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Purification of a Strand Exchange Stimulatory Factor from *Saccharomyces cerevisiae*†

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ABSTRACT: The SEP1 strand exchange protein of *Saccharomyces cerevisiae* catalyzes the formation of heteroduplex DNA joints between single-strand circles and homologous linear duplexes in vitro. Previous work [Kolodner, R., Evans, D. H., & Morrison, P. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5560–5564] showed that the optimal stoichiometry of SEP1 in this reaction was 1 SEP1 monomer per 12–14 nucleotides of single-stranded DNA. The work presented here describes the purification and characterization of a 33 000-dalton yeast protein that permits SEP1 to catalyze joint molecule formation at much lower stoichiometries. In the presence of this second factor, which has been designated SF1 for *s*timulatory *f*actor *1*, the optimal amount of SEP1 dropped to 1 SEP1 monomer per 725 nucleotides of single-stranded DNA. At this concentration of SEP1, the rate of joint molecule formation increased approximately 3-fold over that seen in the unstimulated reaction (no SF1). Titration experiments indicated that when the concentration of SEP1 was reduced over 300-fold to 1 SEP1 molecule per 5800 nucleotides of single-stranded DNA, the stimulated reaction had the same rate and extent of joint molecule formation as the unstimulated reaction. The optimal amount of SF1 was 1 molecule of SF1 per 20 nucleotides of single-stranded DNA. Electron microscopic analysis showed that a bona fide strand exchange reaction produced the joint molecules in the stimulated reaction. The stimulated reaction had requirements that were essentially identical with those seen in the unstimulated reaction, including a lack of dependence on ATP. SF1 aggregated single-stranded and double-stranded DNA. This property of the protein, however, could not account for all of the observed stimulation as it was possible to develop reaction conditions under which strand exchange was still SF1 dependent but the aggregation of double-stranded DNA did not occur.

One of the central intermediates of genetic recombination is the heteroduplex DNA joint [for representative reviews, see Fogel et al. (1981) and Orr-Weaver and Szostak (1985)]. Extensive genetic and biochemical analysis has indicated that a unique class of proteins—the strand exchange proteins—catalyzes the formation of such joints (Radding, 1982; Smith, 1988). The mechanism of action of the strand exchange proteins is best exemplified by the *Escherichia coli* RecA protein (Radding, 1982; Cox & Lehman, 1987; Griffith & Harris, 1988) and T4 bacteriophage UvsX protein (Yonesaki et al., 1985; Formosa & Alberts, 1986; Griffith & Harris,

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1988). These two proteins catalyze strand exchange in reactions that can be conceptually reduced into three stages. In the first stage, known as presynapsis, the proteins bind at high stoichiometries to single-stranded DNA to form a nucleoprotein filament, the active intermediate in the strand exchange reaction. During the second stage of the reaction, known as synapsis, these nucleoprotein filaments scan duplex DNA for homologous regions of DNA. Once a homologous region has been found, the third step of the reaction, known as branch migration, occurs in which the single-stranded DNA hybridizes to its homologous sequence in the duplex, thereby displacing one strand of the duplex DNA. The end product of this reaction is a heteroduplex DNA joint in which the individual single strands originate from two noncontiguous regions of DNA.

Although the RecA and UvsX proteins tend to act as the paradigms for all strand exchange proteins, our laboratory and others have been attempting to analyze eukaryotic strand exchange proteins using the yeast *Saccharomyces cerevisiae* as an experimental model. Our work was initiated with the development of a cell-free system from mitotic yeast cells that was capable of catalyzing genetic recombination (Symington et al., 1983, 1984). Two strand exchange proteins have been purified from this type of extract. The first of these, designated SEP1 for *s*trand *e*xchange *p*rotein *1*, was purified from mitotic cells (Kolodner et al., 1987; Heyer et al., 1988). SEP1 catalyzes strand exchange with the same polarity as RecA and UvsX and, like these proteins, is required at high concentrations (optimal stoichiometry: 1 SEP1 molecule per 12–14 nucleotides of single-stranded DNA). The reaction catalyzed by SEP1 is independent of ATP hydrolysis, a characteristic which was originally thought to distinguish SEP1 from the prokaryotic proteins; recently, however, it has been shown that RecA can also catalyze strand exchange in the absence of ATP hydrolysis (Menetski et al., 1990). A second yeast protein, designated STPα for *s*trand *t*ransfer *p*rotein α, has been purified from extracts of meiotic cells (Sugino et al., 1988). STPα also does not require ATP hydrolysis to catalyze strand exchange. Halbrook and McEntee have reported the purification of a putative third strand exchange protein from mechanically ruptured mitotic yeast cells (Halbrook & McEntee, 1989). This protein, however, has similar biochemical and physical characteristics to SEP1 and therefore may not represent a new activity.

It is unlikely that a single class of proteins catalyzes all aspects of heteroduplex DNA formation in vivo, and indeed other proteins have been shown to play a role in the reaction. For example, RecA catalyzes strand exchange at lower concentrations in the presence of the *E. coli* single-stranded DNA binding protein (Cox & Lehman, 1981; Cox et al., 1983; Muniyappa et al., 1984). RecA-catalyzed heteroduplex DNA formation can also be made to depend in vitro on the helicase activity of the RecBCD nuclease (Roman & Kowalczykowski, 1989). The activity of UvsX is stimulated in vitro by the dda helicase (Kodadek & Alberts, 1987), the gene 32 helix-destabilizing protein (Formosa & Alberts, 1986), and the UvsY protein (Harris & Griffith, 1989; Kodadek et al., 1989; Yonesaki & Minagawa, 1989), three other phage-encoded proteins. SEP1 is stimulated by a yeast single-stranded DNA binding protein (Heyer & Kolodner, 1989), while STPα is stimulated by a series of putative single-stranded DNA binding proteins that are thought to act by aggregating the substrate DNAs (Hamatake et al., 1989).

In this and the following paper (Norris & Kolodner, 1990), we report the purification and characterization of a new factor from mitotic yeast cells which stimulates SEP1, the mitotic strand exchange protein, to an unprecedented degree. This new factor, designated SF1 for *s*timulatory *f*actor *1*, stimulates SEP1 >300-fold, thereby allowing significant strand exchange to occur at SEP1 concentrations as low as 1 molecule of SEP1 per 5800 nucleotides of single-stranded DNA.

Materials and Methods

Strains. *Saccharomyces cerevisiae*, *Escherichia coli*, and bacteriophage strains were the same as described previously (Kolodner et al., 1987).

Enzymes and Chemicals. Enzymes and chemicals were the same as described previously (Kolodner et al., 1987). Single-stranded and double-stranded DNA–cellulose was prepared by the method of Alberts and Herrick (1971) and contained >1 mg of DNA/mL of bed volume [modification as described in Heyer and Kolodner (1989)]. Histone H1 was from Boehringer Mannheim. SEP1, kindly supplied by Dr. W.-D. Heyer, was purified as described previously (Kolodner et al., 1987). The preparation of SEP1 used was greater than 98% pure and had a protein concentration of 1.46 mg/mL.

Nucleic Acids. Viral and RF forms of M13mp19 bacteriophage DNA were obtained as described in the following paper (Norris & Kolodner, 1990). Concentrations of DNA are expressed in terms of moles of nucleotides. Tritium-labeled M13mp19 DNAs had specific activities of 5280 cpm/nmol for double-stranded DNA and 3795 cpm/nmol for single-stranded DNA.

Assay for Strand Exchange Stimulatory Factors. Assays were carried out in 30-μL volumes containing 33 mM Tris-HCl (pH 7.5), 13 mM $MgCl_2$, 1.8 mM dithiothreitol, 88 μg/mL bovine serum albumin, 0.6 nmol of *Eco*RI-cleaved linear duplex DNA (M13mp19 RF DNA), and 0.3 nmol of circular single-stranded DNA (viral M13mp19 DNA). In addition to the above, the standard reaction mixture contained purified SEP1, the yeast mitotic strand exchange protein, at a concentration that was insufficient to catalyze the strand exchange reaction (<250 ng per 30-μL reaction). All reactions were incubated at 30 °C for 20 min unless otherwise noted. Reactions were terminated, and the reaction products were analyzed by agarose gel electrophoresis, as described previously (Kolodner et al., 1987). During the original screen for stimulatory factors, an ATP-regenerating system (Kolodner et al., 1987) was also included in the reaction mixture. As determined by the agarose gel assay, however, the protein described in this paper stimulated joint molecule formation in the absence of ATP as efficiently as it did in the presence of ATP (Table I). The ATP-regenerating system was therefore excluded from the reaction mixtures during the subsequent analysis.

Purification of a Strand Exchange Stimulatory Factor. The strand exchange stimulatory factor (SF1) copurified with the mitotic strand exchange protein (SEP1) through the first four steps in the published purification protocol for SEP1 (Kolodner et al., 1987). These steps, starting with 800 g of cells, included the crude cell extract (fraction I, 3625 mL, 16.95 mg/mL), the ammonium sulfate fraction (fraction II, 1650 mL, 25.60 mg/mL), chromatography on single-stranded DNA–cellulose and subsequent ammonium sulfate precipitation (fraction III, 15 mL, 11.15 mg/mL), and size fractionation on Sephacryl S200 (fraction IV, 75 mL, 0.83 mg/mL). SF1 separated from SEP1 during the next purification step: Fraction IV was applied at 8.5 mL/h to a 0.64 cm^2 × 18.5 cm column of PBE94 (Pharmacia) equilibrated with buffer A [20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 mM PMSF, and 10% w/v glycerol] containing 100 mM NaCl, and then the column was washed

at the same flow rate with buffer A containing 100 mM NaCl. The peak of protein that flowed through the column, as monitored by UV absorption, was collected, yielding 80 mL of a 0.30 mg/mL protein solution (fraction V) (SEP1 binds to PBE94 under these conditions). Fraction V was applied at 18 mL/h to a 1.77 $cm^2 \times 17$ cm column of single-stranded DNA–cellulose equilibrated in buffer A containing 100 mM NaCl. After the column was washed with 30 mL of buffer A containing 100 mM NaCl, the column was eluted, at the same flow rate, with a 300-mL linear gradient from 100 to 600 mM NaCl in buffer A. The active fractions, which eluted at 210–280 mM NaCl, were dialyzed against 1 L of buffer A containing 30 mM NaCl for 2 h at 0 °C. This yielded 33 mL of a 0.37 mg/mL protein solution (fraction VI). Fraction VI was applied at 7 mL/h to a 0.64 $cm^2 \times 4.4$ cm column of double-stranded DNA–cellulose equilibrated in buffer A containing 30 mM NaCl, and then the column was washed at the same flow rate with buffer A containing 30 mM NaCl. The peak of protein that flowed through the column, as monitored by UV absorption, was collected, yielding 30 mL of a 0.29 mg/mL protein solution (fraction VII). Fraction VII was applied at 6.6 mL/h to a 0.64 $cm^2 \times 6$ cm column of hydroxylapatite equilibrated in buffer B [50 mM potassium phosphate (pH 7.3), 10 mM 2-mercaptoethanol, 0.1 mM PMSF, and 10% glycerol]. After being washed with 4 mL of buffer B, the column was eluted, at the same flow rate, with an 80-mL linear gradient from buffer B to buffer B in which the concentration of potassium phosphate (pH 7.3) was 800 mM. The active fractions, eluting at 300–400 mM potassium phosphate, were dialyzed overnight at 0 °C against 1 L of buffer A containing 100 mM NaCl and 60% (w/v) glycerol, yielding 4.5 mL of a 1.5 mg/mL protein solution (fraction VIII). The protein soluion was stored at −20 °C and remained stable for at least 1 year. One microgram of fraction VIII, either in the presence or in the absence of ATP, digested <1 pmol of single-stranded or double-stranded DNA in a 1-h reaction at 30 °C (less than six nucleotides solubilized per DNA end under the conditions of the strand exchange reaction), indicating that fraction VIII did not contain contaminating nuclease activity (data not shown).

Electron Microscopy. Strand exchange assays were terminated by adding ethylenediaminetetraacetic acid, proteinase K, and sodium dodecyl sulfate to final concentrations of 50 mM, 600 μg/mL, and 0.1%, respectively. After incubation at 37 °C for 15 min, reactions were chromatographed over 1.5-mL columns of A-5m (Bio-Rad) in 10 mM Tris, pH 7.5, and 1 mM EDTA, and the eluent was mounted for electron microscopy by the formamide technique (Davis et al., 1971).

DNA Aggregation. DNA aggregation was assayed, with slight modifications, as described previously (Chow & Radding, 1985; Tsang et al., 1985; Heyer et al., 1988). SF1 was added to a 30-μL reaction mixture containing 33 mM Tris-HCl (pH 7.5), 13 mM $MgCl_2$, 1.8 mM dithiothreitol, 88 μg/mL bovine serum albumin, and, where indicated, either 0.6 nmol of *Eco*RI-cleaved M13 RF DNA (^{3}H-labeled or nonradioactive), 0.3 nmol of viral M13 DNA (^{3}H-labeled or nonradioactive), or combinations of both. After incubation at 30 °C for 2 min, the reaction mixtures were centrifuged for 3 min at 12000 rpm in an Eppendorf microcentrifuge. A 9-μL aliquot was removed from the supernatant and added to 100 μL of 0.1% SDS. This was repeated 2 more times. The remaining 3 μL and the pellet were then resuspended in 100 μL of 0.1% SDS. The radioactivity in the aliquots was determined by adding each to 3 mL of Ready Safe liquid scintillation cocktail (Beckman) and counting on a Beckman LS 3801 scintillation counter. The amount of DNA in the total supernatant was estimated from the average radioactivity of the first two aliquots of the supernatant. The amount of radioactivity in the pellet was corrected for the presence of 3 μL of supernatant.

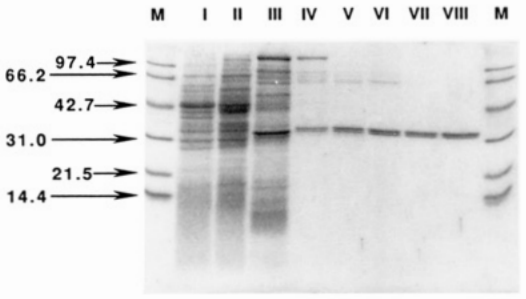

FIGURE 1: Electrophoretic analysis of purified protein fractions. The indicated lanes contained 10, 17.35, 12.75, 1, 0.6, 0.6, 0.5, and 0.5 μg of fractions I, II, III, IV, V, VI, VII, and VIII, respectively. The markers, from top to bottom, were phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. Electrophoresis was on a 0.75-mm-thick, 12% polyacrylamide mini-gel containing $NaDodSO_4$ (Kolodner et al., 1987).

An alternative method was used when DNA aggregation was assayed in conjunction with strand exchange. After a standard strand exchange reaction, the reaction mixtures were centrifuged for 3 min at 12000 rpm in an Eppendorf microcentrifuge. The supernatant was collected, and the pellet was resuspended in 30 μL of 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. The supernatant and the pellet were subsequently electrophoresed on a 1% agarose gel as described above, and the amounts of DNA in both fractions were determined by densitometric scanning.

Results

Purification of a Strand Exchange Stimulatory Factor. A M_r 132000 polypeptide has been purified from mitotic yeast cells which transfers a single strand of DNA from a linear, duplex molecule (linear M13 RF) to a homologous, single-stranded, circular molecule (viral M13) (Kolodner et al., 1987). This protein, which is designated SEP1, catalyzes strand exchange only when present at high stoichiometries [optimal stoichiometry is 1 SEP1 molecule/12–14 nucleotides of single-stranded DNA (Kolodner et al., 1987)]. To identify other proteins involved in the strand exchange reaction, we analyzed mitotic yeast extracts for the presence of factors that allowed SEP1 to function at lower stoichiometries. The purification of a factor with this characteristic is described under Materials and Methods and is summarized in Figure 1.

The end product of the purification was a fraction consisting of two polypeptides with apparent molecular weights, as determined by SDS–PAGE, of 32000 and 33000 (Figure 1, fraction VIII). These two polypeptides, in addition to cochromatographing with the stimulatory activity over the last four purification steps, also cochromatographed with the activity over a CM-HW65 cation-exchange column (Toyosoda; data not shown) and cosedimented with the activity in sucrose gradients (Norris & Kolodner, 1990). The stimulatory activity therefore appears to reside in these polypeptides. Tryptic mapping showed that these two polypeptides were nearly identical (Figure 2), indicating that they were different forms of the same protein, which we designate SF1 for *s*timulatory *f*actor *1*. Five of the tryptic fragments were sequenced, yielding 48 amino acids of unique sequence (data not shown). When these sequences were compared to the PIR data base (release 22), no significant matches were found. Recently, the DNA sequence of the gene encoding the SEP1 strand

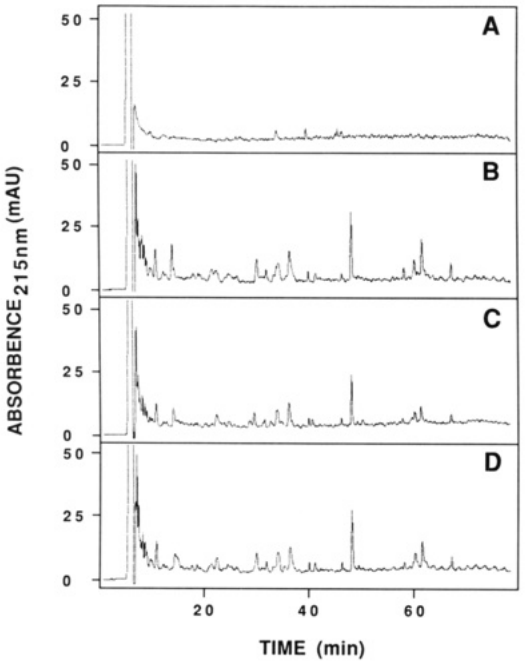

FIGURE 2: Comparative tryptic maps of the M_r 32 000 and the M_r 33 500 polypeptides. Fifteen micrograms of fraction VIII was electrophoresed through a 12% polyacrylamide gel containing $NaDodSO_4$ and then electrophoretically transferred to nitrocellulose. After transfer, the individual polypeptide bands were visualized by staining in Poinceau S, excised with a razor blade, and digested in situ with trypsin (Aebersold et al., 1987). The resulting trypsin fragments were analyzed by high-performance liquid chromatography using an HP1090 chromatograph and a Vydac, 2.1 mm × 25 cm, 5-μm particle size, C4 column essentially as described (Aebersold et al., 1987). (A) Nitrocellulose blank. (B) M_r 33 500 polypeptide fragments obtained from 2 μg of the M_r 33 500 polypeptide applied to the gel. (C) M_r 32 000 polypeptide fragments obtained from 2 μg of the M_r 32 000 polypeptide applied to the gel. (D) Mixture of the M_r 33 500 and the M_r 32 000 polypeptide fragments obtained from 1 μg of each polypeptide applied to the gel.

SEP1	+	+	+	+	+	+	+	+	+	-
SF1	+	+	+	+	+	+	+	+	-	+
TIME (MIN)	0	2	5	10	20	30	40	60	60	60

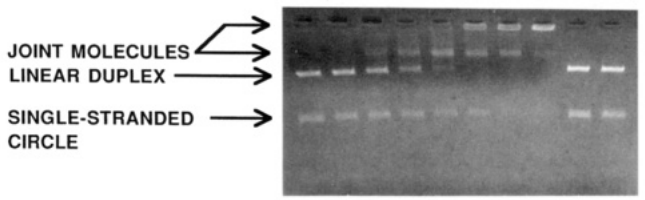

FIGURE 3: Analysis of the stimulated strand exchange reaction by agarose gel electrophoresis. SEP1, where indicated, was present at a concentration of 1 SEP1 molecule per 180 nucleotides of single-stranded DNA. SF1, where indicated, was present at a concentration of 1 SF1 molecule per 20 nucleotides of single-stranded DNA. The reactions were incubated at 30 °C for the indicated times as described under Materials and Methods. At the completion of each reaction, the reactions were terminated with EDTA, deproteinized with proteinase K and $NaDodSO_4$, and electrophoresed through a 1% agarose gel in the presence of 0.5 μg/mL ethidium bromide.

exchange protein has been determined (D. Tishkoff and R. D. Kolodner, unpublished results); the sequences of the tryptic fragments from SF1 did not match any sequences in the SEP1 gene, indicating that SF1 is not a fragment of the mitotic strand exchange protein (SEP1) itself.

Characterization of the Stimulated Strand Exchange Reaction. An agarose gel electrophoresis assay was used to characterize the stimulated in vitro strand exchange reaction

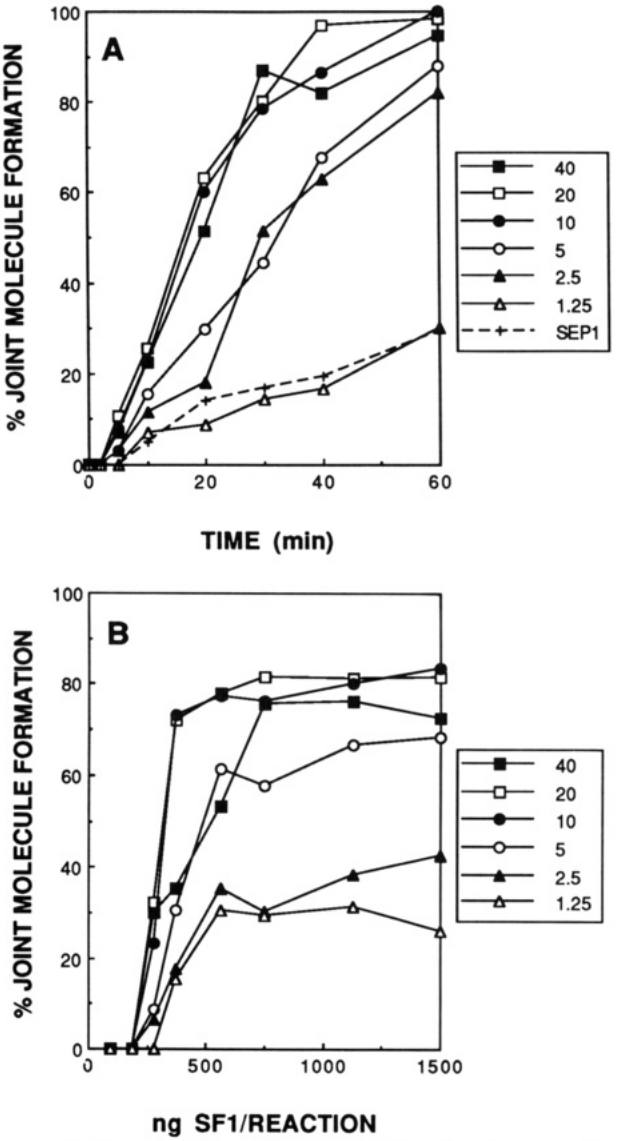

FIGURE 4: Characterization of the stimulated strand exchange reaction. Joint molecule formation was quantitated by densitometric scanning of agarose gels. Each curve represents a reaction run in the presence of a fixed concentration of SEP1. The numbers in the legends indicate the number of SEP1 molecules per single-stranded M13mp19 circle (7250 nucleotides) in each experiment. (A) Kinetics of the stimulated strand exchange reaction. All reactions contained 1 SF1 molecule per 20 nucleotides of single-stranded DNA. The dashed line represents a strand exchange reaction containing no SF1 in which SEP1 was present at a concentration of 480 molecules per single-stranded M13mp19 circular DNA. (B) Titration of SF1 required for stimulation. All reactions were incubated for 20 min.

(Kolodner et al., 1987). An example of an assay is shown in Figure 3. The basic reaction mixture, which contained no SF1 and a low concentration of SEP1 (1 molecule of SEP1 per 180 nucleotides of single-stranded DNA), did not catalyze strand exchange. SF1, when present alone at its optimal concentration for stimulation, was unable to catalyze strand exchange. However, when SF1 and SEP1 were both present, a band appeared within 5 min that migrated where joint molecules are known to migrate (Kolodner et al., 1987). By 30 min, a second type of reaction product appeared which was apparently too large to migrate out of the well. The generation of these two forms of joint molecules was dependent on homology as neither was formed when ΦX174 viral DNA was substituted for M13 viral DNA in the assay (data not shown).

To determine the amount of stimulation conferred on SEP1 by SF1, the time course shown in Figure 3 was repeated using

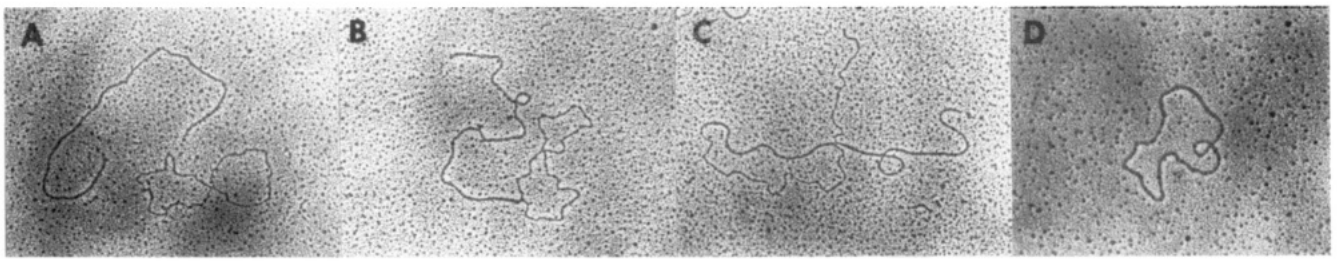

FIGURE 5: Electron microscopic analysis of the products of the stimulated strand exchange reaction. (A) Single-stranded, circular M13mp19 DNA and double-stranded, linear M13mp19 DNA. (B) σ molecule. (C) α molecule. (D) Double-stranded, circular M13mp19 DNA.

decreasing concentrations of SEP1, and the amount of joint molecule formation was determined by densitometry (Figure 4A). When present at its optimal stoichiometry, SF1 stimulated strand exchange such that significant joint molecule formation occurred at concentrations as low as 1.25 SEP1 molecules per single-stranded M13 molecule (1 molecule of SEP1 per 5800 nucleotides of single-stranded DNA). Moreover, joint molecule production at 1.25 SEP1 molecules per single-stranded DNA molecule essentially paralleled that seen in an unstimulated reaction (no SF1) containing an optimal amount of SEP1 (480 SEP1 molecules per single-stranded M13 DNA molecule). Therefore, the amount of SEP1 required to catalyze strand exchange decreased more than 300-fold in the presence of SF1.

The data in Figure 4A showed that the rate and the extent of joint molecule formation increased as the concentration of SEP1 increased from 1.25 to 10 molecules per single-stranded DNA molecule. At concentrations greater than 10 SEP1 molecules per single-stranded DNA molecule, however, no further increases were observed. Therefore, in the presence of optimal amounts of SF1, no more than 10 SEP1 molecules per single-stranded DNA molecule (1 SEP1 molecule per 725 nucleotides) are required for maximal catalysis of strand exchange. Under these conditions, the rate of joint molecule formation increased 3–4-fold over that seen in the unstimulated reaction (Figure 4A).

The optimal amount of SF1 required for stimulation of SEP1 has also been determined (Figure 4B). The results of this analysis indicated that approximately 500 ng of SF1 per 30-μL reaction was required for maximal stimulation. Assuming a monomer molecular weight for SF1 of 33 000 [see Norris and Kolodner (1990)], this is 1 molecule of SF1 per 20 nucleotides of single-stranded DNA. The results also indicated that the amount of SF1 required for stimulation was roughly independent of the amount of SEP1 in the reaction mixture (Figure 4B).

The reaction requirements of the stimulated reaction were determined by using the agarose-gel electrophoresis assay (Table I). The stimulated reaction had no requirement for ATP. The reaction required magnesium ions and was dependent on the presence of at least one reduced sulfhydryl group. These requirements are similar to those for SEP1 alone (Kolodner et al., 1987).

Electron Microscopic Analysis of the Products of the Stimulated Strand Exchange Reaction. It was important to determine whether the end products scored in the electrophoretic assay were bona fide joint molecules. Therefore, several different stimulated strand exchange reactions were examined by electron microscopy. The observed products in these reactions could be grouped into three general catagories of joint molecules. The first class contained σ molecules (Figure 5B), in which the double-stranded linear and single-stranded circle interacted over an extremely small region at the end of the double-stranded linear. The second class contained α molecules (Figure 5C), in which the circle was partially single- and partially double-stranded, and both a double-stranded tail and a single-stranded tail emanated from one end of the joint. The third class contained double-stranded circles (Figure 5D). The distributions of molecules in these three catagories are presented in Table II. Two points can be made from this electron microscopic analysis: first, the existence of α forms indicated that joint molecules in the stimulated reaction were products of a bona fide strand exchange reaction; second, the observed distributions of joint molecules in the stimulated reaction were similar to the distribution seen in an unstimulated reaction at optimal concentrations of SEP1 (Kolodner et al., 1987).

Our attempts to visualize the molecules remaining at the top of the gel after electrophoresis were not completely successful (Figure 3). These molecules failed to enter an agarose A-5m column and, hence, were removed from the reaction mixture prior to spreading (Materials and Methods). Moreover, when this band was cut out of the gel and subjected to the "freeze-squeeze" mounting protocol (Thuring et al., 1975), the molecules observed were standard σ forms, α forms, and double-stranded circles (data not shown). Thus, although the nature of the higher order association remains unclear, this last observation indicated that at least some of the molecules remaining at the top of the gel were composed of standard joint molecules.

Table I: Summary of Reaction Requirements[a]

	% joint molecule formation
complete	100
$-Mg^{2+}$	<2
−BSA	94.6
−DTT	74.7
+1.3 mM ATP	89.6
−dithiothreitol + 5 mM *N*-ethylmaleimide	7.6

[a] Reaction mixtures were incubated at 30 °C for 20 min and contained 1 SEP1 molecule per 180 nucleotides of single-stranded DNA and 1 SF1 molecule per 20 nucleotides of single-stranded DNA. A relative activity of 100% is equivalent to the amount of joint molecule formation in the standard reaction (Materials and Methods), which was, in this experiment, 53.8%.

Table II: Electron Microscopic Analysis of Strand Exchange[a]

no. of SEP1 molecules per viral M13mp19 DNA	time (min)	σ (%)	α (%)	monomer ds circle (%)	total molecules counted
0	60	0	0	0	250
1.25	20	3.5	3.1	0	254
1.25	60	7.5	11.4	3.0	255
20	20	4.5	25.4	4.0	224

[a] Reaction mixtures contained 1 SF1 molecule per 20 nucleotides of single-stranded DNA and the indicated amount of SEP1 per M13mp19 single-stranded circle. Reactions were processed for electron microscopy as described under Materials and Methods. Representative electron micrographs are presented in Figure 6.

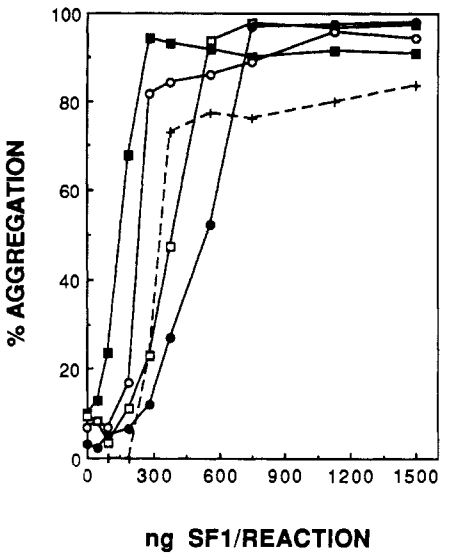

FIGURE 6: Aggregation of single-stranded and double-stranded DNA by SF1. Reaction mixtures (30 μL) contained 33 mM Tris-HCl (pH 7.5), 13 mM $MgCl_2$, 1.8 mM dithiothreitol, 88 μg/mL BSA, and, where indicated, 0.6 nmol of *Eco*RI-cleaved M13mp19 RF DNA (^{3}H-labeled or nonradioactive), and, where indicated, 0.3 nmol of M13mp19 viral DNA (^{3}H-labeled or nonradioactive). Reactions were incubated for 2 min in the presence of the indicated amounts of SF1 and were processed as described under Materials and Methods. (■) ^{3}H-Labeled single-stranded DNA aggregation; (□) ^{3}H-labeled double-stranded DNA aggregation; (○) ^{3}H-labeled single-stranded DNA aggregation in the presence of unlabeled double-stranded DNA; (●) ^{3}H-labeled double-stranded DNA aggregation in the presence of unlabeled single-stranded DNA. Included in the figure (dashed line) is the percent strand exchange catalyzed by SEP1 (1 monomer per 725 nucleotides of single-stranded DNA) and the indicated amounts of SF1 in a 20-min reaction (Figure 4).

Role of DNA Aggregation in the Stimulated Strand Exchange Reaction. Highly basic molecules, such as spermidine or histones, stimulate the in vitro enzymatic activities of several enzymes that interact with DNA, including *E. coli* DNA gyrase (Krasnow & Cozzarelli, 1982) and yeast STPα (Hamatake et al., 1989). These positively charged molecules are stimulatory by virtue of their ability to nonspecifically compact nucleic acids into huge (>25 000 S) aggregates, thereby increasing the effective local concentration of substrate molecules (Krasnow & Cozzarelli, 1982). The mechanism of DNA aggregation is best explained by the theory of counterion condensation proposed by Manning (1978). We attempted to determine what role, if any, this kind of aggregation played in the SEP1–SF1 stimulated strand exchange reaction.

As seen in Figure 6, SF1 actively aggregated single-stranded and double-stranded DNA. Since SF1 also failed to bind to an anion-exchange column (PBE, Pharmacia), but bound well to a cation-exchange column (CM-HW65, Toyosoda), the protein appeared to be basic. This was confirmed by analyzing the electrophoretic behavior of SF1 on the two-dimensional gel system of O'Farrell (1975). When such a gel system was used, SF1 exhibited a p*I* of approximately 9.2 and, therefore, was positively charged under the conditions used for the strand exchange reaction. On the basis of the precedence of spermidine and histones, these results could be interpreted to suggest that SF1 was stimulatory merely as a consequence of its ability to aggregate DNA.

Aggregation, however, did not appear to account entirely for the stimulatory activity of SF1. Included in Figure 6 is a curve showing percent joint molecule formation as a function of SF1 concentration in the presence of 1 SEP1 monomer per 725 nucleotides of single-stranded DNA. As can be seen, high levels of joint molecule formation occurred under conditions

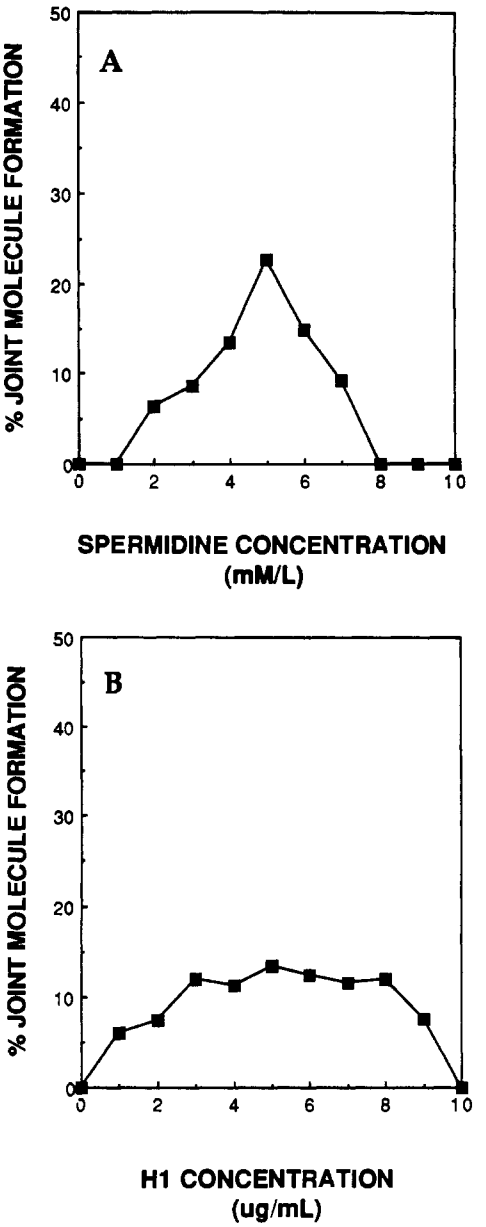

FIGURE 7: Titrations of spermidine and histone H1 in the strand exchange stimulation assay. Reaction mixtures (30 μL) contained 33 mM Tris-HCl (pH 7.5), 13 mM $MgCl_2$, 1.8 mM dithiothreitol, 88 μg/mL BSA, 0.6 nmol of *Eco*RI-cleaved M13mp19 RF DNA, 0.3 nmol of M13mp19 viral DNA, and 250 ng of SEP1. Reactions were incubated for 30 min in the presence of the indicated amounts of stimulant and were analyzed by gel electrophoresis as described under Materials and Methods. (A) Joint molecule formation in the presence of spermidine. (B) Joint molecule formation in the presence of histone H1.

where SF1 did not aggregate double-stranded DNA in the presence of single-stranded DNA. This suggested that SF1 was not acting by the same mechanism as spermidine or histones, since these latter aggregants were stimulatory by virtue of their abilities to aggregate all substrate molecules (Krasnow & Cozzarelli, 1982; Hamatake et al., 1989).

To more directly test this point, we compared the stimulatory effects of SF1, spermidine, and histone H1 in the strand exchange reaction. When present at their optimal concentrations for stimulation—5 mM for spermidine and 5 μg/mL for histone H1 (Figure 7)—spermidine and histone H1 were approximately 15–20-fold less stimulatory than SF1 (Figure 8). In fact, maximal joint molecule formation in the SF1-stimulated reaction could be observed with levels of SEP1 where the spermidine- or histone-stimulated reactions exhibited

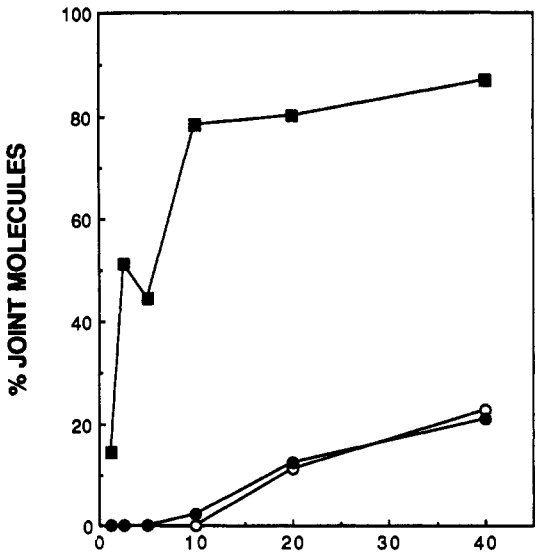

FIGURE 8: Relative stimulation of SEP1 by SF1, spermidine, and histone H1. Reaction mixtures (30 μL) contained 33 mM Tris-HCl (pH 7.5), 13 mM $MgCl_2$, 1.8 mM dithiothreitol, 88 μg/mL BSA, 0.6 nmol of *Eco*RI-cleaved M13mp19 RF DNA, and 0.3 nmol of M13mp19 viral DNA. Reactions were incubated for 30 min in the presence of the indicated amounts of SEP1 and were analyzed by gel electrophoresis as described under Materials and Methods. Reactions were stimulated by (■) SF1 at 1 SF1 monomer per 20 nucleotides of single-stranded DNA, (○) spermidine at 5 mM final concentration, and (●) histone H1 at 5 μg/mL final concentration.

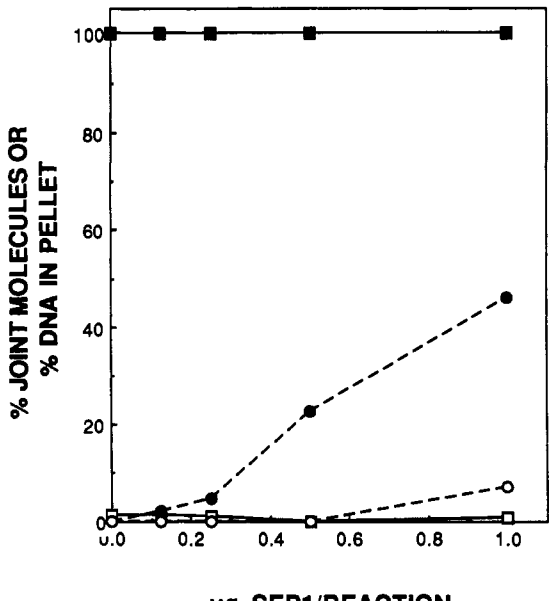

FIGURE 9: Aggregation and strand exchange by SEP1 and SF1 in the presence of 40 mM potassium glutamate. Reaction mixtures (30 μL) contained 33 mM Tris-HCl (pH 7.5), 40 mM potassium glutamate, 13 mM $MgCl_2$, 1.8 mM dithiothreitol, 88 μg/mL BSA, 0.6 nmol of *Eco*RI-cleaved M13mp19 RF DNA, 0.3 nmol of M13mp19 viral DNA, and, where indicated, SF1 at 1 monomer per 20 nucleotides of single-stranded DNA. All incubations were for 30 min in the presence of the indicated amounts of SEP1. The reaction mixtures were centrifuged for 3 min at 12 000 rpm in an Eppendorf centrifuge, and the supernatants and pellets were electrophoresed on a 1% agarose gel. The relative amount of DNA in each fraction was quantitated by densitometry. (■) Percent of circular single-stranded DNA that was in the pellet (SF1 present); (□) percent of linear double-stranded DNA that was in the pellet (SF1 present); (●) percent joint molecule formation in the presence of SF1; all joint molecules were in the pellets; (○) percent joint molecule formation in the absence of SF1; all joint molecules were in the pellets.

no joint molecule formation at all. This suggested that aggregation by itself did not appear to account for the entire stimulation imparted on SEP1 by SF1.

Further support for this notion came from a qualitative examination of the SF1 titration curves shown in Figure 4B. As can be seen in this figure, approximately 500 ng of SF1 per reaction was required for optimal stimulation of SEP1, while the addition of more SF1 had no apparent effect on joint molecule production (Figure 4B). This contrasted with previously published titration curves for spermidine or histones (Krasnow & Cozzarelli, 1982; Hamatake et al., 1989). When these agents were used to stimulate gyrase or STPα, a sharp transition to complete aggregation occurred when approximately 90% of the charge on the DNA molecules was neutralized; this was also the point at which the stimulation occurred (Krasnow & Cozzarelli, 1982). However, when additional spermidine or histones were added, thereby neutralizing more than 90% of the charge, the reactions were completely inhibited, even though the substrate molecules remained aggregated (Krasnow & Cozzarelli, 1982; Hamatake et al., 1989). In other words, the titration curves showed a peak of maximal activity rather than a plateau as seen with SF1 (Figure 4B). Since spermidine and histone H1 also showed this characteristic stimulatory peak for SEP1 (Figure 7), the activity of SF1 did not appear to result wholly from its ability to nonspecifically neutralize negative charges on the DNA molecule.

The results in Figure 6 showed that SF1, when present at an appropriate concentration, stimulated SEP1 in the absence of double-stranded DNA aggregation. Under these conditions, SF1 was not acting merely by increasing the local effective concentration of substrates since only one of the substrates aggregated. The window, however, between single-stranded and double-stranded DNA aggregation was relatively narrow, making exact interpretation of the experiment difficult. We attempted, therefore, to more definitively establish conditions under which double-stranded DNA was not aggregated. To develop such conditions, DNA aggregation was analyzed in the presence of increasing concentrations of salt. The salt used in these experiments was potassium glutamate, chosen primarily because it is likely to be the most common in vivo salt (Measures, 1975) and because it is closely related to another compound, polyglutamic acid, which has been shown to abolish DNA aggregation in an in vitro chromatin assembly assay (Stein & Bina, 1984). Titration experiments indicated that SF1 was unable to aggregate double-stranded DNA in the presence of 40 mM potassium glutamate, although it still fully aggregated single-stranded DNA (Figure 9 and unpublished observations). Importantly, SF1 was still stimulatory under these conditions, although the amount of SEP1 required for activity was higher than in the absence of the salt (compare Figures 4 and 9). Therefore, on the basis of this experiment and the ones described in Figures 6 and 8, we conclude that stimulation by SF1 does not arise merely as a consequence of its ability to induce DNA aggregation.

As a final note, it is important to understand that the aggregates formed by SF1 differ from the large molecules that remain at the top of the gel in the stimulated strand exchange reaction (Figure 4). These latter molecules only appeared after prolonged incubation with *both* SF1 and SEP1, were completely dependent on homology, and were stable after deproteinization. By contrast, the aggregates in Figure 6 formed very rapidly in the *absence* of SEP1, were not dependent on homology, and were sensitive to deproteinization (data not shown).

Discussion

We have purified a factor from yeast cells that stimulated the strand exchange activity of SEP1, the mitotic strand exchange protein. This protein, which has been designated SF1, had no significant homology with any protein present in the PIR data base. SF1 had a molecular weight of approximately 33 000 and existed in solution as an asymmetric monomer (Norris & Kolodner, 1990). In the presence of SF1, the optimal stoichiometry of SEP1 in the strand exchange reaction decreased from 1 molecule of SEP1 per 12–14 nucleotides to 1 molecule of SEP1 per 725 nucleotides of single-stranded DNA. Decreasing the level of SEP1 to 1 molecule per 5800 nucleotides of single-stranded DNA reduced the rate and extent of the reaction to the level observed with saturating amounts of SEP1 in the absence of SF1. The optimal amount of SF1 for stimulation was 1 molecule of SF1 per 20 nucleotides of single-stranded DNA. The stimulated reaction had requirements that were essentially identical with those seen in the unstimulated reaction, including a lack of dependence on ATP.

Electron microscopic analysis indicated that bona fide strand exchange produced the observed joint molecules in the stimulated reaction. The three expected classes of joint molecules, σ forms, α forms, and double-stranded circles, were observed in the stimulated reaction, and the distribution of joint molecules into these three classes approximated the distribution seen in an unstimulated reaction at optimal concentrations of SEP1 (Kolodner et al., 1987). Moreover, the existence of single-stranded tails of DNA on the α joint molecules ruled out any models of stimulation in which SF1 was acting as a nuclease. In particular, SF1 did not appear to act by exposing, on the double-stranded DNA substrate, a single-stranded tail which was used as a substrate for renaturation by SEP1. This conclusion was also supported by the finding that SF1 had no nuclease activity.

Previous studies have shown that basic molecules, such as spermidine or histones, stimulate the activity of proteins that interact with DNA (Krasnow & Cozzarelli, 1982; Hamatake et al., 1989). The stimulatory effect of these agents arises as a consequence of their abilities to compact the substrate DNA molecules into huge aggregates (Manning, 1978). While SF1 was able to aggregate DNA, this characteristic could not explain the quantitative and qualitative properties of stimulation imparted on SEP1 by SF1. For instance, concentrations of SEP1 could be found which produced joint molecules at the maximal rate in the presence of SF1, but which produced no joint molecules in the presence of concentrations of spermidine or histones shown to be optimal for stimulation. Furthermore, it was possible to develop reaction conditions in which strand exchange occurred in an SF1-dependent fashion even though one of the substrate molecules remained in solution. These observations distinguish SF1 from spermidine and histones, thereby supporting the idea that SF1 represents a specific strand exchange stimulatory factor.

It is unclear how SEP1 catalyzes homologous pairing and strand exchange or how SF1 stimulates this reaction. The observation that only 1–2 SEP1 molecules per single-stranded DNA molecule are required in the presence of SF1, while 500 are required in the absence of SF1, clearly suggests that SF1 substantially alters the reaction mechanism of SEP1-mediated strand exchange. It also suggests that certain aspects of the mechanism proposed for RecA and UvsX proteins, in particular the obligatory nucleoprotein intermediate, may not apply to SEP1 (Cox & Lehman, 1987; Griffith & Harris, 1988). In order to understand how SEP1 catalyzes strand exchange and how SF1 stimulates this activity, it is necessary to have a better understanding of the biochemical function of SF1. In the following paper (Norris & Kolodner, 1990), we analyze the properties of SF1 in the absence of SEP1 and propose several ideas about how SEP1 and SF1 might interact to catalyze strand exchange.

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Interaction of a *Saccharomyces cerevisiae* Strand Exchange Stimulatory Factor with DNA†

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ABSTRACT: In the preceding paper (Norris & Kolodner, 1990), we described the purification of a M_r 33 000 polypeptide which dramatically stimulated the activity of SEP1, the yeast mitotic strand exchange protein. In this paper, we characterized this new protein, which was designated SF1, in the absence of SEP1. SF1 had a sedimentation coefficient of 1.7 S and a Stokes radius of 30 Å, which was consistent with a calculated native molecular weight of 31 000, indicating that SF1 existed in solution as a monomer. Filter binding assays showed that SF1 bound preferentially to single-stranded rather than double-stranded DNA. Fluorescence spectroscopy analysis indicated that SF1 occluded approximately eight nucleotides when bound to single-stranded DNA and exhibited a dissociation constant, K_D, of 2.83×10^{-6} M. The binding of SF1 to single-stranded DNA was noncooperative and appeared to involve at least one tyrosine residue. SF1, in the absence of SEP1, stimulated the renaturation of homologous single-stranded DNA, suggesting that it might act directly in some phase of the strand exchange reaction.

In the preceding paper (Norris & Kolodner, 1990), we described the purification of a M_r 33 000 polypeptide which dramatically stimulated the enzymatic activity of the SEP1 protein, the mitotic yeast strand exchange protein (Kolodner et al., 1987; Heyer et al., 1988). In the presence of this new protein, which was designated SF1 for *s*timulatory *f*actor *1*, the optimal amount of SEP1 in the in vitro strand exchange reaction dropped from 1 SEP1 monomer per 10–12 nucleotides to 1 SEP1 monomer per 725 nucleotides of single-stranded DNA. Even at levels as low as 1 monomer of SEP1 per 5800 nucleotides of single-stranded DNA, the rate of joint molecule formation in the presence of SF1 was equivalent to the maximal rate that could be catalyzed by SEP1 alone. The SF1 protein, however, did not appear to change any of the other characteristics of the SEP1-catalyzed reaction: the reaction requirements of the simulated and unstimulated reactions were the same, and the distributions of joint molecule products in the two reactions were nearly identical. To confer the observed levels of stimulation, SF1 had to be present at the relatively high stoichiometry of 1 SF1 monomer per 20 nucleotides of single-stranded DNA.

When compared to other purified strand exchange stimulatory factors (Cox & Lehman, 1981; Cox et al., 1983; Formosa & Alberts, 1986; Hamatake et al., 1989; Harris & Griffith, 1989; Heyer & Kolodner, 1989; Kodadek & Alberts, 1987; Kodadek et al., 1989; Muniyappa et al., 1984; Roman & Kowalczykowski, 1989; Yonesaki & Minagawa, 1989), SF1 imparted a level of stimulation on its cognate strand exchange protein which was unprecedented. Indeed, the observation that joint molecule formation in the presence of SF1 occurred at an SEP1 concentration of 1 monomer per 5800 nucleotides of single-stranded DNA called into question some accepted ideas about how strand exchange proteins work in vivo, particularly with regard to the presynaptic filament, a proposed obligatory intermediate in the strand exchange reactions catalyzed by the RecA and UvsX proteins (Cox & Lehman, 1987; Griffith & Harris, 1988). It was therefore of interest to understand the mechanism by which SF1 stimulated SEP1. As a first step toward this goal, we decided to characterize SF1 in the absence of SEP1. SF1 was found to be a monomer in solution which bound preferentially to single-stranded DNA

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