

## Purification and Characterization of a DNA-pairing and Strand Transfer Activity from Mitotic *Saccharomyces cerevisiae*\*

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An enzyme catalyzing homologous pairing of DNA chains has been extensively purified from mitotic yeast. The most highly purified fractions are enriched for a polypeptide with a molecular mass of approximately 120 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein-dependent pairing of single-stranded DNAs requires a divalent cation ( $Mg^{2+}$  or  $Ca^{2+}$ ) but proceeds rapidly in the absence of any nucleoside triphosphates. The kinetics of reassociation are extremely rapid, with more than 60% of the single-stranded DNA becoming resistant to S1 nuclease within 1 min at a ratio of 1 protein monomer/50 nucleotides. The results of enzyme titration and DNA challenge experiments suggest that this protein does not act catalytically during renaturation but is required stoichiometrically. The protein promotes formation of joint molecules between linear M13 replicative form DNA (form III) containing short single-stranded tails and homologous single-stranded M13 viral DNA. Removal of approximately 50 nucleotides from the ends of the linear duplex using either exonuclease III (5' ends) or T<sub>7</sub> gene 6 exonuclease (3' ends) activates the duplex for extensive strand exchange. Electron microscopic analysis of product molecules suggests that the homologous circular DNA initially associates with the single-stranded tails of the duplexes, and the heteroduplex region is extended with displacement of the noncomplementary strand. The ability of this protein to pair and to promote strand transfer using either exonuclease III or T<sub>7</sub> gene 6 exonuclease-treated duplex substrates suggests that this activity promotes heteroduplex extension in a nonpolar fashion. The biochemical properties of the transferase are consistent with a role for this protein in heteroduplex joint formation during mitotic recombination in *Saccharomyces cerevisiae*.

Insights into the molecular aspects of homology-dependent recombination in eukaryotes have come, in large part, from studies of fungal systems. The ability to isolate and examine the products of individual meioses has been instrumental in developing models for the biochemical events associated with crossing over. For example, the Holliday model (1) and the Meselson-Radding model (2) were proposed to explain the recovery and distribution of genetic markers during meiotic exchange. More recently, the double strand gap repair model (3) has been proposed to explain certain properties of yeast transformation. A common feature of all these models is the formation of obligatory heteroduplex DNA intermediates containing strands from both parent duplexes. The transfer of homologous single-stranded DNA from one parent molecule to another is thought to be one of the earliest events in pairing and has received biochemical support from studies of the RecA protein of *Escherichia coli* (4-7) and a functionally analogous enzyme purified from the fungus *Ustilago maydis* (8). *In vitro*, these enzymes catalyze the transfer of single-stranded DNA into homologous duplexes to form joint molecules containing a limited heteroduplex junction. The nascent heteroduplex is extended by continued action of RecA protein in a polar reaction (3' to 5' with respect to the invading strand) that requires continuous ATP hydrolysis (9-11). The *U. maydis* Rec1 protein catalyzes a similar ATP-dependent strand exchange reaction but with a polarity opposite (5' to 3') that of RecA protein (12). Like the RecA protein, the Rec1 protein requires ATP for heteroduplex formation and elongation. Recently, proteins from human (13, 14), mouse (15), plant (15), and yeast cells (16, 17) have been isolated which have been implicated in strand exchange.

As part of our interest in the molecular mechanisms of recombination, we have investigated whether mitotic yeast cells contain activities that promote formation of heteroduplex molecules from single-stranded and duplex DNA substrates. Although mitotic recombination is approximately 0.1% of that seen in meiotic cells, treatment of yeast with DNA-damaging agents can stimulate this level 100-1000-fold (18). The low level of recombination observed in mitotic cells likely reflects the lack of suitable substrates for strand exchange rather than a paucity of recombination enzymes. This conclusion is supported by the observations of Szostak and co-workers (19) who have shown that recombination between gapped plasmid DNA and homologous chromosomal regions occurs at high frequency in mitotic yeast cells and proceeds through a heteroduplex intermediate. Furthermore, the results of Simon and Moore (20) argue that single-stranded DNA plays an important role in this process.

Using an assay that was employed in the characterization of the *E. coli* RecA protein and in the isolation of the *U. maydis* Rec1 enzyme, we have purified a protein from *Saccharomyces cerevisiae* which efficiently promotes homologous

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pairing of single-stranded DNA chains as well as pairing between single-stranded DNA and homologous duplex molecules containing short single-stranded tails approximately 50 nucleotides in length. Unlike RecA protein or the Rec1 enzyme, the yeast activity was not stimulated by nucleoside triphosphates and appeared to promote rapid heteroduplex extension in a nonpolar fashion. The biochemical properties of this protein suggested that it is likely to play a role in mitotic recombination in *S. cerevisiae*.

## EXPERIMENTAL PROCEDURES

### Materials

Phenylmethylsulfonyl fluoride, pepstatin, leupeptin, soybean trypsin inhibitor, 4-chloro-1-naphthol, and S1 nuclease were purchased from Sigma. Restriction enzymes, exonuclease III, and polymin P were from Bethesda Research Laboratories. Phosphocellulose (P11) and GF/C filters were from Whatman. Hydroxylapatite and high molecular mass markers for SDS<sup>1</sup>-polyacrylamide gels were from Bio-Rad. Spectropore 2 tubing (Spectrum Medical Industries, Los Angeles, CA) was used for dialysis. Red Star bakers' yeast was purchased from Fast Whipped Foods (Long Beach, CA). Phage T<sub>7</sub> gene 6 exonuclease was purchased from United States Biochemical Corp.

### Yeast Strains

The *S. cerevisiae* laboratory strains used were M12B ( $\alpha$  trp1-289 ura3-52 gal2) (R. Davis) and S288c ( $\alpha$  suc2 mal cup1 gal2).

### Bacterial Strains

*S. typhimurium* strains DB7004 and D21 (leuAam414) were provided by Dr. George Weinstock (University of Texas Houston). *E. coli* strain XY5274 was provided by Dr. Dan Ray (University of California, Los Angeles).

### DNAs

P22 DNA was purified using a protocol provided by Dr. George Weinstock. 100-ml cultures of D21 were grown at 37 °C in M9 medium (21) supplemented with 3% vitamin-free casamino acids to  $A_{595} = 0.5$ . Phage P22 (CI<sup>-</sup> 13am H101) was added to a multiplicity of infection = 5 ( $1 \times 10^{11}$  plaque-forming units), and the infected culture was shaken vigorously for 2.5 h. CHCl<sub>3</sub> was added to saturation to lyse cells, and cellular debris was removed by centrifugation at  $10,000 \times g$  for 10 min. The supernatant was centrifuged at  $30,000 \times g$  for 60 min. Phage pellets were resuspended in 6 ml of buffered saline solution (66 mM potassium phosphate, pH 7.1, 0.85% (w/v) NaCl), layered onto CsCl step gradients (densities of 1.7, 1.5, and 1.4 g/ml), and centrifuged at 30,000 rpm for 45 min in a Beckman SW 41 rotor. Phage bands were collected and dialyzed against 10 mM Tris-HCl, pH 7.5, at 25 °C. DNA was extracted with phenol and precipitated with alcohol. Greater than 1 mg of P22 DNA was obtained from a 100-ml culture of infected cells.

For the preparation of P22 [<sup>3</sup>H]DNA, cells were grown in medium containing 250  $\mu$ g/ml 2'-deoxyadenosine. Ten min after infection, [methyl-<sup>3</sup>H]thymidine (1.5 ml, 1 mCi/ml; Amersham Corp.) was added to the culture, and the cells were allowed to grow for 1.5 h. Cells were collected by centrifugation at  $10,000 \times g$  for 15 min, washed with 50 ml of buffered saline solution, lysed, and DNA was purified as described above. The specific activity of the purified DNA was routinely greater than  $5.0 \times 10^4$  cpm/nmol. <sup>32</sup>P-Labeled P22 DNA was synthesized by nick translation using [ $\alpha$ -<sup>32</sup>P]dCTP (ICN) (22).

$\phi$ X174 RF I DNA was purchased from United States Biochemical Corp. Phage M13 RF I DNA was isolated from infected cells and purified by centrifugation in CsCl gradients containing ethidium bromide. Viral M13 DNA was prepared from purified virions as described (23).

Linear duplex molecules possessing single-stranded termini for use in the joint molecule assay were created by a modification of the method of Wu *et al.* (24). In reactions (50  $\mu$ l) containing 50 mM Tris-HCl, pH 8.0, at 23 °C, 100 mM KCl, 5 mM MgCl<sub>2</sub>, and 5 mM  $\beta$ -mercaptoethanol were added to 10  $\mu$ g of linear duplex DNA and 150 units of exonuclease III or 75 units of T<sub>7</sub> gene 6 exonuclease. Reactions

were incubated at 23 °C for 1.5 min followed by the addition of 5  $\mu$ l of 250 mM EDTA, pH 8.0. The DNA was immediately extracted with phenol and precipitated by the addition of 2 volumes of ethanol. For exonuclease III treatment, M13 RF I DNA was digested with *Bam*HI;  $\phi$ X174 RF I DNA was digested with *Ava*I. For T<sub>7</sub> gene 6 exonuclease treatment, M13 RF I DNA was digested with *Hpa*I;  $\phi$ X174 RF I DNA was digested with *Pst*I.

### Enzyme Assays

Standard reactions used to measure reannealing activity during protein fractionation contained, in 30  $\mu$ l, 20 mM Tris-HCl, pH 7.5, 5 mM CaCl<sub>2</sub>, 1 mM DTT, and 40  $\mu$ M heat-denatured P22 [<sup>3</sup>H]DNA. Incubations were performed for 30 min at 37 °C, at which time 3  $\mu$ l of 10% SDS was added, followed by 270  $\mu$ l of S1 nuclease buffer (75 mM sodium acetate, pH 4.6, 150 mM NaCl, 1 mM zinc acetate). Nuclease digestions were carried out by the addition of 50 units of S1 nuclease and incubating an additional 30 min at 37 °C. Reactions were terminated by the addition of 5  $\mu$ l of heat-denatured calf thymus DNA (5 mg/ml) and placed on ice following addition of 1 ml ice cold 10% trichloroacetic acid. After 10 min, reactions were filtered through GF/C filters (prewet with 10% trichloroacetic acid) and washed with 10 ml of 10% trichloroacetic acid and 5 ml of 95% ethanol. Filters were dried, and radioactivity was determined by liquid scintillation.

To calculate the protein-dependent formation of duplex DNA, the amount of spontaneously renatured DNA was subtracted from total duplex DNA generated in reaction mixtures containing added protein. Spontaneous renaturation was measured as the amount of radioactivity resistant to S1 nuclease digestion following incubation in a reaction lacking yeast protein but otherwise complete. Protein-independent DNA renaturation may be due to inter- and/or intramolecular hydrogen bond formation. This background value was always less than 10% of the total input DNA and was usually less than 5%. DNA available for protein-dependent renaturation was represented by the trichloroacetic acid-precipitable radioactivity in reactions not treated with S1 nuclease minus that precipitated in S1-treated reactions (spontaneous renaturation). Therefore, (cpm (protein dependent) - cpm (spontaneous))/(cpm (total) - cpm (spontaneous)) = percent protein-dependent S1-resistant (duplex) DNA formed. One unit of activity is defined as the amount of protein which converts 1 pmol of single-stranded DNA (in nucleotides) to S1 nuclease resistance/min at 37 °C.

DNA-reannealing activity was also determined by analyzing DNA products on agarose gels. Assay mixtures were prepared as above except 40  $\mu$ M heat-denatured P22 DNA and 20  $\mu$ M viral M13 DNA were used as substrates. Reactions were stopped by the addition of 0.10 volume of gel loading buffer (10  $\times$  TAE, 50% glycerol, 10% SDS, and 0.1% bromophenol blue) and electrophoresed in 0.7% agarose gels. DNAs were stained with ethidium bromide and visualized on a UV transilluminator (1  $\times$  TAE buffer contains 40 mM Tris acetate, 2 mM EDTA).

Filter binding reactions contained 20 mM Tris-HCl, pH 7.5, at 37 °C, 10 mM MgCl<sub>2</sub>, 1 mM DTT, and DNA at concentrations indicated in the Table V legend. Unless otherwise indicated, reactions were performed for 10 min at 37 °C, and aliquots (30  $\mu$ l) received one of the following treatments: (i) addition of 3  $\mu$ l of 10% SDS; (ii) addition of 90  $\mu$ l of 8 M guanidine HCl; (iii) addition of 3  $\mu$ l of 10% SDS and incubation for 2 min at 100 °C; or (iv) no treatment. Reaction mixtures were diluted with 1  $\times$  SSC (1 ml; 1  $\times$  SSC contains 0.15 M NaCl and 15 mM sodium citrate) and passed over alkali-treated nitrocellulose filters (Millipore) (25), previously washed and stored in 1  $\times$  SSC, and washed with 1  $\times$  SSC (10 ml), dried, and radioactivity was determined by liquid scintillation counting. Treatment of the yeast protein-DNA complexes with either SDS or guanidine HCl reduced the amount of labeled P22 DNA bound to the filter by 40%. This same treatment completely abolished binding of viral M13 DNA to alkaline-treated filters.

Joint DNA molecule formation was measured in the same buffer that was used to measure heteroduplex DNA. Typical reaction mixtures contained 20  $\mu$ M resected linear duplex DNA and 10  $\mu$ M viral single-stranded DNA. Reactions were stopped by the addition of agarose gel loading buffer (3  $\mu$ l) and analyzed by agarose gel electrophoresis.

### Electron Microscopy

Reaction mixtures containing 20  $\mu$ M T<sub>7</sub> gene 6 exonuclease-treated or exonuclease III-treated M13 RF III DNA, 20  $\mu$ M single-stranded

<sup>1</sup> The abbreviations used are: SDS, sodium dodecyl sulfate; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PEI, polyethyleneimine; ssDNA, single-stranded DNA.

M13 DNA, and 17.5  $\mu$ g of fraction VI in 100  $\mu$ l of joint molecule assay buffer were incubated for 15 min at 37°C. Reactions were terminated by the addition of SDS to a final concentration of 1%. The sample was applied to a Sepharose CL-4B-200 column (0.5 ml) that had been equilibrated in annealing buffer, and 50- $\mu$ l fractions were collected. Fractions containing DNA were pooled, and an aliquot was made 50% in formamide and analyzed by gel electrophoresis in 1% agarose. The formamide treatment reduced the recovery of joint molecules by as much as 50%. The remaining DNA was spread and prepared for electron microscopy as described (29). The grids were examined, and DNA molecules were photographed using a JEOL JEM-1200EX microscope.

#### Polyacrylamide Gel Electrophoresis

Proteins were electrophoresed in 15-cm polyacrylamide gels (0.75-mm thickness) (30, 31). Samples of protein were mixed with 0.33-volume gel loading buffer (15% (w/v) glycerol, 15% (v/v) 2-mercaptoethanol, 9% SDS (Bethesda Research Laboratories), 200 mM Tris-HCl, pH 6.8, and 0.15% bromophenol blue) which was preheated at 100°C and incubated at 100°C for 5 min prior to loading onto the gel. Electrophoresis was typically performed for 4.5 h at 45-mA constant current in 15-cm polyacrylamide gels (0.75 mm thick). Gels were soaked in 50% (v/v) methanol, and proteins were visualized by silver staining (32).

#### Protein Assay

Protein concentration was determined by the method of Bradford (33).

#### Enzyme Purification

All procedures were carried out at 4°C. Preparative centrifugations were performed using a Beckman J2-21 centrifuge.

**Buffers Used for Protein Purification**—The pH values of all buffers are given as those measured at 4°C. PMSF (100 mM in isopropyl alcohol) was added immediately prior to use. Lysis buffer: 100 mM Tris-HCl, pH 7.5, 1 mM Na<sub>3</sub>EDTA, 0.5 mM DTT, 10% glycerol, 1 mM benzamidine HCl, 1 mM NaHSO<sub>3</sub>, 1 mM PMSF, 4  $\mu$ g/ml pepstatin, 2  $\mu$ g/ml leupeptin, and 2  $\mu$ g/ml soybean trypsin inhibitor. P buffer: 20 mM potassium phosphate, pH 6.8, 0.5 mM Na<sub>3</sub>EDTA, 0.5 mM DTT, 5% glycerol, and 0.5 mM PMSF. P400 buffer: the same as P buffer except 400 mM potassium phosphate. TCa buffer: 20 mM Tris acetic acid, pH 7.5, 5 mM calcium acetate, 0.5 mM DTT, 5% glycerol, and 0.5 mM PMSF. T buffer: 20 mM Tris-HCl, pH 7.5, 0.5 mM Na<sub>3</sub>EDTA, 0.5 mM DTT, 5% glycerol, and 0.5 mM PMSF.

**Cell Lysis**—Red Star bakers' yeast (116 g) was thawed on ice overnight. Cells were suspended in lysis buffer to a final volume of 160 ml and passed twice through an ice-cold French pressure cell (18,000 p.s.i., American Instrument Company, Silver Springs, MD) at a flow rate of approximately 2 ml/min. Aliquots (40-ml) of cells were lysed at one time, and disrupted material was collected into 50-ml centrifuge tubes on ice. Under these conditions, approximately two-thirds of the cells were disrupted as judged by light microscopy. The lysate was centrifuged at 30,000  $\times$  g for 30 min to remove intact cells and debris (fraction I).

**PEI and Ammonium Sulfate Fractionation**—Lysis buffer was added to the crude lysate to a final volume of 160 ml. While stirring, a solution of 10% PEI-HCl, pH 7.5, was added dropwise to the crude lysate to a final concentration of 1%. The suspension was stirred 30 min and centrifuged at 20,000  $\times$  g for 15 min. The pellet was resuspended in 160 ml of lysis buffer containing 200 mM ammonium sulfate, stirred 30 min, and the insoluble material was removed by centrifugation. Solid ammonium sulfate was added to the supernatant to a final concentration of 0.49 g/ml and allowed to sit on ice for 3 h. The precipitate was collected by centrifugation and resuspended in 100 ml of P buffer containing 500 mM KCl and dialyzed against the same buffer until the conductivity equaled that of P buffer containing 500 mM KCl, 23 mmho (fraction II).

**Phosphocellulose Chromatography**—Fraction II was applied to a 240-ml column (6.4  $\times$  8 cm) equilibrated in P buffer containing 500 mM KCl and washed with the same buffer at a flow rate of 100 ml/min. Fractions containing protein were pooled and precipitated by the addition of solid ammonium sulfate to 0.49 g/ml and placed on ice for at least 3 h. Precipitated protein was collected by centrifugation, resuspended in TCa buffer, and dialyzed against the same buffer until the conductivity equaled that of TCa buffer, 0.8 mmho (fraction III).

**ssDNA-cellulose Chromatography**—Fraction III was applied to a

60-ml ssDNA-cellulose column (3.2  $\times$  7 cm) (34) equilibrated in TCa buffer. The column was washed with 3 volumes of the same buffer and eluted with a linear salt gradient (0–400 mM KCl in TCa buffer, 600 ml) at a flow rate of 50 ml/h. DNA-reannealing activity was eluted at 25 mM NaCl. Fractions containing DNA-reannealing activity were pooled, dialyzed initially against 7 liters of T buffer overnight (to reduce the Ca<sup>2+</sup> concentration), and then dialyzed exhaustively against P buffer (fraction IV).

**Hydroxylapatite Chromatography**—Fraction IV was applied to a 25-ml column (3.0  $\times$  3.7 cm) equilibrated with P buffer. The column was washed with 3 column volumes of buffer and developed with a linear gradient (20–400 mM KPO<sub>4</sub> in P buffer, 250 ml) at a flow rate of 50 ml/h. This step separates the annealing activity into many fractions throughout the column profile. The fractions were divided into several pools and dialyzed against T buffer to remove phosphate ions until the conductivity equaled 0.65 mmho. Pooled fractions eluting between 300 and 400 mM phosphate possessed the highest specific activity as well as the largest amount of total activity and was designated fraction V.

**Fast Protein Liquid Chromatography**—Fraction V was applied to an HR5/5 Mono Q column equilibrated in T buffer. The column was washed with T buffer (10 ml) and developed with a 15-ml linear gradient (0–600 mM NaCl in T buffer). One major protein peak eluted at 360 mM NaCl. This peak was pooled and dialyzed against T buffer until the conductivity equaled 0.65 mmho (fraction VI). Fraction VI possessed greater than 80% of the total DNA-reannealing activity loaded onto the column and was enriched for a protein with *M*<sub>r</sub> 120,000.

**Storage of Fraction VI**—Three methods were used for storage of fraction VI. Protein was stored on ice in T buffer, made 50% (v/v) in glycerol, and stored at –20°C, or made 25% (v/v) in glycerol, frozen in a dry ice-ethanol bath, and kept at –70°C. Storage at 4°C did not prevent proteolysis and led to a loss of 50% activity after 4 months. Fraction VI maintained at either –20 or –70°C retained more than 85% of its activity after 18 months.

**Notes on Protein Purification**—Chromatography resins that did not yield increases in purification or were not reproducible were PBE 94, phenyl-Sepharose CL-4B, heparin-Sepharose CL-4B, Sephacryl 300, QAE-Sephadex, Sephadex G-50, Sephadex G-200, (Pharmacia LKB Biotechnology Inc.), carboxymethyl-cellulose, and DEAE-cellulose (Whatman).

## RESULTS

**Purification of a Yeast Protein Catalyzing Homologous Pairing of DNA**—Both the RecA protein of *E. coli* and the Rec1 protein of *U. maydis* catalyze reassociation of denatured DNA. An important feature of annealing catalyzed by RecA protein is that the reaction occurs relatively efficiently over a significant range of protein/DNA ratios. By contrast, the strand exchange reaction in which RecA protein pairs single-stranded molecules with homologous duplex DNA is considerably more sensitive to the ratio of RecA protein to DNA (36–38). For these reasons, we used both the S1 nuclease resistance assay and the agarose gel assay (see "Experimental Procedures") to investigate whether extracts prepared from mitotic yeast cells contained DNA-reannealing activity. Crude extracts prepared by high pressure disruption of yeast cells did not contain a significant amount of renaturing activity as judged by the conversion of <sup>3</sup>H-labeled denatured P22 DNA to S1-resistant duplex. Moreover, using the agarose gel assay, we detected a considerable amount of nuclease activity that rapidly degraded the substrate (data not shown).

When Ca<sup>2+</sup> was substituted for Mg<sup>2+</sup> in the reaction, a significant level of reassociation was detected, but nuclease interference was significantly reduced. This observation allowed us to measure activity in partially purified fractions and to use DNA-cellulose affinity chromatography earlier in the purification, which is summarized in Table I. The activity bound to PEI and was eluted with 200 mM ammonium sulfate. Residual PEI and nucleic acid were removed by phosphocellulose chromatography. The activity was retained on ssDNA cellulose and could be eluted with 25 mM NaCl. Chromatog-

TABLE I  
Summary of enzyme purification

Fractions		Volume	Protein concentration	Total protein	Total units $\times 10^{-2}$	Specific activity
		ml	mg/ml	mg		units/ $\mu$ g
I.	Crude extract	160	30.3	4,850	— <sup>a</sup>	—
II.	PEI wash	109	11.0	1,200	—	—
III.	Phosphocellulose pool	45	16.4	737	3,537	0.48
IV.	ssDNA-cellulose pool	62	1.8	110	5,016	4.6
V.	Hydroxylapatite pool	33	0.41	13.0	625 <sup>b</sup>	4.8
VI.	Mono Q pool	3	1.0	3.0	3,960	132

<sup>a</sup> DNA renaturation activity could not be determined in fractions I and II (see "Experimental Procedures").

<sup>b</sup> This value represents only the activity of the material eluting between 300 and 400 mM phosphate.

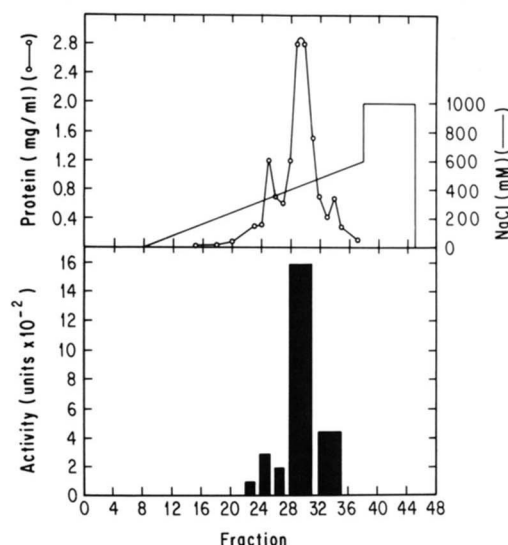


FIG. 1. Purification of the yeast DNA-pairing activity by Mono Q chromatography. Fraction V was applied to a Mono Q column equilibrated in T buffer, and protein was eluted with a linear NaCl gradient (0–600 mM). Top, protein concentration of fractions. Bottom, DNA renaturation activity. Fractions were pooled as indicated, dialyzed against T buffer, and assayed as described under "Experimental Procedures."

raphy on hydroxylapatite resolved three major peaks of activity eluting at 150, 200, and 300 mM potassium phosphate buffer, pH 6.8. The active fractions eluting near 300 mM phosphate were pooled (fraction V) and applied to a Mono Q column. Activity eluted between 370 and 450 mM NaCl, which coincided with the major protein peak. Fig. 1 shows the activity profile on the Mono Q-fractionated material. Fractionation by fast protein liquid chromatography on the Mono Q column provided the largest single step increase in specific activity. Because the activity peak coeluted with the majority of protein, however, it was likely that the primary effect of the Mono Q chromatography was to remove inhibitors of the annealing reaction. Consistent with this idea, Fig. 2 shows that the Mono Q column separated a  $Mg^{2+}$ -dependent nuclease (present in fractions 16–27) from the pairing activity (beginning in fraction 28). This nuclease activity was not detected in the presence of  $Ca^{2+}$ . The results shown in Fig. 2 also demonstrated that the DNA-pairing activity required sequence homology since the M13 viral DNA did not become associated with the DNA aggregates formed from denatured P22 DNA.

Proteins in the more highly purified fractions were separated by electrophoresis in polyacrylamide gels and stained with silver (Fig. 3). Fraction VI was highly enriched for a single protein with an approximate molecular mass of 120 kDa which accounted for 85–90% of the protein in this frac-

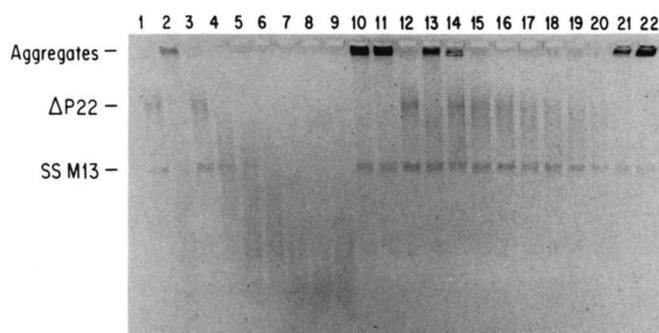


FIG. 2. Mono Q chromatography separates nuclease from the yeast-reannealing activity. Column fractions from Mono Q chromatography were assayed for DNA renaturation activity in the presence of  $Mg^{2+}$  (lanes 1–11) or  $Ca^{2+}$  (lanes 12–22) using agarose gel (0.7%) electrophoresis as described under "Experimental Procedures." Lanes 1 and 12, no protein; lanes 2 and 13, fraction V (6  $\mu$ g); lanes 3 and 14, column fraction 12 (1  $\mu$ g); lanes 4 and 15, fraction 16 (1  $\mu$ g); lanes 5 and 16, fraction 18; lanes 6 and 17, fraction 20 (2  $\mu$ g); lanes 7 and 18, pooled fractions 22 and 23 (4.5  $\mu$ g); lanes 8 and 19, pooled fractions 24 and 25 (4.5  $\mu$ g); lanes 9 and 20, pooled fractions 26 and 27 (4.5  $\mu$ g); lanes 10 and 21, pooled fractions 28–31 (4.5  $\mu$ g); lanes 11 and 22, pooled fractions 32–35 (4.5  $\mu$ g). The positions of denatured P22 ( $\Delta$ P22) and single-stranded M13 DNA are indicated. The annealed DNA forms large aggregates that migrate extremely slowly in agarose gels.

tion. Low molecular mass contaminants were observed after prolonged storage of the material at 0°C, and these likely resulted from proteolysis (data not shown).

**pH Optimum and Cofactor Requirements for Enzyme-catalyzed DNA Pairing**—The pH dependence of DNA renaturation catalyzed by the fraction VI enzyme was measured, and the activity was optimum between pH 6.5 and 7.5. At pH 6 and 8, the amount of reassociation was approximately half that measured at pH 7.0 (data not shown).

The cofactor requirements for DNA pairing are presented in Table II. There was an absolute requirement for a divalent cation which could be satisfied by either  $Mg^{2+}$  or  $Ca^{2+}$ . No activity was detected (<1%) when  $Zn^{2+}$  was substituted in the reaction (data not shown). The maximal rate of reassociation was observed in the presence of 10 mM  $Mg^{2+}$  or 5 mM  $Ca^{2+}$  (data not shown). No ATP hydrolysis was detected when fraction VI was incubated with [ $^3H$ ]ATP in the presence or absence of DNA (data not shown), and ATP (or dATP) partially inhibited the activity (Table II). The renaturation reaction was sensitive to salt and was inhibited 65% in the presence of 200 mM NaCl. Treatment with SDS or proteinase K as well as heating to 100°C for 2 min inactivated the protein.

**Kinetic Characterization of Enzyme-catalyzed DNA Pairing**—The dependence of reassociation on enzyme concentration was examined, and the results are presented in Fig. 4. A



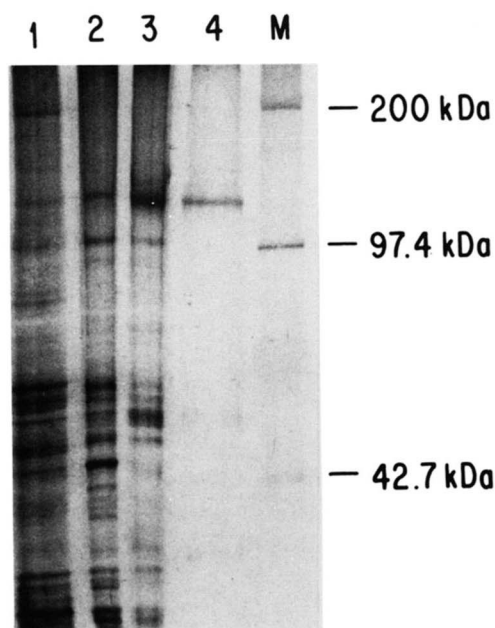


FIG. 3. Analysis of protein fractions by SDS-polyacrylamide gel electrophoresis. Aliquots of protein fractions from each step in the purification (see Table I) were separated by electrophoresis in a polyacrylamide gel (8%) containing SDS and stained with silver nitrate as described under "Experimental Procedures." Lane 1, fraction III (25  $\mu$ g); lane 2, fraction IV (14  $\mu$ g); lane 3, fraction V (10  $\mu$ g); lane 4, fraction VI (4  $\mu$ g). The positions of molecular mass standards (lane M) are indicated.

TABLE II

## Reaction requirements for ssDNA renaturation

Renaturation of heat-denatured P22 DNA was determined as described under "Experimental Procedures." The relative value of 100% represents 65% of the input-denatured P22 DNA becoming S1 resistant.

Conditions	Relative activity
	%
Complete reaction mixture	(100)
-Enzyme	0
-Mg <sup>2+</sup> or Ca <sup>2+</sup>	3
-DTT	71
+200 mM NaCl	35
+ATP or dATP (1 mM)	67
+0.5% SDS	0
+Proteinase K	5
+100 °C, 2 min	0

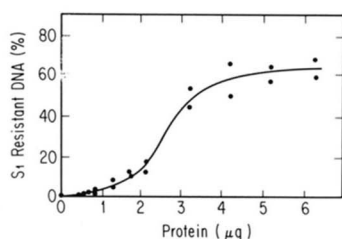


FIG. 4. DNA reassociation shows a sigmoidal dependence on yeast protein concentration. The indicated amount of fraction VI was added to an otherwise complete reaction (30  $\mu$ l) containing <sup>3</sup>H-labeled denatured P22 DNA (40  $\mu$ M). After incubation at 37°C for 30 min, the reaction was stopped by the addition of SDS, and the amount of duplex DNA was measured using S1 nuclease (see "Experimental Procedures"). The results of two independent experiments are shown.

maximum extent of 60–70% of the input DNA became double-stranded in the presence of 4–6  $\mu$ g of fraction VI enzyme. However, only approximately 10% of the DNA was renatured when the reaction contained 2  $\mu$ g of fraction VI protein. These results suggested that cooperative interactions were important during catalysis of heteroduplex formation.

The kinetics of renaturation catalyzed by the yeast enzyme were compared with the *E. coli* RecA protein using the S1 nuclease resistance assay (Fig. 5A). Assuming fraction VI to be 85% pure, the maximal amount of duplex DNA (60%) was formed within 2 min of incubation at 37°C at a ratio of approximately 50 mol of DNA nucleotide/protein monomer. At a ratio of 13 mol of DNA nucleotide/RecA monomer, a comparable amount of duplex DNA was produced after 10–15 min of incubation. When the amount of RecA protein was reduced below a ratio of 40 nucleotides/monomer of enzyme, only a slow rate of reassociation was observed. These results demonstrate that the yeast enzyme promoted an extremely rapid reassociation reaction with single-stranded P22 DNA which was approximately 50-fold more rapid than ATP-dependent renaturation catalyzed by RecA protein under identical reaction conditions.

Both the rate and the extent of DNA renaturation were strongly dependent on the amount of enzyme present in the reaction (Fig. 5B). A very low level of reannealing was detected using 0.09 and 0.18  $\mu$ M fraction VI protein. Above this concentration, there was a significant increase in the rate of pairing, and the final extent of renaturation also increased with increasing protein. The addition of more fraction VI protein to a reaction that had been incubated for 10 min

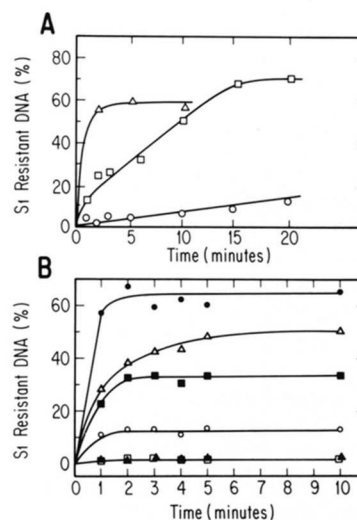


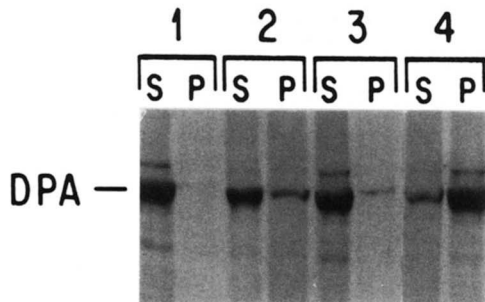
FIG. 5. The dependence of the rate and extent of renaturation upon yeast protein concentration. A, reaction mixtures (160  $\mu$ l) contained 40  $\mu$ M <sup>3</sup>H-labeled heat-denatured P22 DNA, 20 mM Tris-HCl, pH 7.5, 5 mM CaCl<sub>2</sub>, 1 mM DTT, and the indicated concentration of fraction VI protein. For measurements of RecA protein-catalyzed renaturation, the CaCl<sub>2</sub> was replaced with 10 mM MgCl<sub>2</sub> and supplemented with 1 mM ATP. At the indicated time, aliquots (20  $\mu$ l) were removed, and S1-resistant DNA was measured.  $\Delta$ , fraction VI, 9  $\mu$ g (50:1 ratio of nucleotide to protein);  $\square$ , RecA protein, 18  $\mu$ g (13:1 ratio of nucleotide to protein). B, reaction mixtures (as above) containing the indicated amount of fraction VI protein were incubated at 37°C. At the indicated times, aliquots (20- $\mu$ l) were removed, and the amount of S1 nuclease-resistant DNA was measured.  $\square$ , 2  $\mu$ g (445 nucleotides/protein monomer);  $\blacktriangle$ , 4  $\mu$ g (222 nucleotides/protein monomer);  $\circ$ , 8  $\mu$ g (111 nucleotides/protein monomer);  $\blacksquare$ , 12  $\mu$ g (82 nucleotides/protein monomer);  $\Delta$ , 16  $\mu$ g (55 nucleotides/protein monomer);  $\bullet$ , 20  $\mu$ g (45 nucleotides/protein monomer).

resulted in a further increase in the extent of renaturation up to a final extent of approximately 70% (data not shown). These results suggested that the yeast protein performed a limited amount of DNA renaturation and did not turn over or turned over extremely slowly to initiate additional rounds of DNA pairing.

*The Yeast DNA-pairing Activity Binds Tightly to Single-stranded DNA*—The kinetic data suggested that the yeast protein dissociated slowly from single-stranded DNA or from one or more intermediates in the pairing reaction. In order to investigate this possibility, we examined the association of this protein with DNA by sedimentation analysis and filter binding.

Following incubation of the DNA-pairing activity with heat-denatured P22 DNA, the reaction mixture was centrifuged in an Eppendorf microcentrifuge (5 min, 12,000 × *g*), and the supernatant and pellet fractions were analyzed by electrophoresis in polyacrylamide gels (Fig. 6). Omitting divalent cation (*lane 1*) or addition of either SDS (1%; *lane 2*) or EDTA (25 mM; *lane 3*) resulted in the yeast protein being recovered almost exclusively in the supernatant fractions. However, in reaction mixtures centrifuged without SDS treatment, almost all the protein was recovered in the pellet fraction (*lane 4*). These results demonstrated that the yeast protein becomes associated with fast sedimenting complexes during renaturation.

In order to examine the sedimentation properties of DNA during renaturation promoted by the yeast protein, we used <sup>3</sup>H-labeled P22 DNA and measured the recovery of radioactivity in both supernatant and pellet fractions after brief centrifugation. The results are presented in Table III. In the absence of yeast protein, the P22 DNA was recovered almost



**FIG. 6. The DNA-pairing activity cosediments with DNA complexes during renaturation.** Reaction mixtures (30  $\mu$ l) containing 40 M heat-denatured P22 DNA, 20 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM DTT, and 3  $\mu$ g of fraction VI protein were incubated at 37 °C for 10 min and centrifuged in an Eppendorf microcentrifuge (4 °C, 15 min). Supernatant and pellet fractions were carefully removed, and each was examined by electrophoresis in a polyacrylamide gel (8%) containing SDS. Proteins were visualized by silver staining. *Lane 1*, reaction without Mg<sup>2+</sup>; *lane 2*, complete reaction stopped with EDTA (30 mM) before centrifugation; *lane 3*, complete reaction stopped with SDS (1%) before centrifugation; *lane 4*, complete reaction, no treatment. S, supernatant; P, pellet; DPA, DNA-pairing activity.

TABLE III

Sedimentation analysis of fraction VI and heat-denatured P22 DNA

Protein	Reaction terminated by	Radioactivity	
		Supernatant	Pellet
% of total			
—		98	2
+		7	93
+	EDTA	26	74
+	1% SDS	54	46
+	1% SDS; 95 °C, 5 min	98	2

TABLE IV

Sedimentation of RecA protein and heat-denatured P22 DNA

Protein	Reaction terminated by	Radioactivity	
		Supernatant	Pellet
% of total			
—		94	6
+		30	70
+	—ATP	90	10
+	EDTA	48	52
+	1% SDS	53	47
+	1% SDS; 95 °C, 5 min	96	4

TABLE V

Challenge of DNA renaturation with homologous and heterologous DNAs

In A, reaction mixtures (90  $\mu$ l) contained yeast-pairing activity (5  $\mu$ g of fraction VI) and 40  $\mu$ M heat-denatured <sup>3</sup>H-labeled P22 DNA. After incubation at 37 °C for 10 min, an aliquot (30  $\mu$ l) was removed and filtered through alkaline-treated nitrocellulose as described under "Experimental Procedures." To the remaining reaction mix, denatured <sup>32</sup>P-labeled P22 DNA was added to a final concentration of 40  $\mu$ M, incubated for 10 min, and an aliquot (30  $\mu$ l) was removed and filtered as above. The DNA retained was the ratio of the radioactivity retained on the filter to the total radioactivity applied. In B, the yeast protein was incubated with <sup>3</sup>H-labeled viral M13 ssDNA in the initial reaction, and <sup>32</sup>P-labeled denatured P22 DNA was added after 10 min.

Reaction mixture	Reaction time	Challenge time	DNA retained	
			Initial	Challenge
	<i>min</i>	<i>min</i>	<i>%</i>	
A	10	0	100	
	20	10	95	4
B	10	0	39	
	20	10	42	3

exclusively in the supernatant, whereas more than 90% of the label was recovered in the pellet when the fraction VI protein was included. Treatment with either SDS (1%) or EDTA (25 mM) resulted in the recovery of 54 and 26% of the label in the supernatant, respectively. Almost all the label was recovered in the supernatant fraction following heat treatment in the presence of SDS. These results argue that the fast sedimenting complexes are held together by protein-DNA interactions as well as through interstrand hydrogen bonding.

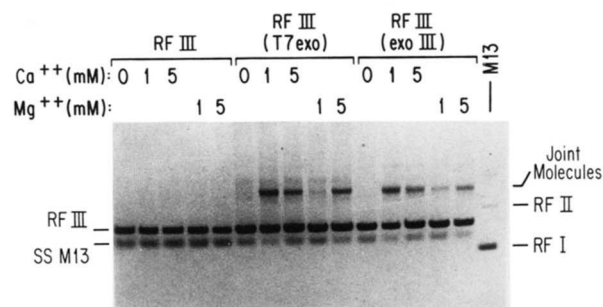
Important intermediates in RecA protein-catalyzed renaturation are DNA networks containing bound protein and multiple interchain junctions. As shown in Table IV, the properties of DNA in fast sedimenting complexes containing RecA protein are remarkably similar to those promoted by the yeast DNA-pairing activity. As expected, these complexes do not form efficiently when ATP is omitted from reactions containing RecA protein.

The interaction of the yeast DNA-pairing activity and single-stranded DNA was also investigated using alkaline-treated nitrocellulose filters that have been used in studies of RecA protein (25). The alkaline treatment prevents the nitrocellulose from binding single-stranded DNA directly, but DNA can be retained by virtue of protein bound to it or when it is associated with large DNA networks that are formed as a result of rapid DNA pairing. In this latter case, the DNA aggregates are unable to pass through the pores of the filter because of their large size.<sup>2</sup> Table V shows the results of DNA binding and challenge experiments with two different DNA substrates. In part A, fraction VI protein was incubated for 10 min with heat-denatured <sup>3</sup>H-labeled P22 DNA. All of the

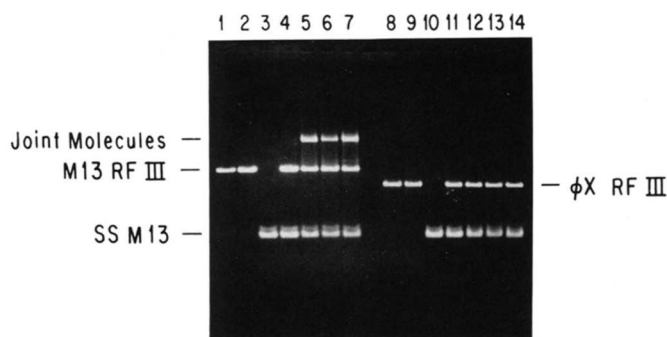
<sup>2</sup> J. Halbrook, unpublished results.

radioactivity was retained on alkaline-treated filters. This value reflects both protein-DNA complexes as well as DNA contained in large networks that form during reassociation. If an equal amount of challenge DNA (heat-denatured  $^{32}\text{P}$ -labeled P22 DNA) was added after a 10-min incubation with the  $^3\text{H}$ -labeled substrate, and the incubation was continued for an additional 10 min and filtered, most of the  $^3\text{H}$ -labeled DNA was retained as expected, but only a small fraction of the challenge DNA was bound to filters. In a second experiment, yeast protein was incubated with  $^3\text{H}$ -labeled viral M13 DNA for 10 min, and 39% of the input DNA was retained on alkaline-treated filters. The subsequent addition of  $^{32}\text{P}$ -labeled heterologous heat-denatured P22 DNA for 10 min did not alter this value, and only 3% of the challenge DNA was retained. Since the viral M13 DNA could not undergo renaturation, all of this substrate was retained by virtue of the bound yeast protein. Taken together, these results argue that the yeast protein bound tightly to single-stranded DNA and did not dissociate or dissociated slowly under these reaction conditions. The possibility that the protein was functionally inactivated during the first 10 min of incubation appeared unlikely since incubating the enzyme at  $37^\circ\text{C}$  for up to 40 min in the absence of DNA did not reduce its ability to renature labeled P22 DNA that was added after this heat pretreatment (data not shown).

**Strand Displacement Catalyzed by the Yeast DNA-pairing Activity**—Many of the purified enzymes that have been implicated in heteroduplex formation during recombination are capable of catalyzing strand exchange between a linear duplex and homologous single-stranded circular DNA *in vitro*. These DNA substrates are well suited for the strand exchange reactions since they migrate differently than joint molecules, and the products can easily be separated on agarose gels. We investigated the ability of the purified yeast protein to promote strand transfer using linear duplex M13 DNA (RF III) and homologous single-stranded viral DNA. Although a variety of reaction conditions and cofactors was used, no strand exchange was detected when the products were analyzed by agarose gel electrophoresis. Because of the high affinity of the yeast DNA-pairing activity for single-stranded DNA, we briefly treated the RF III DNA with either *E. coli* exonuclease III to produce 5' tails or phage  $T_7$  gene 6 exonuclease to produce 3'-single-stranded tails. The conditions used for digestion favored the production of short single-stranded tails of uniform length. The average length of exonuclease III-generated tails, determined by end filling using DNA polymerase I Klenow fragment and nucleoside triphosphates containing [ $\alpha$ - $^{32}\text{P}$ ]dCTP, was measured to be 50 nucleotides at each end of the resected DNA. Furthermore, restriction analysis of end-filled DNA demonstrated that all of the label was incorporated into short terminal fragments (data not shown). For each resected molecule, only one of the two single-stranded termini could form a hybrid with the viral M13 strand. As shown in Fig. 7, although blunt ended RF III was not converted to joint molecules with the M13 viral DNA, each of the resected DNAs reacted efficiently. Based upon densitometry of the gels, approximately 30–70% of the substrate RF III disappeared and was replaced by the slower migrating joint molecules. These joint molecules migrated more slowly than RF II DNA, the product of a complete strand transfer reaction, and their formation required a divalent cation, either  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$ . Furthermore, the formation of the joint structures required that the reacting DNAs be homologous. When either  $\phi\text{X174}$  RF III DNA or resected  $\phi\text{X}$  RF III DNA was incubated with M13 viral DNA, no joint molecules were formed (Fig. 8, lanes 11–14). However, substi-



**FIG. 7. Agarose gel assay of joint molecule formation.** Standard reaction mixtures ( $30\ \mu\text{l}$ ) contained 1 mM DTT, 20 mM Tris-HCl, pH 7.5, 10 mM viral M13 single-stranded DNA, and either 20  $\mu\text{M}$  linearized M13 RF DNA (RF III), 20  $\mu\text{M}$  M13 RF III DNA treated with  $T_7$  gene 6 exonuclease (3'-tailed) or with exonuclease III (5'-tailed). Fraction VI ( $3.4\ \mu\text{g}$ ) and the indicated concentration of  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  were added and incubated at  $37^\circ\text{C}$  for 30 min. Reactions were terminated by the addition of SDS and analyzed on an agarose gel (0.7%). The positions of M13 viral DNA, and M13 RF I, RF II, and RF III are indicated for reference. Note that joint molecules migrate as a discreet band behind the RF II marker DNA.



**FIG. 8. Nucleotide sequence homology is required for joint molecule formation.** Reaction mixtures ( $30\ \mu\text{l}$ ) contained 20  $\mu\text{M}$  viral M13 DNA and 20  $\mu\text{M}$  linear duplex M13 DNA (lanes 1–7) or 20  $\mu\text{M}$  linear duplex  $\phi\text{X174}$  DNA (lanes 8–14) and 2.5  $\mu\text{g}$  of fraction VI protein (lanes 5–7 and 12–14). Unless otherwise indicated, reactions were for 15 min at  $37^\circ\text{C}$ . Lane 1, blunt ended M13 RF III DNA; lane 2, exonuclease III-treated M13 RF III DNA; lanes 3 and 10, single-stranded M13 viral DNA. Lanes 4–7, viral M13 DNA plus M13 RF III DNA treated with exonuclease III, no enzyme (lane 4), incubated with enzyme for 5 min (lane 5), for 10 min (lane 6), or for 15 min (lane 7). Lane 8, blunt ended  $\phi\text{X174}$  RF III DNA; lane 9,  $\phi\text{X174}$  RF III DNA treated with exonuclease III; lanes 11–14, single-stranded M13 viral DNA and exonuclease III-treated  $\phi\text{X174}$  RF III DNA incubated without enzyme (lane 11), incubated for 5 min (lane 12), for 10 min (lane 13), and for 15 min (lane 14) with enzyme. Reactions were analyzed by electrophoresis in a 0.7% agarose gel as described under "Experimental Procedures."

tutions of  $\phi\text{X174}$  viral single-stranded DNA resulted in joint molecule production (data not shown).

Joint molecules were resistant to treatment with SDS (1%) or protease K but were dissociated by treatment at elevated temperatures (data not shown), indicating that these structures were held together by interwinding of the DNA chains.

**Structure of the Joint Molecules Formed by the Yeast DNA-pairing Activity**—The joint molecules formed by the yeast-pairing activity could be due to a simple "end annealing" between the resected duplex and circular single-stranded DNA or they could represent a true strand exchange intermediate in which heteroduplex extension around the circle is accompanied by displacement of the noncomplementary strand of the duplex. In order to investigate their structure, joint molecules were prepared by incubating  $T_7$  gene 6 exonuclease-treated and exonuclease III-treated M13 RF III

DNA with circular M13 viral DNA in the presence of yeast enzyme. Following incubation, the DNAs were prepared for electron microscopy as described under "Experimental Procedures." Examples of joint molecules formed with the two different duplex substrates are presented in Fig. 9, *A* and *B*. In both cases, the major classes of joint products were  $\alpha$ -structures containing a partially duplex circle, a linear duplex segment, and a displaced single-stranded arm. In addition, simpler structures designated  $\sigma$ -forms were observed which contained a linear duplex joined at one of its ends to a circular

single strand (Fig. 9, *A* and *B*). Because no joint molecules were formed using RF III DNA lacking single-stranded tails, we conclude that the  $\sigma$ -structure represented the initial association between the DNA substrates. The predominant products using either the 5'- or 3'-tailed M13 RF III DNA substrates were  $\alpha$ -structures, whereas the  $\sigma$ -forms were present at approximately 25–35% of the levels of  $\alpha$ -forms (Table VI). Taken together, these results demonstrate that the yeast DNA-pairing activity catalyzed extensive strand exchange between the resected duplex molecule and its circular single-

**FIG. 9. The purified yeast DNA-pairing activity promotes strand transfer between circular single strands and resected homologous duplex DNAs.** Reaction mixtures containing fraction VI protein, resected M13 RF III DNA, and single-stranded M13 viral DNA were incubated for 15 min in assay buffer and prepared for electron microscopy as described under "Experimental Procedures." Electron micrographs of joint molecules were photographed at a final magnification of  $\times 30,000$ . *A*, joint molecules formed using T<sub>7</sub> gene 6 exonuclease-treated M13 RF III. Interpretive drawings show regions of duplex DNA (thick line) and single-stranded DNA (thin line). A " $\sigma$ -structure" is shown in the bottom left panel. *B*, joint molecules formed using exonuclease III-treated M13 RF III.

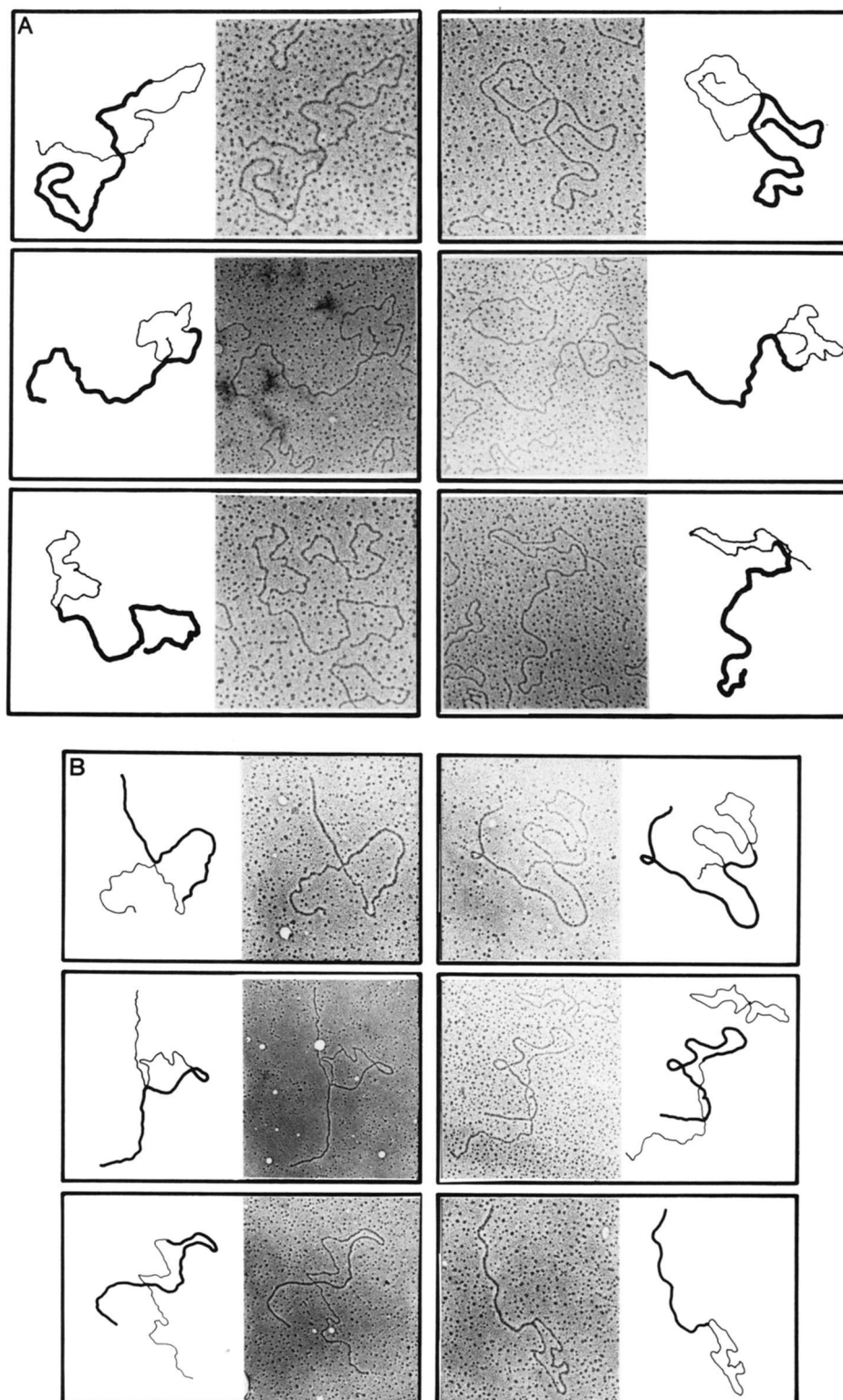




TABLE VI

The yeast DNA-pairing activity promotes strand transfer at 5'- and 3'-overhanging ends

Duplex DNA	Treatment of RF III	
	T <sub>7</sub> gene 6 exonuclease	Exonuclease III
$\alpha$ -Forms	32% (12/38)	53% (9/17)
$\sigma$ -Forms	8% (3/38)	18% (3/17)
Linear RF III	60% (23/38)	29% (5/17)

stranded homolog. Measurements of five molecules from each reaction indicate that the heteroduplex regions were extended up to approximately 3–5 kilobases. Based upon the results that the yeast DNA-pairing activity promoted joint molecule formation efficiently with the T<sub>7</sub> exonuclease- and exonuclease III-resected molecules, we conclude that there was no obligatory directionality for the strand transfer activity.

### DISCUSSION

A simple DNA renaturation assay has been used to purify extensively a DNA-pairing activity from mitotic *S. cerevisiae*. The utility of this assay was based upon the observations that the *E. coli* RecA protein (35), the *U. maydis* Rec1 protein (8, 12), phage  $\lambda$ -encoded  $\beta$ -protein (39, 40), and the phage T<sub>4</sub> uvsX protein (41, 42), all of which participate in homology-dependent recombination and presumably catalyze similar DNA-pairing steps *in vivo*, efficiently drive the association of complementary DNA chains. The advantages of this renaturation assay are that it is rapid, quantitative, and it is not as sensitive to changes in protein concentration as the strand transfer reaction. Unfortunately, DNA-binding proteins and nuclease activities interfere with the assay leading to overestimates or underestimates of the specific activity, respectively. Thus, during purification it is difficult to determine accurately specific activity at early steps in the fractionation. As indicated in Table I, we were unable to measure the amount of renaturing activity in the crude supernatant fraction or in the high salt eluate of the PEI precipitate. Only after phosphocellulose chromatography was it possible to determine a reliable specific activity. During subsequent steps in the purification, in particular during chromatography on hydroxylapatite, multiple peaks of renaturing activity were detected. Column fractions corresponding to the protein peak having the highest specific activity were pooled, dialyzed against T buffer, and applied to a Mono Q column (fraction V). In addition to providing a 27-fold purification of the renaturation activity, the Mono Q column separated nuclease activity from the DNA-pairing enzyme. The fluctuations in activity measured during the latter stages of purification are likely due to the presence of factors that inhibit DNA pairing in addition to several chromatographically distinct activities that promote DNA renaturation. Nevertheless, each step in the purification resulted in an increase in specific activity.

Fraction VI was devoid of single-stranded DNA exonuclease and endonuclease, nicking activity, and double-stranded DNA exonuclease and endonuclease (data not shown). As shown in Fig. 3, fraction VI was highly enriched for a protein with a molecular mass of approximately 120 kDa. No single contaminant was present in amounts greater than 2% of the total protein. We estimate from densitometry of silver-stained gels of fraction VI that the 120-kDa protein was 85–90% homogeneous. Based upon the characteristics of the DNA-pairing reaction promoted by fraction VI (see Fig. 4), it is likely that the 120-kDa protein was the only polypeptide present at concentrations high enough to promote renaturation. Furthermore, we have found the 120-kDa protein associated with

fast sedimenting DNA aggregates that were formed during reassociation of denatured P22 DNA (Fig. 6). Similar DNA networks have been observed as intermediates in RecA protein-catalyzed DNA-pairing reactions (35) and are produced by multiple synaptic events between a DNA strand and several partners. The resulting heteroduplex regions joining these strands are relatively short, and their extension appears to be slow under the conditions of enzymatic renaturation.

DNA pairing catalyzed by the yeast protein was rapid and proceeded in the absence of nucleoside triphosphates. Furthermore, fraction VI was devoid of ATPase or dATPase activities, either in the presence or absence of DNA. Thus, this yeast protein differed significantly from RecA protein of *U. maydis* Rec1 protein, both of which require or are stimulated by ATP (6, 35). The only cofactor requirement for the yeast enzyme was a divalent cation such as Ca<sup>2+</sup> or Mg<sup>2+</sup>.

With heat-denatured P22 DNA as substrate, the yeast enzyme promoted renaturation extremely rapidly. Typically, the reaction was complete in 1–2 min. Moreover, as shown in Fig. 5B, the extent of the reaction was also dependent upon yeast protein concentration, a result that indicated that the protein might not be turning over during pairing. In order to examine this possibility, we performed a series of challenge experiments using both renaturable (denatured P22 DNA) and nonrenaturable (M13 viral single-stranded DNA) substrates. Our results indicate that once the protein bound to single-stranded DNA, it was unavailable to bind additional substrate molecules. Although the protein was not inactivated upon binding, it appeared that the protein dissociated extremely slowly from single-stranded regions. By contrast, RecA protein that had been bound to single-stranded DNA associated readily with "challenge" DNA. However, if RecA protein-DNA complexes were formed in the presence of ATP and *E. coli* single-stranded DNA-binding protein, the protein did not migrate to challenge DNA (37, 43). These results demonstrate that other proteins, in this example, ssDNA-binding protein, can modulate the binding of RecA protein to DNA substrates. It is possible that in yeast, other proteins modulate the binding properties of the transferase such that its dissociation rate is increased due to specific interactions with other components of a recombination-repair complex. Additional characterization of the yeast protein-DNA interaction should provide details of the mechanism of DNA pairing and the possible involvement of other proteins.

Surprisingly, the yeast DNA-pairing activity was unable to promote strand transfer between blunt ended linear duplex and circular single-stranded DNA substrates. However, if the duplex DNA contained short single-stranded tails, approximately 50 nucleotides long, there was rapid association between the different substrate DNAs to form joint molecules. The only requirements for joint molecule formation were sequence homology and a divalent cation, either Mg<sup>2+</sup> or Ca<sup>2+</sup>. Our results indicated that joint molecules were formed rapidly with M13 RF III DNA resected with either exonuclease III or T<sub>7</sub> gene 6 exonuclease to produce short 5' or 3' tails, respectively. Both DNA substrates were efficiently used by the yeast transferase with more than 50% of the duplex becoming associated with the single-stranded circles within 15 min. Electron microscopic examination of the joint molecules indicated that the major joint molecular products were  $\alpha$ -structures containing a displaced linear single strand and a smaller proportion of  $\sigma$ -structures in which the end of the linear duplex was annealed to the homologous circle. The displaced single-stranded tail of the  $\alpha$ -structures indicated that the "heteroduplex" region had been extended into the duplex portion of the linear molecule. Based upon the length of the

duplex portion of the circular molecule, we estimate the heteroduplex regions were extended up to approximately 3–5 kilobases in length.

The yeast DNA-pairing activity described in this report is the third "strand transferase" purified from *S. cerevisiae*: Sugino *et al.* (17) have recently purified a DNA-pairing activity from  $a/\alpha$  cells undergoing meiosis, and an enzyme fraction purified from vegetative yeast has been described by Kolodner *et al.* (16, 44). There are several similarities between the enzyme described by this latter group and the DNA strand transferase reported in this work. Both enzymes are large proteins (132 and 120 kDa) that promote DNA pairing in the absence of nucleoside triphosphates. Large aggregates of protein and DNA are produced during renaturation by both activities, and both the rate and extent of renaturation depend upon the amount of protein in the reaction. Despite these similarities, the yeast DNA-pairing activity that we have purified cannot initiate strand transfer at duplex ends but requires a contiguous single-stranded region. The activity described by Kolodner appears to promote strand exchange in the absence of single-stranded tails. We conclude that the yeast activity described in this report binds to a single-stranded DNA terminus between 4 and 50 nucleotides in length and initiates strand transfer and heteroduplex extension. It is worth noting that at least one nuclease activity copurified with the DNA-pairing activity and was separated in the final chromatographic step (see Fig. 2). It is possible that this tight biochemical association is important for the function of the yeast strand transferase *in vivo*.

What, if any, is the role of this strand transfer activity in recombination? The biochemical properties of the purified DNA-pairing activity suggest that this enzyme may participate in heteroduplex formation and extension in a region of DNA containing a gap. In this model, an invading single strand is produced on one homolog by a helicase that unwinds from a nick or small discontinuity in this duplex. The yeast transferase binds to the single strand and rapidly anneals it to the complementary sequences within the gap. This nascent heteroduplex region may be quite small, 50 base pairs or less. However, once pairing has initiated within the gap, the yeast transferase can extend the heteroduplex into the neighboring duplex region thereby displacing the resident strand. A unique feature of this heteroduplex extension suggested by our results is that DNA on both sides of the gap could be displaced due to the apparent lack of polarity of the transferase. This displaced strand might be degraded or "invade" the other homolog leading to either a single heteroduplex region or asymmetric heteroduplexes and crossing over, respectively. Mitotic recombination in yeast is stimulated up to 1000-fold by exposing cells to a wide variety of DNA-damaging agents including UV light (18). It is likely that short gaps are produced during repair of these lesions, and these gaps could serve as the initiation sites for exchange.

Although a role for this protein during mitotic recombination is suggested by its biochemical properties, a more definitive demonstration of its *in vivo* role requires the isolation of

mutants lacking this enzyme. We have recently isolated the gene encoding the DNA-pairing activity and have constructed strains carrying disruption of this region. A detailed characterization of this gene and its function are in progress.

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