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Purification and characterization of an activity from *Saccharomyces cerevisiae* that catalyzes homologous pairing and strand exchange

(mitotic cells/genetic recombination/electron microscopy/cooperativity/strand transfer)

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ABSTRACT **An activity that catalyzes the formation of joint molecules from linear M13mp19 replicative form DNA and circular M13mp19 viral DNA was purified 1000- to 2000-fold from mitotic *Saccharomyces cerevisiae* cells. The activity appeared to reside in a M_r 132,000 polypeptide. The reaction required that the substrates be homologous and also required Mg^{2+}. There was no requirement for ATP. The reaction required stoichiometric amounts of protein and showed a cooperative dependence on protein concentration. Electron microscopic analysis of the joint molecules indicated they were formed by displacement of one strand of the linear duplex by the single-stranded circular molecule. This analysis also showed that heteroduplex formation started at the 3′-homologous end of the linear duplex strand followed by extension of the hybrid region toward the 5′-homologous end of the linear duplex strand (3′-to-5′ direction).**

Genetic recombination is thought to occur by many different mechanisms (1–4). Common to the initiation of genetic recombination as postulated by many models is the pairing of DNA molecules at regions of homology followed by exchange of single strands to yield joint molecules containing regions of heteroduplex DNA (1–5). Strand exchange is probably the most extensively studied aspect of the enzymology of genetic recombination. Much of our knowledge comes from studies with the *Escherichia coli* RecA protein, which is required for genetic recombination, repair of DNA damage, and the SOS response (6–9). The RecA protein can catalyze the formation of a variety of joint DNA molecules (reviewed in refs. 9 and 10). Analysis of this process has shown it involves the pairing of a single-stranded DNA molecule with a homologous duplex DNA molecule (9, 11–15). Initially, stoichiometric amounts of RecA protein bind to single-stranded DNA, and this RecA protein–single-stranded DNA complex appears to be an active intermediate in the strand-exchange process (15–21). Stable joint formation appears to initiate in both the 3′-to-5′ and 5′-to-3′ directions; however, extensive heteroduplex formation is polar and proceeds in the 3′-to-5′ direction (22, 23). The bacteriophage T4 UvsX protein appears to catalyze strand-exchange reactions in a similar fashion to the RecA protein (24–27).

Proteins that catalyze strand exchange have been purified from eukaryotes. The best studied example is the *Ustilago maydis* Rec1 protein (28, 29). One difference between the Rec1 and RecA proteins is the Rec1 protein catalyzes heteroduplex formation in the 5′-to-3′ direction, which is opposite to that catalyzed by RecA (22, 23, 28, 29). It is unclear if the Rec1 protein acts by forming an active protein–single-stranded DNA complex because 20–40 times less Rec1 protein is required to catalyze strand exchange compared to RecA protein (28, 29). Proteins that catalyze strand exchange have also been purified from human mitotic cells and from mitotic and meiotic mouse and lilly cells (30, 31). The human protein catalyzes 100–200 nucleotides of strand exchange in the 5′-to-3′ direction and does not require ATP. The polarity of the strand exchange catalyzed by the mouse and lilly proteins has not yet been determined. In this communication we report preliminary studies on the purification and characterization of an activity from mitotic *Saccharomyces cerevisiae* cells that catalyzes extensive strand exchange in the 3′-to-5′ direction as does the *E. coli* RecA protein.

EXPERIMENTAL PROCEDURES

Strains. The *S. cerevisiae* strain BJ926 **a**/α,*trp1/TRP1,HIS1/his1,prc1-126/prc1-126,pep4-3/pep4-3,prb1-1122/prb1-1122,can1/can1* was from D. Hinkle (University of Rochester, Rochester, NY). The *E. coli* strain AB259 HfrH,*thi-1,rel-1* and bacteriophage M13mp19 were from laboratory stocks. M13mp19 *recJ* contains a 2.3-kilobase (kb) *Eco*RI–*Sal* I fragment of *E. coli* DNA inserted between the *Eco*RI and *Sal* I sites of M13mp19 and was from S. T. Lovett of this laboratory. All M13 phage stocks were propagated on *E. coli* AB259.

Enzymes and Chemicals. Restriction endonucleases were obtained from New England Biolabs and used as suggested by the manufacturer. Creatine phosphokinase (type I), creatine phosphate, and phenylmethanylsulfonyl fluoride were from Sigma, and methyl methanesulfonate and 2-mercaptoethanol were from Kodak. Zymolyase-100T was from Miles Biochemicals (Naperville, IL). Single-stranded DNA-cellulose was prepared by the method of Alberts and Herrick (32).

Nucleic Acids. To purify M13 viral DNA, M13 phage were obtained by standard methods (33) and purified by centrifugation in CsCl density gradients (33). The viral DNA was then purified by phenol extraction and dialyzed against TE buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA). Crude M13 covalently closed, supercoiled replicative form (RFI) DNA was purified from phage-infected cells (33, 34). The replicative form (RF) fraction was chromatographed on a column (10 ml/liter of infected cells) of nitrocellulose (Hercules, Wilmington, DE) to remove contaminating single-stranded DNA and further purified by centrifugation in CsCl density gradients containing ethidium bromide (35, 36). ϕX174 viral DNA and RFI DNA were from New England Biolabs. Linear duplex DNA was prepared by digestion of RFI DNA with a restriction endonuclease followed by extraction with phenol and precipitation with ethanol. DNA concentrations are

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Abbreviations: RF DNA, replicative form DNA; RFI DNA, covalently closed, supercoiled RF DNA.

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expressed as mol of nucleotides using an ε_{260} of 6800 and 8500 for double-stranded and single-stranded DNA, respectively.

Assay for Strand Exchange. Assays were carried out in 30 μl containing 33 mM Tris·HCl (pH 7.5), 13 mM $MgCl_2$, 1.8 mM dithiothreitol, 1.3 mM ATP, 3 mM creatine phosphate, bovine serum albumin (88 μg/ml), creatine kinase (10 units/ml), 0.6 nmol of *Eco*RI-cleaved linear duplex DNA, and 0.3 nmol of circular viral DNA and were incubated at 30°C for 20 min unless otherwise indicated. Then 0.5 M EDTA (pH 8.0), proteinase K (20 mg/ml) (Beckman Instruments, Palo Alto, CA), and 10% (wt/vol) $NaDodSO_4$ were added to final concentrations of 50 mM, 600 μg/ml, and 0.1%, respectively, and the reaction mixtures were incubated at 37°C for 10 min. Then 4 μl of 0.25% bromophenol blue, 10 mM EDTA (pH 8.0), and 60% (wt/vol) sucrose was added, and each sample was analyzed by electrophoresis through an 0.8% agarose slab gel in buffer containing 40 mM Tris acetate (pH 7.9), 1 mM EDTA, and ethidium bromide at 0.5 μg/ml. The gels were photographed on Polaroid type 665 positive/negative film and quantitated using an LKB Ultroscan XL laser densitometer.

Purification of the Strand-Exchange Activity. *S. cerevisiae* strain BJ926 was grown in YPD media (35) in 10-liter carboys at room temperature with constant stirring. When the cell suspension reached an A_{600} of 1.0, methyl methanesulfonate was added to 0.01% (subsequent experiments showed that this addition did not alter the yield of enzyme). Then 2.5 hr later 20 g of dextrose per liter was added, and after 2.5 hr the cells were harvested by centrifugation. The cells were washed once with buffer W [50 mM Tris·HCl (pH 7.5), 10% (wt/vol) sucrose, and 1 mM EDTA], resuspended in 1 ml of buffer W per g of cells, frozen in liquid N_2, and stored at −75°C. After thawing the cells at room temperature all of the steps described below were performed at 0–4°C.

To 420 ml of cell suspension, 630 ml of buffer W, 105 ml of 4 M KCl, 10.5 ml of 0.5 M spermidine hydrochloride, 2.1 ml of 0.5 M EDTA (pH 8.0), 1.05 ml of 2-mercaptoethanol, and 450 mg of Zymolyase-100T were added. After 2.5 hr on ice 10.5 ml of 10% (wt/vol) Brij 58 and 1.05 ml of 0.1 M phenylmethylsulfonyl fluoride (dissolved in ethanol) were added. After 20 min the suspension was centrifuged for 25 min at 40,000 rpm in a Beckman 60Ti rotor, and the supernatants were pooled to give 1.09 liters of a solution of ≈10 mg/ml of protein (fraction I). To fraction I was added 529 g of ammonium sulfate with stirring over a 30-min period. After stirring an additional 30 min, the precipitate was harvested by centrifugation for 30 min at 9000 rpm in a Sorvall GS3 rotor. The precipitate was resuspended with 400 ml of buffer A [20 mM Tris·HCl (pH 7.5), 0.1 mM EDTA, 10% (wt/vol) glycerol, 10 mM 2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride], and the solution was first dialyzed for 2.5 hr against 4 liters of buffer A and then dialyzed for 1.5 hr against 2 liters of buffer A. The solution was diluted with buffer A to a conductivity equivalent to buffer A/85 mM NaCl to yield 710 ml (fraction II). Fraction II was applied at 200 ml/hr to a 12.6 cm^2 × 10 cm column of single-stranded DNA-cellulose equilibrated with buffer A/70 mM NaCl. After washing with 450 ml of buffer A/70 mM NaCl, the column was eluted with 1.4 liters of a linear gradient from 70 mM NaCl to 2.0 M NaCl in buffer A. The active fractions were eluted at 250 mM NaCl and yielded 101 ml of a protein solution (1.27 mg/ml) (fraction III). Fraction III was concentrated by precipitation with 52.1 g of ammonium sulfate and resuspended with 4.5 ml of buffer A. The protein was chromatographed on a 2 cm^2 × 70 cm column of Sephacryl S200 (Pharmacia) in buffer A/0.1 M NaCl at 11 ml per hr. The activity was eluted with ≈0.42 column volume and yielded 14.5 ml of a protein solution (1.08 mg/ml) (fraction IV). Fraction IV was diluted with one volume of buffer A and applied at 13 ml/hr to a 0.64 cm^2 × 3.3 cm column of PBE94 (Pharmacia) equilibrated with buffer A/50 mM NaCl. Then the column was washed at 8 ml/hr with 3 ml of buffer A/50 mM NaCl, and activity was eluted with a 60-ml linear gradient from 50 mM NaCl to 1 M NaCl in buffer A. The

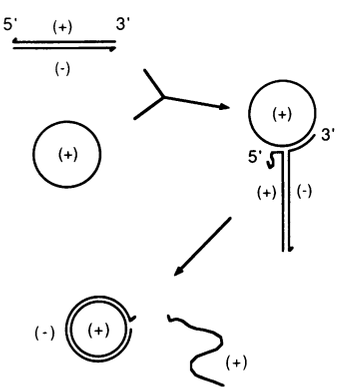

FIG. 1. Illustration of the formation of joint molecules from linear, duplex M13mp19 DNA and M13mp19 viral DNA. +, Viral strand; −, complementary strand. The 3′ and 5′ ends are indicated according to observed polarity of the reaction as discussed below.

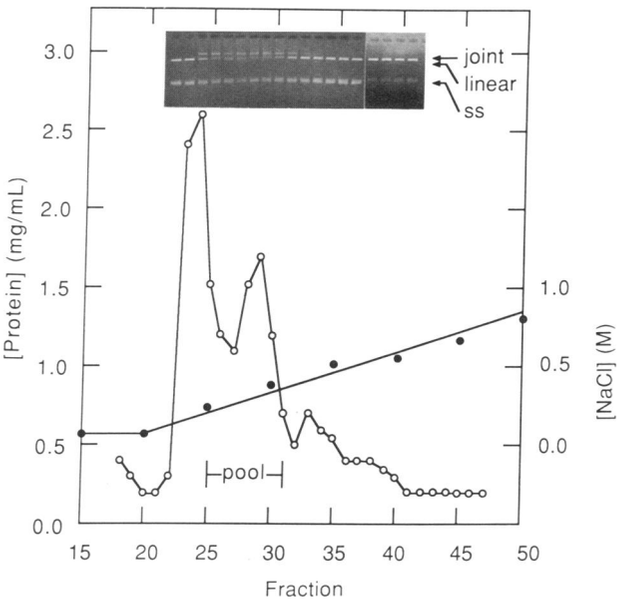

FIG. 2. Detection of an activity that catalyzes the formation of joint molecules after chromatography on single-strand DNA-cellulose. A 2-μl aliquot of each 16-ml fraction was assayed for activity. Nicked, circular duplex molecules comigrate with the joint molecules under the electrophoretic conditions used here. ○, Protein concentration; ●, NaCl concentration.

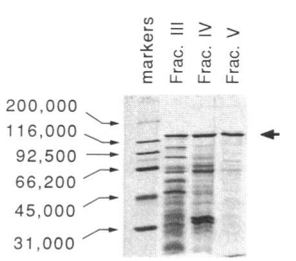

FIG. 3. Electrophoretic analysis of purified protein fractions. The indicated lanes contained 50 μg, 4.5 μg, and 1.5 μg of fractions III, IV, and V, respectively. The markers were myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin, and carbonic anhydrase. Electrophoresis was on a 10% polyacrylamide mini-gel containing $NaDodSO_4$. Arrow, M_r 132,000 polypeptide.

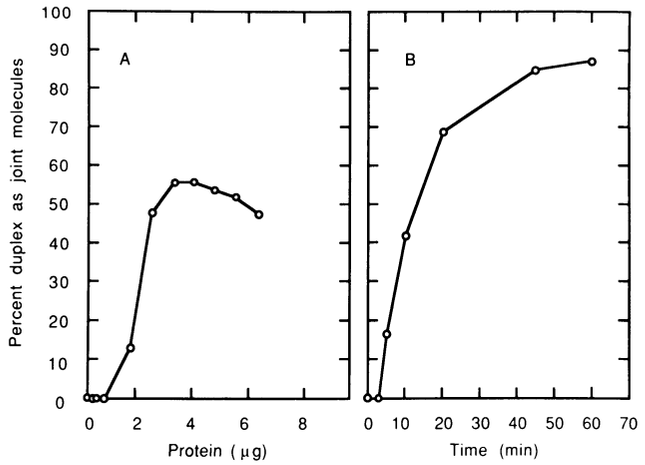

FIG. 4. Protein requirement and kinetics of joint molecule formation. (*A*) Reaction mixtures contained the indicated amounts of fraction V and were incubated for 20 min. (*B*) Reaction mixtures contained 3 μg of fraction V and were incubated for the indicated times.

activity was eluted with ≈270 mM NaCl, and the active fractions (4 ml) were pooled and dialyzed overnight against 1 liter of buffer A containing 60% (wt/vol) glycerol and 0.1 M NaCl to yield 1.2 ml of a protein solution (3 mg/ml) (fraction V). The preparation lost ≈20% of its activity during storage for 2 months at −20°C. Protein fractions were diluted in buffer containing 10 mM Tris·HCl (pH 7.5), 10 mM 2-mercaptoethanol, and bovine serum albumin at 0.5 mg/ml. Protein concentrations were determined by the method of Lowry (37), and protein samples were analyzed by NaDodSO$_4$/PAGE (38).

Electron Microscopy. For electron microscopy, 90-μl reaction mixtures were carried out through the above procedure through the proteinase K digestion step and then extracted once with phenol, and the aqueous phase was chromatographed on a Pasteur pipet column of agarose A5M (Bio-Rad) in TE buffer/0.1 M NaCl. Then the DNA was mounted for electron microscopy by the formamide technique (39).

RESULTS

Detection and Purification of a Strand-Exchange Activity. To detect a *S. cerevisiae* strand-exchange activity, we used the single-stranded circular by double-strand linear pairing reaction (Fig. 1) developed in studies with the *E. coli* RecA protein (22, 23). We did not detect a strand-exchange activity in either crude extracts or ammonium sulfate fractions of mitotic *S. cerevisiae* cells but were able to detect an activity after chromatography on a column of single-stranded DNA cellulose (Fig. 2). This activity was further purified by gel filtration through Sephacryl S200 and chromatography on PBE94. Fraction V represents ≈0.03% of the total soluble protein. Assuming a 50- to 100-fold purification by DNA-cellulose chromatography based on the recovery of 1% of the applied protein and an assumed 50% recovery of activity, and an ≈5-fold purification with a 50% recovery at each of the subsequent two steps, we estimate that the activity has been purified 1000- to 2000-fold. Analysis of fractions III, IV, and V by NaDodSO$_4$/PAGE indicated that a single M_r 132,000 polypeptide was enriched by each purification step (Fig. 3). If this polypeptide contains the activity, then we estimate the enzyme preparation is >90% pure.

Characterization of the Strand-Exchange Reaction. The formation of joint molecules (Fig. 4*A*) required stoichiometric amounts of protein, the reaction showed a high degree of cooperativity and, as with RecA, excess protein inhibited the reaction (12, 15–22). Maximal joint molecule formation was found at 2.5–3 μg of protein per 0.3 nmol of single-stranded M13mp19 DNA, which is about 1 M_r 132,000 polypeptide per 12–14 nucleotides of single-stranded DNA assuming that all of the protein molecules were active. Similar titration experiments containing either 0.2 or 0.6 nmol of single-stranded M13mp19 DNA also required 1 M_r 132,000 polypeptide per 12–14 nucleotides for maximal activity (data not shown). The reaction was linear for 20 min at 30°C with nearly quantitative joint molecule formation occurring near the reaction limit (Fig. 4*B*).

The formation of joint molecules required homology. Joint molecules were formed in reactions containing either M13mp19 viral DNA and linear M13mp19 RF DNA (Fig. 5*A*, lane 4) or φX174 viral DNA and linear φX174 RF DNA (Fig.

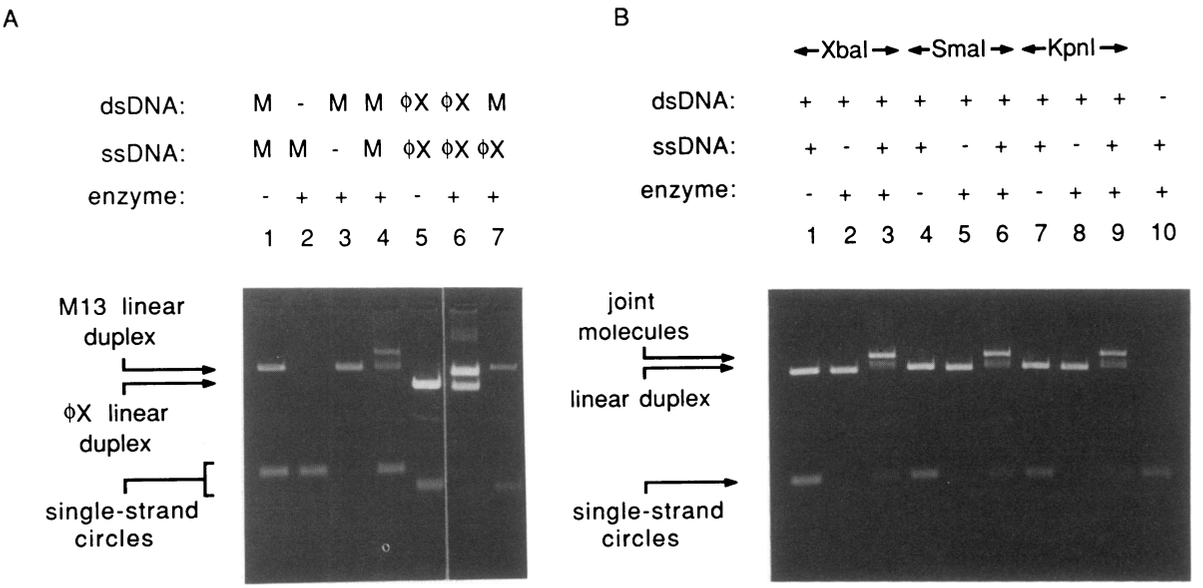

FIG. 5. Effect of homology and ends on strand transfer. All procedures were carried out as described in Fig. 2. (*A*) Where indicated reaction mixtures contained M13mp19 viral or linear, duplex DNA designated by M, φX174 viral or linear, duplex DNA designated by φX, and 3 μg of fraction V. (*B*) Reaction mixtures contained M13mp19 viral DNA and where indicated 3 μg of fraction V and M13mp19 RFI DNA digested with *Xba* I, *Sma* I, or *Kpn* I.

Table 1. Reaction requirements

	Relative activity, %
Complete reaction mixture	100
− Enzyme	<2
− Mg^{2+}	<2
− Dithiothreitol	89
− Dithiothreitol/+ 5 mM *N*-ethylmaleimide	<2
− ATP regenerating system*/− ATP	230
+ 200 mM NaCl	<2

Reaction mixtures were incubated for 20 min and contained 3 μg of fraction V and viral and linear, duplex M13mp19 substrates. A relative activity value of 100% is equivalent to 26% of the linear, duplex substrate DNA converted to joint molecules.
*Omission of creatine phosphokinase and creatine phosphate.

Table 2. Electron microscopic analysis of strand-exchange products

Linear substrate	% duplex DNA molecules present as a given form: Monomer linear	α form	σ form	Monomer circle	No. of molecules scored
M13mp19/ *Eco*RI	74	16	7	3	675
M13mp19*recJ*/ *Eco*RI	87	9	4	<0.2	525
M13mp19*recJ*/ *Hin*dIII	88	0.3	12	<0.3	337

Experimental procedures were carried out as described in Fig. 6, and reaction mixtures contained M13mp19 viral DNA and the indicated linear, duplex substrate. Representative electron micrographs are presented in Fig. 6.

5*A*, lane 6). Joint molecules were not formed in reactions containing φX174 viral DNA and linear M13mp19 RF DNA (Fig. 5*A*, lane 7) or M13mp19 viral DNA and linear φX174 RF DNA (data not shown). Joint molecules were formed with equal efficiency if the homologous, linear duplex had been linearized by digestion with *Xba* I (4 base pairs of a 5′-single-stranded overhang; Fig. 5*B*, lane 3), *Sma* I (blunt end; Fig. 5*B*, lane 6) or *Kpn* I (4 base pairs of a 3′-single-stranded overhang; Fig. 5*B*, lane 9). No detectable joint molecules were formed in the absence of homologous, single-stranded DNA (Fig. 5*B*, lanes 2, 5, and 8).

The requirements for different components in the reaction were determined (Table 1). The formation of joint molecules required Mg^{2+}. The reaction was inhibited by *N*-ethylmaleimide and 200 mM NaCl. There was also no requirement for ATP or an ATP regenerating system.

Structure of the Joint Molecules and Polarity of the Strand-Exchange Reaction. In reaction mixtures containing M13mp19 viral DNA and linear M13mp19 RF DNA the major class of joint molecules observed by electron microscopy (Fig. 6 and Table 2) were α forms consisting of a partially duplex circle with a duplex linear branch and a single-strand linear branch both attached to the circle at the same end of the duplex segment of the circle (Fig. 6 *A*, *B*, and *C*). The average extent of strand exchange was 4.1 kb, and the range of strand exchange observed was 1.1 kb to 7.1 kb. Less-abundant joint molecules observed were σ forms consisting of a full-length linear duplex attached to a single-stranded circle that showed no sign of strand exchange (Fig. 6*G*) and full-length duplex circular molecules (Fig. 6 *E* and *F*). These results indicate that the majority of the joint molecules are formed by a strand-exchange mechanism.

To determine the polarity of strand exchange, experiments were carried out in which a linear duplex substrate containing a 2.3-kb segment of nonhomologous DNA attached to one end or the other was added to the reaction mixture. When M13mp19 *recJ* RFI DNA was cleaved with *Eco*RI only the 3′ end of the linear duplex was complementary to the M13mp19 viral DNA. Reactions with these substrates yielded predominantly α-form joint molecules (Table 2). When M13mp19 *recJ* RF DNA was cleaved with *Hin*dIII only the 5′ end of the linear duplex was complementary to the M13mp19 viral DNA. Reactions with this pair of substrates yielded only σ forms (Table 2). These results show that initially joint molecules can be formed in both the 5′-to-3′ and 3′-to-5′ directions, but heteroduplex formation is polar and occurs only in the 3′-to-5′ direction.

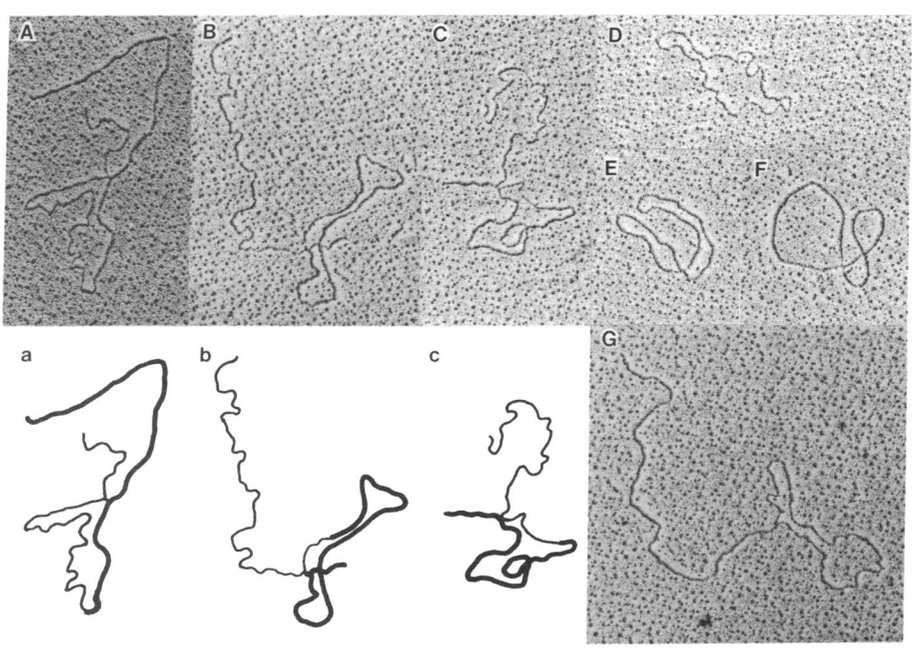

FIG. 6. Electron microscopic analysis of the structure of joint molecules. Reaction mixtures (90 μl) contained M13mp19 viral and linear, duplex DNA and 9 μg of fraction V. *A*, *a*, *B*, *b*, *C*, and *c*, α forms; *D*, single-stranded, circular M13mp19 DNA; *E* and *F*, circular, duplex M13mp19 DNA; *G*, σ form.

DISCUSSION

We have described the purification of an activity from mitotic *S. cerevisiae* cells that catalyzes the transfer of a single strand from a linear, duplex DNA molecule to a homologous, single-stranded, circular DNA molecule. The protein preparation that we have described primarily contains a M_r 132,000 polypeptide. While only this species copurifies with the strand-exchange activity, we do not have any other evidence that it is responsible for the strand-exchange activity or that trace amounts of additional proteins are not also required. Further studies will be required to resolve this point. Fraction V is slightly contaminated with exonuclease activity and will release no more than 40–50 nucleotides per single-stranded or double-stranded linear substrate under the conditions of strand exchange, but it lacks detectable endonuclease activity on single-stranded and double-stranded substrates. The exonuclease activity cannot account for the strand-exchange reaction for the following four reasons: (*i*) The specific activity of the exonuclease activity decreases at every step of the purification. (*ii*) It is less stable than the strand-exchange activity. (*iii*) There is insufficient exonuclease activity to account for the extent of heteroduplex formation. (*iv*) The formation of α forms clearly occurs by pairing of the complementary 3′ end of the linear duplex with the single-stranded circle in combination with displacement, rather than degradation, of the 5′ end of the linear duplex. We cannot eliminate the possibility that limited degradation is required for the initiation of pairing. Also, it is possible that the σ forms that are formed by pairing of the 5′ end of the linear duplex with the single-stranded circle result from limited digestion of the 3′ end of the linear duplex followed by the annealing of the single-stranded end to the single-stranded circle.

The *S. cerevisiae* strand-exchange activity shares several properties with other strand-exchange proteins, most notably the *E. coli* RecA and T4 UvsX proteins. It catalyzes the linear, duplex by single-strand circle strand-exchange reaction by pairing the 3′-homologous end of the linear duplex strand with the single-stranded circle followed by extension of the hybrid region toward the 5′ end of the linear duplex strand (3′-to-5′ direction) whereas all presently known eukaryotic proteins catalyze this reaction in the 5′-to-3′ direction (22, 23, 27–30). Also, it requires a high protein/single-stranded DNA molar ratio approaching that required by the RecA and UvsX proteins, and the reaction shows a high degree of cooperativity (12, 15–22). This suggests that it may catalyze strand exchange by first forming an active, single-stranded DNA–protein complex. That other eukaryotic strand-exchange proteins act in this fashion is unclear at present. One surprising finding is the lack of an ATP requirement. The only other strand-exchange activity that lacks an ATP requirement is the human activity, which has only been purified to a limited extent (30). Possibly further studies will demonstrate that the *S. cerevisiae* activity requires a high-energy cofactor under different conditions or that it has been purified as an activated protein intermediate as can occur with *E. coli* DNA ligase (40). If the *S. cerevisiae* activity does not require a high-energy cofactor, then mechanistic studies with this protein may provide some insight into the role of ATP in other strand-exchange reactions.

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