Yeast cell-free system that catalyses joint-molecule formation in a Rad51p- and Rad52p-dependent fashion

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One of the central reactions of homologous recombination is the invasion of a single strand of DNA into a homologous duplex to form a joint molecule. Here we describe the isolation of a cell-free system from meiotic yeast cells that catalyses joint-molecule formation *in vitro*. The active components in the system required ATP and homologous DNA and operated in both 0.5 and 13 mM MgCl₂. When the cell-free system was prepared from *rad51/rad51* and *rad52/rad52* mutants and joint-molecule formation was assayed at 0.5 mM MgCl₂, the specific activity decreased to 6% and 13.8% respectively of the wild-type level. However, when the same mutant extracts were premixed, joint-molecule formation increased 4–8-fold, i.e. the mutant extracts exhibited complementation *in vitro*. These results demonstrated

that Rad51p and Rad52p were required for optimal joint-molecule formation at 0.5 mM MgCl₂. Intriguingly, however, Rad51p and Rad52p seemed to be more dispensable at higher concentrations of MgCl₂ (13 mM). Further purification of the responsible activity has proven problematical, but it did flow through a sizing column as a single peak (molecular mass 1.2 MDa) that was co-eluted with Rad51p and RFA, the eukaryotic single-stranded DNA-binding protein. All of these characteristics are consistent with the known properties of the reaction *in vivo* and suggest that the new cell-free system will be suitable for purifying enzymes involved in homologous recombination.

Key words: DNA repair, heteroduplex DNA, recombination.

INTRODUCTION

Homologous recombination is the intracellular process that moves genetic information between chromosomal loci that share DNA sequence homology. The central intermediate in this process is heteroduplex DNA (hDNA). hDNA forms when a single-stranded tail, generally produced by exonucleolytic degradation at the end of a broken chromosome, invades a homologous duplex and pairs with its complementary strand [1] (see Figure 1a). The formation of such an hDNA joint aligns the recombining duplexes, brings them into physical association and serves as a substrate for later steps in the reaction.

Given the important role of hDNA in homologous recombination, its formation has been the subject of intensive enzymological analyses in prokaryotes and eukaryotes. For the yeast *Saccharomyces cerevisiae*, the most successful approaches have relied on the overexpression and purification of known recombination gene products, in particular those encoded by genes in the *RAD52* epistasis group (*RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57* and *DMC1*). From these studies it has been demonstrated that Rad51p, which has homology with the RecA protein of *Escherichia coli*, catalyses joint-molecule formation *in vitro* between circular single-stranded DNA (M13 or ϕ X174 viral DNA) and a homologous duplex (linearized M13 or ϕ X174 replicative form) [2,3]. This reaction requires ATP hydrolysis and RFA, the eukaryotic single-stranded DNA-binding protein. The interaction of Rad51p with a nucleoprotein complex consisting of RFA and single-stranded DNA is stimulated by a Rad55p/Rad57p heterodimer [4] and by Rad52p [5–8], although the latter protein has also been demonstrated to stimulate the renaturation of oligonucleotides in isolation [9], suggesting a more direct role

in hDNA formation. These biochemical studies argue that proteins encoded by genes in the *RAD52* epistasis group cooperate to form hDNA.

Genetic analysis, however, indicates that this simple model is insufficient to account for all hDNA formation *in vivo* [10]. In two separate studies, meiotic hDNA was shown to form in *rad52* and *rad57* mutants at significant levels: (1) 33% wild-type levels at the *MAT* locus [11], and (2) 50–100% wild-type levels at the *HIS4* locus [12]. Recombinational repair of broken chromosomes has also been shown to occur in *rad51* strains [12a]. Moreover, the mitotic recombination reaction that switches mating type in yeast occurred in the absence of genes in the *RAD52* epistasis group (with the exception of *RAD52* itself) if the silent donor loci were carried on plasmids [13]. Finally, proteins encoded by genes outside the *RAD52* epistasis group also have a role in hDNA formation (although whether this role is direct or indirect remains unclear in some cases). As an example, yeast mutants lacking Sep1p, a protein that forms joint molecules *in vitro* between homologous single-stranded and double-stranded substrates [14], exhibited altered intrachromosomal recombination rates, particularly in backgrounds that also lacked Rad51p [15]. These results strongly suggest that the enzymology of hDNA formation *in vivo* encompasses unelucidated complexities.

Standard chromatographic fractionation has been a powerful experimental method for analysing complex nucleic acid processes, such as DNA replication and transcription. By this approach, enzymes that catalyse an appropriate model reaction *in vitro* are purified from a crude cellular extract by column chromatography. Here we describe a yeast cell-free system for hDNA formation that should be appropriate for this type of

Abbreviations used: hDNA, heteroduplex DNA; PIC, protease-inhibitor cocktail.

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approach. The system, which is easily prepared from meiotic yeast extracts, catalyses a joint-molecule reaction *in vitro* that is a stringent and faithful model reaction of hDNA formation *in vivo*. Moreover, like recombination *in vivo*, the system *in vitro* is partly, but not completely, dependent on Rad51p and Rad52p. As expected, the reaction also requires ATP and homologous DNA for optimum activity. These properties indicate that the new system will be a suitable starting point for the fractionation of activities involved in hDNA formation, the central reaction of homologous recombination.

MATERIALS AND METHODS

D-loop assay

Standard assays (30 μ l) contained 33 mM Tris/HCl, pH 7.5, 13 mM MgCl₂, 1.8 mM dithiothreitol, 3 mM creatine phosphate, 1.3 mM ATP, 0.67 unit/ μ l creatine kinase, 1 μ M EcoRI-linearized pBlueScript SK⁺, 0.0275 μ M end-labelled oligonucleotide and 40–125 μ g of Fraction III or later fractions. (DNA concentrations are expressed as molar concentrations of nucleotides; the ratio of oligonucleotide to duplex substrate was 2.5:1). The homologous oligonucleotide was a 51-mer (5'-CGC CTC CAT CCA GTC TAT TAA TTG TTG CCG GGA AGC TAG TTC GCC AGT TAA-3') complementary to positions 2201–2261 of pBlueScript. The non-homologous oligonucleotide was a 60-mer (5'-AGT CTC TAT GCC CAT ATC CTC TAC TAG GAG TAG GGA CAC ACG TCC TAC CCT TGT CGA AGT-3') designed by using the algorithm of Abbasi and Sengupta [16], which generates a DNA sequence with minimum similarity to another defined sequence. All reactions were incubated for 20 min at 30 °C and terminated by the addition of 5 μ l of stop solution [5% (w/v) SDS/0.5% Bromophenol Blue/25% (v/v) glycerol]. The terminated reactions were subjected to electrophoresis at 8 V/cm for 2–2.5 h through 0.9% agarose gels containing 45 mM Tris/borate, pH 8.0, 1 mM EDTA and 1 mM MgCl₂. The bottom of each gel was cut off to remove untransferred oligonucleotides; the remaining portion was fixed in 7% (w/v) trichloroacetic acid for 30 min. After fixation, the gel was dried under vacuum at 50 °C for 2–3 h; joint-molecule formation was monitored by autoradiography.

Preparation of meiotic extracts

The *S. cerevisiae* strain was DN1064 (*MATa/MAT α* , *HO/HO*, *lys2/lys2*, *ura3 Δ ::hisG/ura3 Δ ::hisG*, *leu2::hisG/leu2::hisG*), an SK-1 derivative [17]. A fresh overnight culture of DN1064 in 5 ml of YPD medium [1% (w/v) Bacto Yeast Extract/1% (w/v) Bacto Peptone/2% (w/v) dextrose] was diluted into 50 ml of YPD medium and incubated overnight at 30 °C. Cells were harvested by centrifugation, washed in YPA medium [1% (w/v) Bacto Yeast Extract/1% (w/v) Bacto Peptone/1% (w/v) potassium acetate], and resuspended in 8 litres of YPA medium. After growth overnight at 30 °C, cells were harvested by centrifugation, washed in SPM medium [1% (w/v) potassium acetate], and resuspended in 8 litres of SPM. Cells were incubated at 30 °C for 4.5 h, i.e. the period of maximal induction of meiotic recombination [18]. The cells were harvested by centrifugation and washed in resuspension buffer [1 M NaCl/50 mM Tris/HCl (pH 7.5)/10 mM EDTA/10 mM 2-mercaptoethanol/1.0 mM dithiothreitol/0.2 mM PMSF/2 mM benzamidine/HCl/1 mM MgCl₂]. The final cell pellet was resuspended in 20 ml of resuspension buffer, frozen in liquid nitrogen and stored at –80 °C.

Isolation of the cell-free system

All operations were performed at 4 °C. Cells were thawed overnight at 4 °C and transferred into a BioSpec BeadBeater fitted with a 400 ml stainless-steel chamber containing 50% (v/v) acid-washed glass beads (0.5 mm in diameter). Before addition of the cells, the beads were washed three times with distilled water, twice with resuspension buffer and once with lysis buffer [resuspension buffer plus 10% (w/v) sucrose]. After transfer of cells into the BeadBeater, the remaining volume of the chamber was filled with lysis buffer, at which point 400 μ l of protease-inhibitor cocktail (PIC; 1 mg/ml each of aprotinin, antipain, chymostatin, pepstatin A and leupeptin) was added. Cells were lysed by nine bursts of 1 min each, separated by 2 min cooling periods; the chamber was cooled by an NaCl/ice-water mixture. The cell lysate was collected and 100 μ l of 0.1 M PMSF, 100 μ l of 1.0 M benzamidine/HCl and 200 μ l of PIC were added. The cell lysate was centrifuged in a Beckman 45Ti rotor for 60 min at 95000 g (35000 rev./min); the supernatant was collected and designated Fraction I (5.27 mg/ml, 328 ml). (NH₄)₂SO₄ (141.04 g) was added over a 30–40 min period to Fraction I to bring the final concentration to 65% satn. After being stirred for an additional 30 min, the precipitate was collected by centrifugation in a Beckman 45Ti rotor for 30 min at 31000 g (20000 rev./min). The precipitate was resuspended in 175 ml of dialysis buffer [20 mM Tris/HCl (pH 7.5)/1 mM EDTA/1 mM dithiothreitol/10 mM 2-mercaptoethanol/0.1 mM PMSF/2 mM benzamidine/HCl/10% (v/v) glycerol/1 mM MgCl₂] and the solution was dialysed against several changes of buffer until the conductivity was equivalent to that of buffer A [25 mM Tris/HCl (pH 8.0)/1 mM EDTA/0.01% (v/v) Nonidet P40/1 mM dithiothreitol/1 mM MgCl₂/10% (v/v) glycerol/0.2 mM PMSF/2 mM benzamidine/HCl, plus PIC] containing 10 mM NaCl. The dialysate was designated Fraction II (6.44 mg/ml; 175 ml). Fraction II was applied at 24 ml/h to a 1.77 cm² × 9 cm column of DEAE-Sephacel (Pharmacia) pre-equilibrated with buffer A containing 10 mM NaCl. The column was washed with buffer A containing 10 mM NaCl until the UV absorbance (254 nm) of the eluate reached background levels. The column was then developed with a 240 ml linear gradient from 10 mM to 1.0 M NaCl in buffer A. The fractions containing joint-molecule activity, which were eluted at 280–450 mM NaCl, were combined and concentrated in an Ultrafree-15 Centrifugal Filter Device (Millipore). Buffer A was added to the retained material and then reconcentrated; this procedure was repeated until the conductivity was approx. 40 mM. The resulting fraction was designated Fraction III (6.4 mg/ml; 50 ml). Fraction III was applied at 1 ml/min to a 5 ml HiTrap Q column (Pharmacia) pre-equilibrated in buffer A containing 10 mM NaCl; the column was washed in the same buffer until the UV absorbance (254 nm) of the eluate reached background levels. The column was then developed with a 100 ml linear gradient from 10 mM to 1.0 M NaCl in buffer A. The fractions containing joint-molecule activity, which were eluted at 260–470 mM NaCl, were dialysed and concentrated as described for Fraction III. The resulting fraction was designated Fraction IV (40.91 mg/ml; 0.6 ml).

RESULTS

D-loop assay for hDNA formation

In many published studies, hDNA formation has been analysed with a model reaction *in vitro* known as the strand exchange assay [19]. This simple electrophoretic assay, which monitors joint-molecule formation between a circular single-stranded DNA (M13 or ϕ X174 viral DNA) and a linear homologous

Figure 1 A model reaction *in vitro* for joint-molecule formation

(a) D-loop formation *in vivo*. During homologous recombination, double-stranded breaks are excised by exonucleases to generate single-stranded tails. These tails subsequently invade homologous DNA to generate D-loops [1]. (b) D-loop assay *in vitro*. The model reaction *in vitro* monitored the invasion of an end-labelled single-stranded oligonucleotide into the middle of linearized pBlueScript SK⁺ DNA. The star indicates the radioactive end-label.

duplex (M13 or ϕ X174 replicative-form DNA), has been used with great success to elucidate the molecular mechanism by which the *E. coli* RecA and T4 UvsX proteins catalyse hDNA formation. Nevertheless, we opted not to use the strand exchange assay, because some non-recombination proteins have been demonstrated to catalyse product formation in this reaction as well [20]. These undesired activities seem to operate by renaturing the single-stranded substrate to the ends of the linear duplex, a reaction that is not predicted by our current models of homologous recombination.

Instead, hDNA is thought to form when a single-stranded tail at the end of a double-stranded break invades the middle of a homologous duplex [1] (Figure 1a). To model this reaction *in vitro*, we used a D-loop assay that monitored the invasion of an end-labelled single-stranded oligonucleotide (60-mer) into the middle of a 3 kb linear duplex molecule (pBlueScript SK⁺) (Figure 1b). The product of this reaction can be detected by agarose-gel electrophoresis followed by autoradiography. So far, only genuine recombination enzymes have been shown to catalyse this (or related) reactions, indicating that it is more discriminatory than the strand exchange reaction against artifacts. For instance, the only known yeast protein that catalyses the invasion of a single-stranded molecule into the middle of a duplex is Rad51p, although it does so inefficiently and only when the single strand has a specific sequence [21].

The product of the D-loop assay *in vitro* might formally be triplex DNA. However, given the stringent deproteinization conditions at the end of the reaction (SDS and proteinase K) and the fact that the joint molecules are subsequently subjected to agarose gel electrophoresis, this likelihood is relatively small.

Figure 2 Biochemical characterization of joint-molecule formation by Fraction IV

(A) Joint-molecule formation is ATP- and homology-dependent. D-loop assays were performed with the homologous (Homol) or non-homologous (Non-homol) oligonucleotides (see the Materials and methods section). In addition to 33 mM Tris/HCl, pH 7.5, 1.8 mM dithiothreitol and an ATP-regeneration system, the reactions contained 1.3 mM ATP plus 13 mM MgCl₂ (complete), 1.3 mM ATP plus 0.5 mM MgCl₂ (low MgCl₂), 13 mM MgCl₂ (—ATP) or 0.5 mM MgCl₂ (low MgCl₂, —ATP). Also shown are end-labelled pBlueScript SK⁺ DNA (end) and D-loop formation in the absence of Fraction IV (no protein). (B) Fraction IV transfers the homologous oligonucleotide into a supercoiled duplex substrate. Supercoiled pBlueScript DNA was used as a substrate in the D-loop assay. Lanes are designated as in (A). The migration of supercoiled (SC) and open circular (OC) pBlueScript is shown by the arrows. The bands between the supercoiled and open circular DNA migrated with linear pBlueScript DNA.

Preparation of a cell-free system that catalyses hDNA formation

By using the D-loop assay, we isolated an (NH₄)₂SO₄ fraction from yeast extracts that catalysed hDNA formation (see the Materials and methods section). The extract was from large-scale cultures of diploid SK1 cells [17] prepared 4.5 h after entrance into the sporulation pathway, i.e. during the stage of meiosis at which hDNA formation is maximally induced [18]. Because the preparation of meiotic extracts involves several inconvenient changes of media, we also examined whether extracts prepared in a similar way from mitotic cells could catalyse the D-loop assay *in vitro*. Unfortunately, although we found some activity in mitotic extracts, the levels were much lower and the consistency of the extracts was much less satisfying, i.e. only a fraction of the mitotic extracts had any activity. We therefore chose to analyse exclusively the meiotic extracts.

The activity in the (NH₄)₂SO₄ fraction from the meiotic extracts was then further purified by anion-exchange chromatography through DEAE-Sephacel (Pharmacia) and Mono Q (Pharmacia) (see the Materials and methods section). The final fraction, designated Fraction IV, was analysed in more detail to determine the parameters of the transfer reaction (Figure 2A). In the standard assay, which contained 13.75 fmol of end-labelled oligonucleotide, 0.4 fmol of oligonucleotide was typically transferred into products in a 20 min reaction, i.e. 3% of the

Figure 3 Rad51p and Rad52p are required for optimal joint-molecule formation

Fraction IV was prepared from wild-type (*RAD51, RAD52*), *rad51Δ/rad51Δ* (*rad51*), *rad52Δ/rad52Δ* (*rad52*) and *rad51Δ/rad51Δ, rad52Δ/rad52Δ* (*rad51, rad52*) cells. Each fraction was then made to the same protein concentration (8.3 mg/ml). Numbers represent the quantity (in μ l) of Fraction IV from the designated strains that were used in the standard D-loop assays. The first lane, which is unmarked, was end-labelled pBlueScript DNA.

oligonucleotide substrates. Because the molar ratio of oligonucleotide to duplex substrates was 2.5:1, this translated into 7.5 % of double-stranded molecules converted into product. The joint-molecule products were sensitive to heat and formamide (results not shown), demonstrating that the monitored band arose from hDNA rather than from the transfer of the radioactive phosphate into a covalent linkage on the duplex.

The joint-molecule activity in Fraction IV displayed the following biochemical requirements. First, product formation was absolutely dependent on the presence of ATP (Figure 2A). Secondly, optimal activity required an oligonucleotide that was homologous with the double-stranded DNA substrate (Figure 2A). When a non-homologous oligonucleotide was used, joint molecules formed at less than 4 % of wild-type levels (Figure 2A). Thirdly, the activity catalysed the invasion of the oligonucleotide into supercoiled pBlueScript duplexes (Figure 2B). Therefore the activity neither required duplex ends for the reaction nor operated through a nicked or nucleolytically degraded intermediate, because the product continued to migrate on an agarose gel with supercoiled DNA. Finally, joint-molecule formation occurred in 13 mM $MgCl_2$ /0.5 mM $MgCl_2$ (Figure 2A). Therefore the activity is functional over a broad range of $MgCl_2$ concentrations, unlike purified Rad51p, which exhibited strand-exchange activity only within a very narrow range [3].

Optimal joint-molecule formation in the cell-free system requires Rad51p and Rad52p

The preceding characteristics of the activity were expected for a genuine recombination activity. To provide further evidence for this conclusion, we examined whether it also required Rad51p and Rad52p. To do so, Fraction IV was purified by the same protocol from a *rad51/rad51* mutant, a *rad52/rad52* mutant and a *rad51/rad51, rad52/rad52* double mutant. The resulting fractions were then made to the same protein concentration and analysed in the D-loop assay *in vitro* at 0.5 mM $MgCl_2$ (Figure 3). The results clearly showed that the mutant fractions had lower specific activities than the wild-type fraction. For instance, when we used 10 μ l of each fraction, product formation in the *rad51/rad51* and *rad52/rad52* extracts occurred at 6 % and 13.8 % respectively of wild-type levels.

However, it was possible that Rad51p and/or Rad52p were not involved in the joint-molecule formation itself, but were instead responsible (directly or indirectly) for lowering the level of an unrelated activity. To test this possibility, we premixed 5 μ l each of the *rad51/rad51* and *rad52/rad52* extracts and repeated the analysis. If Rad51p and/or Rad52p affected the expression of

Figure 4 Rad51p and Rad52p are more important for joint-molecule formation at low concentrations of $MgCl_2$

Fraction IV was prepared from wild-type (*RAD51, RAD52*), *rad51Δ/rad51Δ* (*rad51*), *rad52Δ/rad52Δ* (*rad52*) and *rad51Δ/rad51Δ, rad52Δ/rad52Δ* (*rad51, rad52*) cells. Each fraction was then made to the same protein concentration (8.3 mg/ml); 15 μ l of each was assayed for D-loop formation *in vitro* at a high [$MgCl_2$] (13 mM $MgCl_2$) and a low [$MgCl_2$] (0.5 mM $MgCl_2$). Also shown is end-labelled pBlueScript SK⁺ DNA (end).

the activity but were not directly involved in the reaction itself, then the mixed fraction would be expected to form 6–13.8 % of the products seen in the wild-type reaction. Instead, the mixed fraction formed 49.3 %, a stimulation of 4–8-fold (Figure 3). The mutant activities therefore exhibited complementation *in vitro*, demonstrating that Rad51p and Rad52p were functionally required for activity at 0.5 mM $MgCl_2$.

Intriguingly, the requirements for Rad51p and Rad52p were less stringent at higher concentrations of $MgCl_2$. Therefore when the same extracts were analysed at 13 mM $MgCl_2$, the specific activity decreased by less than 50 % in comparison with the wild-type extract (Figure 4). Further enzymological analysis will be required to explain the different sensitivities, but the latter effects in 13 mM $MgCl_2$ were more in keeping with the known effects of *rad51* and *rad52* mutations on hDNA formation *in vivo* [11,12]. We therefore conclude from the analyses of mutant extracts that Rad51p and Rad52p are important, but not absolutely essential, for joint-molecule formation in the cell-free system, as is true of hDNA formation *in vivo*.

Further purification of the joint-molecule activity

The joint-molecule activity in Fraction IV required ATP, homologous DNA, Rad51p and Rad52p for optimal activity. These characteristics, which were expected of a recombination activity, encouraged us to purify the proteins responsible more extensively. However, this proved to be problematical, because the activity seemed to separate into multiple fractions on different matrices (results not shown). Nevertheless, if the protein concentration of Fraction IV was kept relatively high, we found that the activity flows through a Sephacryl HR300 column without separation into multiple fractions. The profile from such a column, which fractionates proteins according to Stokes radii, showed that the activity was exceptionally large, being eluted after the void volume at 1.5 MDa but before thyroglobulin at 669 kDa (Figure 5). With the method of Laurent and Killander [22], these results indicate that the D-loop activity was a complex with a molecular mass of 1.2 MDa. Whether the complex consists solely of proteins or is a mixture of proteins and nucleic acids remains unclear. However, the fact that Fraction IV was prepared from a 65 %-(NH_4)₂SO₄ precipitate [2.7 M (NH_4)₂SO₄] argues against a nucleoprotein complex, a conclusion consistent with the observation that no nucleic acids were observable in the fraction when assayed by agarose-gel electrophoresis (results not shown).

Figure 5 Analysis of the joint-molecule activity in Fraction IV by gel-exclusion chromatography

Fraction IV was chromatographed over a Sephacryl S300-HR sizing column, and the resulting fractions (designated by numbers) were analysed by the D-loop assay [Activity (I)]. Fractions from activity (I) were subjected to Western-blot analysis with antibodies against RFA1 and RFA2 (RFA1, RFA2). Fraction IV was prepared from a different batch of cells and chromatographed over Sephacryl S300-HR. The resulting fractions (designated by numbers) were analysed by the D-loop assay [Activity (II)]. Fractions from activity (II) were subjected to Western blot analysis with antibodies against Rad51p. Also shown are end-labelled pBlueScript DNA (end), the D-loop activity of the load (IV) and the elution points for blue dextran at 1.5 MDa and thyroglobulin at 669 kDa (arrows). RFA1p and RFA2p were purified from a bacterial overexpression system [31] and used as controls for the top two Western blots (RFA1p, RFA2p). Fraction IV was prepared from a *rad51Δ/rad51Δ* mutant and used as a control for the bottom Western blot (*rad51Δ*).

Finally, we used the fractions from the sizing column to determine whether RFA, the heterotrimeric single-stranded DNA-binding protein of eukaryotes, was co-eluted with the joint-molecule activity. To do so, we performed a Western-blot analysis on the eluted fractions with antibodies directed against the middle and small subunits of RFA. The activity and RFA were co-eluted perfectly (Figure 5). This does not constitute formal proof that the single-stranded DNA binding protein is in the activity, but is nevertheless consistent with this hypothesis, as predicted by published genetic [23,24] and biochemical studies [3]. As predicted by our earlier experiments, Rad51p was also co-eluted perfectly with the activity from the sizing column (Figure 5).

DISCUSSION

Here we have described the isolation of a cell-free system from meiotic yeast cells that catalyses joint-molecule formation *in vitro*, i.e. the invasion of a single-stranded DNA molecule into the middle of a homologous duplex. Optimal activity required ATP, homologous DNA, Rad51p and Rad52p. The responsible

activity(s) in the fraction worked over a range of $MgCl_2$ concentrations (0.5–13 mM) and, at high protein concentration, were co-eluted on a sizing column with Rad51p and the RFA single-stranded DNA-binding protein. These properties were consistent with known characteristics of recombination in the yeast *S. cerevisiae* [25].

Four characteristics of this cell-free system should make it an attractive starting point for the future purification of recombination proteins. First, the system catalyses a biologically relevant reaction that has proved to be a stringent and faithful model of hDNA formation *in vivo*. Secondly, it catalyses this reaction relatively efficiently (7.5% of duplex substrate is converted into product). Thirdly, it catalyses the reaction in a robust manner, generating products over a range of $MgCl_2$ concentrations and after different orders of addition of the various reaction components (results not shown). Fourthly, it exhibits a dependence on Rad51p and Rad52p that mimics recombination *in vivo*, i.e. the reaction *in vitro* is partly, but not completely, dependent on these two proteins [11,12]. This last point in particular encourages us to believe that the cell-free system recapitulates at least some of the enigmatic complexities of the reaction *in vivo*.

Work in this laboratory has now begun on purifying the responsible proteins from Fraction IV by standard chromatographic methodologies. Our preliminary attempts have convinced us that this will be a long-term project. Nevertheless, we have developed appropriate conditions that allow the activity to flow through a sizing column as a large complex. It remains to be determined whether this complex is biologically significant or is a non-specific aggregate of unrelated proteins. However, it is interesting to note that several laboratories have posited the existence of a recombinosome, a large recombination machine that catalyses important subreactions of homologous recombination [26–30].

Much of the current work on recombination enzymology has analysed known recombination gene products purified from overexpression systems. Typically, these proteins have been characterized, alone or in combination with other purified gene products, in reactions *in vitro* that model specific steps of homologous recombination. Fundamentally, this approach is viable only if one already has a hypothesis about the function of a particular protein; moreover, gene products that have not been identified by genetic analysis will be missed. In contrast, the chromatographic fractionation of a complex cell-free system has none of these disadvantages. That is not to say that the wealth of genetic information on recombination in *S. cerevisiae* is to be ignored; in fact, results obtained from the cell-free system can and should be validated by examining extracts from recombination mutants, as demonstrated here with *rad51/rad51* and *rad52/rad52* extracts. Rather, it is our hope that the cell-free system described here will provide a valuable and complementary tool for elucidating the enzymology of homologous recombination.

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