during pairing reactions Sep1 binds to the ssDNA and dsDNA substrates independently. These protein-DNA complexes then aggregate through protein-protein interactions facilitating pairing by the high local DNA concentration in these aggregates. This is unlike RecA-mediated pairing reactions in which renaturation is inhibited by saturating amounts of RecA protein and the formation of an active protein-DNA filament on the single-stranded circle is necessary and sufficient to promote pairing with a free dsDNA molecule (Tsang et al., 1985).

It appears that the most significant difference between Sep1 and RecA is in their mechanisms of pairing. The proposal that the formation of stable joint molecules by Sep1 involves annealing of single-stranded regions is supported by several observations. Sep1 appears to have a requirement for ends on dsDNA molecules that can serve as points of entry for the dsDNA exonuclease activity. Pairing requires the intrinsic exonuclease of Sep1 or an exonuclease provided in trans. Furthermore, the digestion of the duplex ends seems to be the rate limiting step in Sep1-promoted pairing and the single-stranded tails produced must be homologous with the target circular ssDNA (Johnson and Kolodner, 1994). These results are quite different from RecA-promoted reactions in which there is not a requirement for exonuclease digestion or ends on the dsDNA substrate molecule and stable pairing of homologous DNA appears to be initiated by the formation of a paranemic joint. We have not been able to demonstrate pairing between closed circular ssDNA and dsDNA using D-loop assays or using a Topo 1-coupled assay for paranemic joints (data not shown). However, the possibility that Sep1 can promote such reactions under some conditions and that such pairing precedes the single-strand annealing event required for the formation of stable
joint molecules cannot be ruled out definitively.

Our results bear on the mechanism of pairing by Sep1 but they do not address how strand displacement and branch migration occur. We showed previously that these two steps occur in the absence of nuclease activity. In addition, other examples of strand exchange proteins exist in which strand exchange depends on exonuclease activity but is functionally or physically separated from exonuclease activity. These include a yeast DNA-pairing activity (Halbrook and McEntee, 1989), the Droso-

phila RecA Recombination repair protein, Rrp1 (Sander et al., 1993), and E. coli RecE and RecT proteins (Hall and Kolodner, 1993). This uncoupling of nuclease and branch migration is further supported by the observation that branch migration proceeds at rates of up to 18 bases/s which is 50 times greater than the rate of digestion of dsDNA by Sep1 of 20 nucleotides/min (Johnson and Kolodner, 1991). Last, because branch migration proceeds ahead of exonuclease digestion and Sep1 digests ssDNA faster than duplex DNA, an increased rate of exonuclease digestion would be expected on the displaced ends relative to the undisplaced ends and such enhanced degradation is observed. Although the exonuclease is dispensable for strand exchange, it is possible that recognition of a duplex end at the exonuclease active site leads to partial melting of the duplex end, initiating strand displacement. Exactly how extensive directional branch migration and strand exchange is promoted by Sep1, or even RecA is unclear and clearly further work is required to understand this aspect of the strand exchange reaction. In some respects the reaction catalyzed by RecA is more complex. Pairing and strand exchange may occur simultaneously as different aspects of the same reaction (Adzuma, 1992) or may be separated by the formation of a triple helix intermediate in the pairing process prior to strand displacement. In the case of Sep1, pairing and strand exchange are separate reactions and may allow analysis of the mechanism of strand displacement as an isolated reaction.

Acknowledgments—We thank E. Alani for providing etheno-DNA, D. Norris for performing the sucrose density gradient analysis, and D. Tishkoff, E. Alani, and A. Page for careful review of the manuscript. We thank the Molecular Biology Core Facility, Dana-Farber Cancer Institute, supported by National Institutes of Health Grant CA06516, for the synthesis of all oligonucleotides.

REFERENCES


