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Plasmid Recombination Intermediates Generated in a *Saccharomyces cerevisiae* Cell-Free Recombination System

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We have developed an assay utilizing *Saccharomyces cerevisiae* cell extracts to catalyze recombination in vitro between homologous plasmids containing different mutant alleles of the *tet* gene. Electrophoretic analysis of product DNA indicated that a number of novel DNA species were formed during the reaction. These species migrated through agarose gels as distinct bands with decreased electrophoretic mobility compared with the substrate DNA. The DNA from each individual band was purified and shown to be enriched 5- to 100-fold for tetracycline-resistant recombinants by using a transformation assay. The structure of the DNA molecules present in these bands was determined by electron microscopy. Recombination between circular substrates appeared to involve the formation and processing of figure-eight molecules, while recombination between circular and linear substrates involved the formation of molecules in which a circular monomer had a monomer-length linear tail attached at a region of homology.

Genetic recombination is the process by which DNA molecules undergo rearrangement to generate new combinations of genes or parts of genes. Because meiotic recombination in *Saccharomyces cerevisiae* generally yields 6:2, 2:6, 5:3, and 3:5 segregants and rarely yields aberrant 4:4 segregants, recombination models involving the formation of asymmetric heteroduplex DNA have been proposed (18). This is in contrast to the original model proposed by Holliday, which hypothesizes the formation of symmetric regions of heteroduplex DNA (13). Asymmetric strand exchange could be initiated at the site of single-strand breaks in DNA. In support of this model, it has been shown that single-strand breaks in DNA accumulate during meiosis at the time of commitment to recombination (24). Double-strand breaks in DNA have been shown to be efficiently repaired by a recombination reaction in *S. cerevisiae* (20, 21, 25). These data have led to the proposal of a model in which recombination initiates at the site of double-strand breaks and results in the formation of a joint molecule containing two Holliday junctions (28). This model can explain certain features of yeast meiotic recombination, such as the predominance of 5:3, 3:5, 6:2, and 2:6 segregants over aberrant 4:4 segregants and the observation that initiation of recombination frequently occurs on the chromatid that is the recipient of genetic information during gene conversion events (11, 12). In contrast to meiotic recombination, mitotic recombination events often appear to involve symmetrical regions of heteroduplex DNA and can be most easily explained by models such as the original Holliday model (13). The intermediate proposed by Holliday initiates by breakage and rejoining of an appropriate pair of single strands at the same site to produce a single crossed-strand junction covalently joining two homologous duplexes (Holliday intermediate). This intermediate can branch migrate to generate heteroduplex regions located symmetrically on both participating duplexes.

Although most proposed recombination models are derived from data obtained from experiments with eucaryotes, there is considerable evidence from experiments with procaryotic plasmids and bacteriophages to support the existence of the Holliday intermediate. One would predict that during recombination of circular genomes, the Holliday intermediate would have a figure-eight geometry. Branched DNA molecules having this type of structure have been visualized among recombining molecules by electron microscopy (15, 32). Similarly, molecules of this configuration have been isolated from cells infected with the small circular phages S13, ϕX174, and G4 (4, 10, 29, 30) and also from plasmid-containing cells (14, 22). Although DNA molecules that look like figure-eights could arise during DNA replication, their virtual absence from cells containing mutations in genes whose products are required for recombination suggests that they are intermediates in a recombination reaction (14, 22). Furthermore, the observation that the cross-strand junction of a figure-eight can branch migrate to form heteroduplex DNA and electron microscopy studies visualizing the junction are consistent with the figure-eights being recombination intermediates rather than replication intermediates (22, 29). DNA molecules resembling figure-eights have also been detected in plasmid populations incubated with cell extracts from *Escherichia coli* or with purified *recA* protein (7, 16, 23, 35).

There is also physical evidence to support the existence of Holliday intermediates in eucaryotic cells. Branched molecules have been observed in cells infected with adenovirus type 2 (36) and also in a cell-free system derived from *Xenopus* oocytes (3). Branched forms of the *Saccharomyces cerevisiae* plasmid 2μm that appear to contain a Holliday junction have been observed during meiosis (1). Furthermore, several types of X-shaped structures, including eye loops and fused junctions, have been identified in total cellular DNA isolated from pachytene-arrested yeast cells (2).

While it seems clear that Holliday junctions are involved in recombination, there is a limited amount of information available about the enzymes that act during the formation and resolution of these junctions. As a first step in the study of this problem we developed an in vitro system that uses cell extracts prepared from *S. cerevisiae* cells to catalyze recombination between homologous plasmids containing dif-

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ferent mutant alleles of either the *S. cerevisiae ARG4* or the pBR322 *tet* gene (27). In these experiments, a transformation assay was used to quantitate the formation of recombinant ARG^+ or Tc^r DNA molecules. The structure of recombinant plasmids recovered from transformants was analyzed by restriction mapping and was found to be consistent with a recombination reaction involving both gene conversion and crossing over. Recombination products were also observed directly by fractionating reaction mixtures by electrophoresis on agarose gels. Although these results indicated that recombinant monomers were formed during the recombination reaction in vitro, it is also possible that a variety of recombination products and intermediates were formed, including some that were subsequently processed into mature recombinants after transformation into *E. coli*. Here we describe the isolation and characterization of several such DNA species and consider their role in the recombination reaction.

MATERIALS AND METHODS

Bacterial and yeast strains. *E. coli* JC10287 Δ(*slrR-recA*)*304 thr-1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-5 proA2 his-4 argE3 rpsL31 tsx-33 supE44*, used to propagate most plasmids, was obtained from A. J. Clark. *E. coli* RK1388 and RK1389 are derivatives of JC10287 that had been transformed to ampicillin resistance with pRDK35 and pRDK39, respectively (9). *E. coli* RDK1400 *thr leuB6 thi-1 thyA trpC117 hsdR12 hsdM12 str recA13* has been described previously (26). *S. cerevisiae* TA405 **a**/α, *his3-11,-15/his3-11,-15, leu2-3,-112/leu2-3,-112, can1/can1*; DA185 **a**/α, *his3-11,-15/his3-11,-15, leu2-3,-112/leu2-3,-112, can1/can1*, 2μm° were obtained from A. Murray. The haploid *S. cerevisiae* strain LBL1/n α, *ade2, ade5, ade6, lys2, his7-1, tyr1-2, cly8, lys2-2, ura3-1, met13-C, trp5-d* was obtained from M. Esposito.

Preparation of DNA. Plasmid DNA substrates pRDK35 and pRDK39 were purified essentially as described previously (14); this procedure included amplification of plasmid DNA by growth in the presence of 250 μg of chloramphenicol per ml and two cycles of equilibrium centrifugation in CsCl-ethidium bromide density gradients. The substrate DNAs were purified further by phenol extraction and ethanol precipitation to remove residual ethidium bromide and to concentrate the DNA samples. The linear substrate DNA was pRDK35 digested with *Bam*HI as described by the manufacturer (New England BioLabs, Inc., Beverly, Mass.) and then purified by phenol extraction and ethanol precipitation. Restriction endonuclease cleavage site maps of these plasmid substrates are shown in Fig. 1.

Transformation of *E. coli*. The preparation of frozen competent cells and procedures for the transformation of RK1400 have been described elsewhere (26).

Growth of yeast cells and preparation of cell extracts. Cultures of yeast strains TA405 and DA185 were grown in YPD medium (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 2% [wt/vol] glucose) at 30°C with shaking until they reached a cell density of 5×10^7/ml. Strain LBL1/n was grown at 21 to 23°C, as the mutation *cly8* confers temperature sensitivity above 25°C. Cell extracts were prepared essentially as described previously (26).

Recombination assays. Assays were generally carried out in 25-μl volumes containing 35 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.8); 10 mM $MgCl_2$; 1 mM dithiothreitol; 2 mM spermidine; 5 mM ATP; 0.2 mM each CTP, GTP, and UTP; 0.01 mM each dATP, dCTP, dGTP, and dTTP; 1 mM NAD; 100 μg of bovine serum albumin per ml; and 0.5 μg of each substrate DNA. Large-scale reactions used for gel fractionation assays were carried out in 100-μl volumes as described above but with 2 μg of each substrate DNA. The yeast cell extract was present at the optimal concentration, which ranged from 0.4 to 0.8 mg/ml. After incubation at 30°C for the designated time, EDTA (pH 8.0) was added to a final concentration of 20 mM and the DNA was purified by two phenol extractions followed by precipitation with ethanol. The plasmid DNA was suspended in an appropriate volume of 10 mM Tris (pH 8.0)–1 mM EDTA and analyzed by transformation or gel electrophoresis.

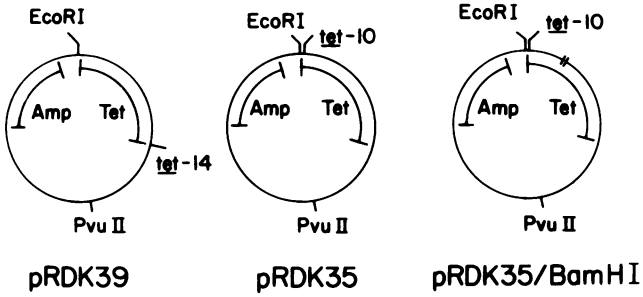

FIG. 1. Restriction endonuclease cleavage site maps of recombination substrates. The position of the double-strand break in pRDK35-*Bam*HI is indicated. All of the substrates are derivatives of pBR322 and have been described previously (9, 26).

Analysis of plasmid DNA. DNA samples were analyzed by electrophoresis on agarose slab gels run in Tris-acetate-EDTA buffer containing 0.5 μg of ethidium bromide per ml. To purify DNA species for analysis by tranformation into *E. coli*, the product DNA was fractionated by electrophoresis through agarose gels made with low-gelling-temperature agarose, and the DNA was extracted essentially as described previously (34). In some experiments, regular agarose was used and the DNA was extracted by the freeze-and-squeeze method (31). For all electron microscopy experiments, regardless of the gel composition, the DNA was purified from gel slices by the freeze-and-squeeze method.

Electron microscopy. Preparation and spreading of samples for electron microscopy was essentially as described previously (14). Restriction endonuclease digestion of plasmid DNA before spreading was performed at 10°C to inhibit branch migration of cross-strand exchange forms (33).

RESULTS

We demonstrated that cell extracts from *S. cerevisiae* catalyze a substantially complete recombination reaction between homologous plasmids. This reaction had kinetics and cofactor requirements typical of many enzymes that act on DNA. During incubation of circular (or circular and linear) plasmid substrates with the yeast extract, the frequency of Tc^r transformants increased 100- to 500-fold over a 2 h period (Fig. 2). The reactions proceeded with almost linear kinetics up to 90 min and then plateaued or decreased slightly. Up to 90 min, the number of Ap^r transformants obtained remained fairly constant, while the number of Tc^r transformants increased dramatically. After longer times, the total number of transformants started to decline, presumably due to DNA degradation by nucleases present in the extract.

Although the transformation assay is convenient and sensitive for detecting recombinants, it provides little insight

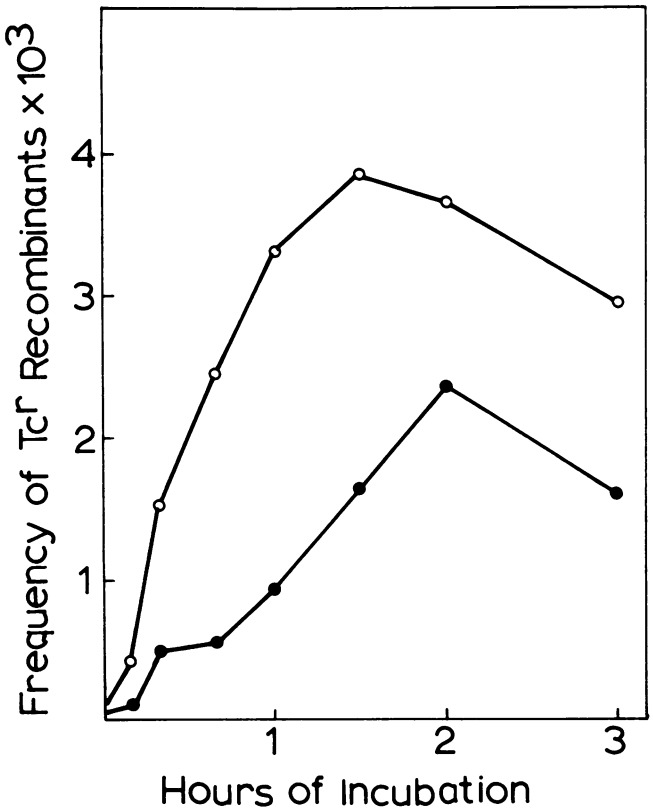

FIG. 2. Time course of recombination. Recombination reactions containing linear and circular substrates (○) or circular substrates (●) were incubated at 30°C for the indicated times and analyzed by using the transformation assay essentially as described in Materials and Methods. The recombination frequency is the fraction of Ap^r transformants that are also Tc^r.

into the DNA intermediates formed during the reaction because only mature recombinant products are recovered from the transformants. A clue to the nature of intermediates formed was provided by the observation that DNA which had been incubated with the yeast extract contained a number of novel high-molecular-weight species when fractionated by electrophoresis through agarose gels. We were interested in determining whether the DNA species present in these bands represented intermediates in the recombination reaction. In an experiment in which circular plasmid DNA was incubated with the yeast extract for different times and then fractionated by electrophoresis, a characteristic pattern of bands indicating species having a slower mobility than the monomeric substrates was observed (Fig. 3, experiment 1). In an analogous experiment with circular and linear substrates, a similar pattern of bands was observed; however, the kinetics of formation of some bands differed (Fig. 3, experiment 2). During both reactions the substrate DNA was not subject to extensive degradation. However, form I DNA (supercoiled) was rapidly converted to form II (nicked), with form III (linear) DNA accumulating more slowly. Linear duplex DNA appeared to be fairly stable in the extract and remained as a discrete band for up to 60 min before any significant degradation by nucleases was observed. The bands labeled B, D, and E in Fig. 3 were present at 5 to 30 min during both reactions; the DNA species present in these bands then either were processed into some other kind of intermediate or product or were degraded. The bands labeled A and C were present at early times in the reaction between circular and linear substrates and persisted throughout the reaction. In contrast, these bands did not appear until later in the reaction between circular substrates (10 to 20 min). Of particular relevance to the formation of these bands is the observation that circular DNA was converted to linear DNA during incubation with yeast extract. After 60 min of incubation, almost 50% of the circular substrate DNA was converted to the linear form. The appearance of bands A and C paralleled the formation of linear DNA, suggesting that they were the products of a reaction between circular and linear substrates. This is consistent with the observation that bands A and C were present at early times during the reaction between pRDK39 and pRDK35-*Bam*HI substrates. Presumably, bands B, D, and E represent products of the reaction between circular substrates, although it is also possible that these species could be generated in a reaction between circular and linear substrates. Their intensity was decreased in the reaction between circular and linear substrates, as only half of the substrate DNA was present in the circular form.

During the course of our experiments, we have observed two types of variability. These are illustrated in Fig. 4, which shows a recombination reaction between pRDK35 and pRDK39 that was analyzed by electrophoresis through a

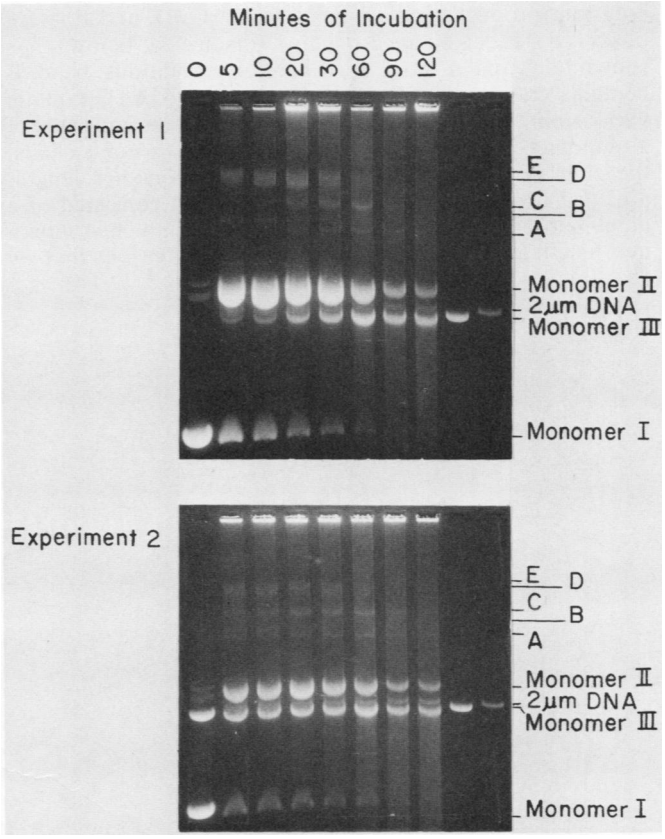

FIG. 3. Analysis of recombination in vitro by electrophoresis through agarose gels. Recombination reactions were incubated at 30°C for the indicated times, and the product DNA was fractionated by electrophoresis through a 1% agarose gel essentially as described in Materials and Methods. Experiment 1, circular substrates; experiment 2, linear and circular substrates. Bands are indicated at the right.

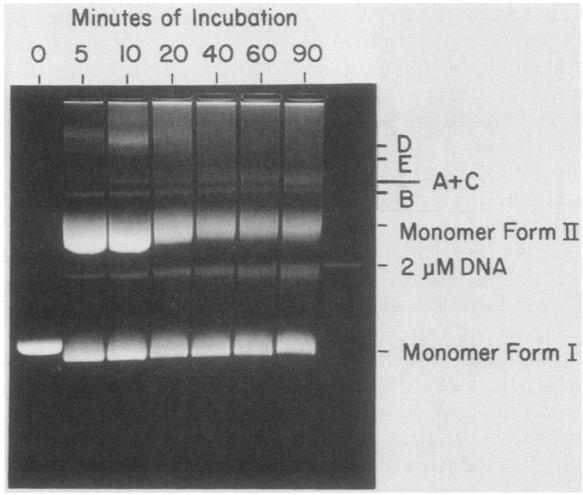

FIG. 4. Fractionation of product DNA by electrophoresis through low-gelling-temperature agarose. Reactions containing the circular substrates were carried out and processed essentially as described in the legend to Fig. 2 except that low-gelling-temperature agarose was used. Bands are indicated at the right.

low-gelling-temperature agarose gel. In this experiment, and in all experiments using low-gelling-temperature agarose, only four product bands (labeled B, A+C, D, and E) were observed. Electron microscopy (discussed below) has shown that under both fractionation conditions band B contains catenanes, while bands A, C, and A+C contain various mixtures of α and σ forms. The α forms consisted of a monomer-length circular molecule with two duplex tails, the sum of whose lengths equaled the monomer length, attached to the same site, and the σ forms consisted of a monomer-length circular molecule with an attached monomer-length tail (see below). Apparently, for reasons that we do not yet understand, the use of low-gelling-temperature agarose does not allow the separation of α and σ forms into two different mixtures. Also in this experiment, electron microscopy showed that the circular dimer band (Fig. 4, band E) migrated in front of the figure-eight band (Fig. 4, band D). In all experiments the bands are labeled so that the DNA species they contain correspond to the DNA species present in the analogously labeled bands shown in Fig. 3. Figure 4 also shows that in some reactions between pRDK35 and pRDK39, the α and σ molecules (band A+C) appeared earlier, in this case beginning to be slightly visible at 5 min. This is unlike some other experiments, such as experiment 2 in Fig. 3, in which bands A and C began to be visible at 10 to 20 min. To determine whether the DNA species present in these various bands represent products or intermediates formed during a recombination reaction, we analyzed them further by using electron microscopy and transformation assays.

Electron microscopy analysis of DNA species present in isolated intermediate bands. To examine the molecular configuration of the DNA species present in a particular band, the DNA was purified from an excised gel slice and mounted for electron microscopy. Several different types of molecules were detected in each band. Of these, one particular class usually predominated and was characteristic of that band (Fig. 5). The major contaminating species present in each band examined were monomeric circular DNA and linear molecules of various lengths. This contamination was most likely due to overloading of the gel with substrate DNA, which was necessary to obtain sufficient amounts of the different minor-product species. The numbers of molecules of each class present in each band were scored (Table 1).

Band A contained several different classes of DNA molecules, many (30%) of which were very tangled and were not scored. One of the major classes of molecules consisted of monomer circles interacting with a monomer-length linear molecule to produce molecules that looked like either α or σ

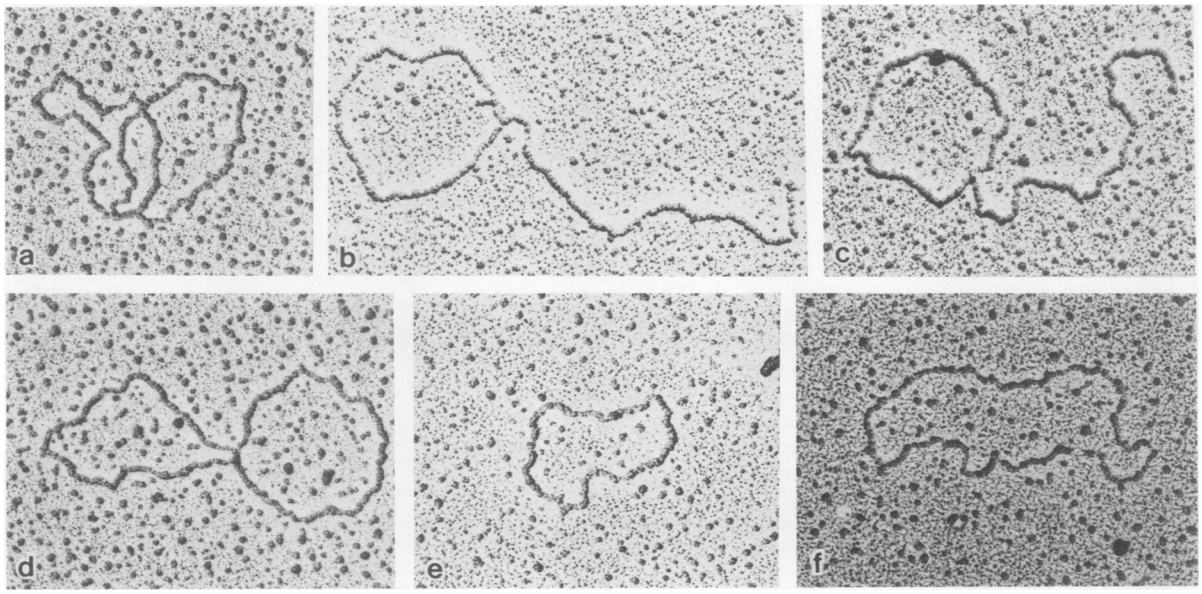

FIG. 5. Electron micrographs of DNA species present in bands isolated from agarose gels (see Fig. 3 and 4) (a) Catenated dimer from band B. (b) α form from band A. (c) σ form from band C. (d) Figure-eight molecule from band D. (e) Monomer from band D. (f) Circular dimer from band E.

TABLE 1. Electron microscopic analysis of DNA molecules present in intermediate forms

Band[a]	n[b]	% Circular monomers	% Circular dimers	% Catenanes and figure-eights[c]	% σ forms	% α forms
A	160	48.8	ND[d]	3.1	15.0	33.1
B	147	34.0	ND	66.0	ND	ND
C	171	18.1	ND	1.8	53.8	26.3
D	90	32.2	1.1	66.7	ND	ND
E	103	8.7	73.8	17.5	ND	ND

[a] DNA was isolated from a 5-min reaction containing pRDK35 and pRDK39 substrate DNA. Fractionation and labeling of bands were as described in the legend to Fig. 2

[b] Number of molecules scored.

[c] Figure-eights and catenanes were grouped together as it was not always possible to distinguish unambiguously between the two forms. See Table 2 for further analysis of these molecules.

[d] ND, Not detected.

forms (Fig. 5b and c). Also present in band A were linear molecules and monomer-length circles. These latter molecules could be present due to contamination with substrate DNA or could conceivably be due to unstable intermediates that branch migrated apart during the processes of DNA extraction and mounting for electron microscopy.

In band B, the predominant class of molecules consisted of two interacting monomer-length circles (Fig. 5a). Some of these molecules appeared to have a figure-eight configuration, while others appeared to be overlapping or interlocked circles. To determine if these molecules were two fused monomers or were catenated (linked) monomer circles, it was necessary to digest the DNA from band B with a restriction endonuclease that recognizes a single site on each monomeric unit. The DNA was cleaved with *Eco*RI at 10°C to inhibit branch migration and then examined by electron microscopy. The results from this analysis indicated that these molecules were most likely catenated monomers because *Eco*RI digestion converted them to unit-length linear monomers (Table 2). Catenanes could arise by a recombination mechanism or could be generated by the action of topoisomerases present in the cell extract.

The classes of molecules observed in band C were very similar to those seen in band A (Table 1). The major class of molecules consisted of a monomer-length circle interacting with a monomer-length linear molecule to form a σ structure (Fig. 5c). To determine if the position of the junction was located within a region of homology, these molecules were digested with *Eco*RI and then analyzed by electron microscopy. This digestion generated T forms that contained two arms of equal length, and the sum of the length of one of these arms and the length of the third arm was equal to the monomer length (Fig. 6b). This is the structure expected if the σ forms contained the linear monomer attached to the circular monomer at a region of homology (Table 2).

TABLE 2. Electron microscopy analysis of DNA molecules from intermediate bands after cleavage with *Eco*RI

Band[a]	n[b]	% X forms	% T forms	% Linear
B	43	4.7	4.7	90.7[c]
C	45	4.4	22.2	73.3
D	26	38.5	7.7	53.8[c]

[a] The DNA samples were those described in Table 1.

[b] Number of molecules analyzed.

[c] Cleavage of one catenated dimer yields two linear monomers.

Band D was characterized by a large proportion of molecules that looked like a figure-eight (Table 1 and Fig. 5d). These structures could represent two monomers joined to each other at a region of homology, or they could be catenated dimers. To distinguish between these possibilities, the DNA was digested with *Eco*RI and then examined by electron microscopy. This treatment will convert catenanes to unit-length linear molecules and will convert figure-eight molecules to X forms that have two pairs of equal-length arms with the sum of the lengths of the two different arms being equal to monomer length (Fig. 6a). This analysis indicated that at least 60% of the molecules present in band D consisted of two monomers joined at a region of homology (Table 2).

The major class of DNA molecules present in band E appeared to be circular dimers (Table 1 and Fig. 5f). These molecules were converted to unit-length monomers when they were digested with *Eco*RI, which is consistent with their being circular dimers.

Transformation analysis. To determine if the different DNA species described above contained recombinant DNA molecules, DNA purified from individual bands was used to transform *E. coli* RDK1400 to determine the frequency and proportion of Tc^r (recombinant) DNA molecules present in each band. We have carried out 38 different experiments in which one of two substrate combinations was incubated with a yeast cell extract for different times (5 to 90 min) and analyzed by electrophoresis through low-gelling-temperature agarose gels and subsequent transformation into *E. coli* RDK1400. The use of low-gelling-temperature agarose gels allowed good recovery of the required DNA species but had the disadvantage that some of the DNA species were not well separated. Thus, as discussed above, the two different bands A and C containing the α and σ forms were not separated from each other in most experiments and were present as a single band designated A+C. These experiments are also complicated by the possibility that complex DNA molecules might transform *E. coli* with lower efficiencies and thus lead to an underestimate of the frequency and proportion of Tc^r DNA molecules present in some of the minor bands.

The results of a typical experiment in which the DNA species formed in a 5-min reaction containing pRDK35 and

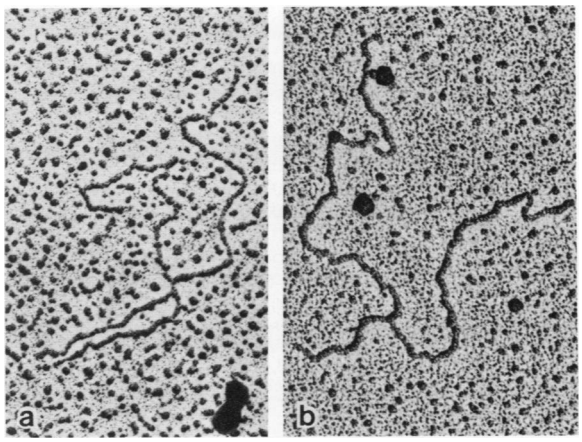

FIG. 6. Electron micrographs of purified DNA species after digestion with *Eco*RI. (a) X form obtained by digesting DNA from band D with *Eco*RI. (b) T form obtained by digesting DNA from band C with *Eco*RI.

pRDK39 as substrate DNA (Fig. 4, 5-min lane) were analyzed are summarized in Table 3. All of the different DNA species observed contained some Tc^r DNA molecules. The supercoiled monomer band contained very few Tc^r DNA molecules, and the proportion of Tc^r DNA molecules found in this band did not increase with longer incubation times. Thus, the reaction did not appear to produce a substantial proportion of supercoiled products. The nicked monomer band contained a large proportion of Tc^r DNA molecules, and the proportion of Tc^r DNA molecules increased throughout the reaction. This is consistent with our previous results that demonstrated that the major class of Tc^r recombinant molecules consisted of monomeric wild-type pBR322 plasmids (26). All of the bands that migrated more slowly than the nicked monomer band were enriched for Tc^r DNA molecules as compared with the unfractionated starting material, and the figure-eight band (band D) had the highest enrichment of Tc^r DNA molecules. Similar results were obtained in experiments that used pRDK35-*Bam*HI and pRDK39 as substrate DNA except that the band containing α and σ forms (band A+C) contained a greater proportion of the total number of Tc^r DNA molecules (35 to 55%). In all of the experiments carried out, the frequency of Tc^r DNA molecules obtained for all of the different purified minor DNA species was enriched 5- to 100-fold over the unfractionated starting material; the highest frequency of Tc^r DNA molecules obtained was 1.7%. It is important to note that the frequency of Tc^r DNA molecules observed in these experiments is not necessarily lower than expected for purified recombination intermediates and products because not all recombinant molecules having the same gross physical configuration need be Tc^r.

The structure of Tc^r plasmid DNAs obtained after transformation of the different purified DNA species into *E. coli* was also investigated (data not shown). Plasmid DNA obtained from the supercoiled and nicked monomer bands, band B, and band A+C all gave rise to > 95% Tc^r monomers. In contrast, the plasmid DNA obtained from band D gave rise to a mixture of 76% Tc^r monomers and 24% Tc^r dimers. DNA obtained from band E was not examined. All of these data are consistent with the DNA molecules present in bands A through E being either recombination products or intermediates.

TABLE 3. Frequency of recombination obtained from DNA present in intermediate bands[a]

Source of DNA	Frequency of Tc^r recombinants	% of Tc^r transformants recovered[b]	Enrichment[c]
Starting material[d]	1.38×10^{-4}		
Band			
Monomer form I	1.44×10^{-5}	6.3	0.1
Monomer form II	4.87×10^{-4}	40.4	3.5
A+C	3.57×10^{-3}	4.3	25.9
B	2.12×10^{-3}	14.9	15.4
D	6.36×10^{-3}	29.6	46.1
E	1.42×10^{-3}	4.3	10.3
Total from gel	1.85×10^{-4}[e]		

[a] The reaction mixture analyzed was the 5-min reaction shown in Fig. 4. The substrates were pRDK39 and pRDK35.
[b] Tc^r transformants from each band/total number of Tc^r transformants obtained from the gel.
[c] Frequency of Tc^r recombinants from each band divided by 1.38×10^{-4}, the frequency of Tc^r recombinants in the starting material.
[d] DNA before fractionation.
[e] Tc^r recombinants recovered from the gel/total number of transformants recovered from the gel. The total recovery of transformants after the fractionation step in this experiment was 16.2%.

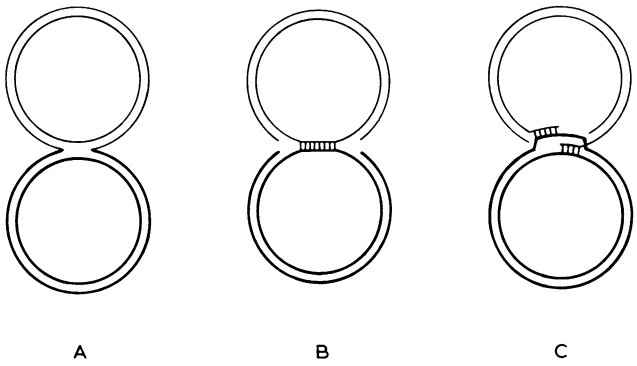

FIG. 7. Interpretation of the observed figure-eight molecules. (A) Holliday recombination intermediate. (B) Broker-Lehman recombination intermediate. (C) Double-strand break repair recombination intermediate. For additional details about these structures, see the Discussion section.

DISCUSSION

We previously described an in vitro system that used cell extracts of *S. cerevisiae* to catalyze homologous recombination events between pairs of *tet* mutant plasmids or between pairs of *arg4* mutant plasmids as measured with a transformation assay (26). The frequency of recombination events catalyzed by this cell-free system was shown to be decreased when the cell extracts were prepared from several different yeast *rec* mutants, suggesting that the in vitro recombination reaction is related to recombination in vivo (26, 27). Here we describe the results of a detailed analysis of the DNA molecules that are formed during the in vitro recombination reaction and subsequently give rise to recombinants when transformed into a *recA E. coli* strain. We have identified two DNA species, nicked circular monomers and nicked circular dimers, whose configurations are consistent with their being mature recombination products or recombination products containing a region of heteroduplex DNA. Three other DNA species that were enriched in recombinants, i.e., figure-eights, α forms and σ forms, were formed during the reaction and we demonstrated that at least two of these species, figure-eights and σ forms, consisted of two substrate molecules joined to each other at a region of homology. The configuration of these DNA molecules and their kinetics of formation were consistent with their being recombination intermediates. This is the first time that joint DNA molecules formed in a crude cell-free recombination system have been demonstrated to be recombinant in a genetic sense, and this finding provides further evidence that the in vitro system we have developed does catalyze homologous recombination events. Masukata et al. (17) have demonstrated that *E. coli recA* protein can pair DNA molecules to form joint molecules that can be processed into biologically active recombinants. While we have been unable to obtain sufficient amounts of these DNA species to allow us to determine their exact structure, our data provide some insight about their structure and the possible mechanisms by which they are formed.

One of the major classes of intermediates generated are molecules with a figure-eight configuration. Our data indicate that the fusion point between the two participating duplexes lies within a region of homology, but the precise

nature of the DNA strands within this region has not been determined. There are several mechanisms by which these figure-eight molecules could be generated. The first type of mechanism produces structures like those proposed by the classical Holliday model, in which the duplexes are covalently joined by one crossed-strand junction (Fig. 7A). This structure is free to branch migrate, thus producing hybrid DNA on both duplexes; correction of mismatches within the region of heteroduplex DNA would lead to a gene conversion or restoration event. Alternatively, the initial pairing could occur between intact circular and gapped circular substrates. Branch migration of the paired region could lead to the formation of a figure-eight molecule containing two closely linked Holliday junctions (for details, see reference 7). Another mechanism that will produce a similar type of figure-eight molecule involves the topoisomerase-mediated pairing of two covalently closed circular molecules (6). The figure-eight recombination intermediates can be resolved by cleavage of the crossed strands in either plane to generate monomeric or dimeric products (crossover event). Analysis of the plasmid DNA present in individual Tc^r transformants recovered after transformation of the figure-eight DNA into *E. coli* indicated that 76% of the products were monomeric plasmids and 24% were dimeric (data not shown). This is consistent with data from others showing that a figure-eight intermediate is resolved in *recA E. coli* strains to generate approximately 40% dimers and 60% monomers (35) and is also consistent with the idea that the figure-eight molecules we identified contained at least one Holliday junction.

Molecules that look like figure-eight structures could also be formed by the combined activity of nucleases, single-stranded-DNA annealing proteins, and topoisomerases. If participating monomers are subject to nicking by endonucleases followed by exonuclease digestion, gapped molecules will result. If the gaps span homologous regions, these molecules could pair, possibly aided by the action of single-stranded-DNA annealing proteins and a topoisomerase (Fig. 7B). This structure would also be free to branch migrate to produce structures of the type described by Broker and Lehman (5) or figure-eight molecules having long heteroduplex junctions if an appropriate nuclease was present to degrade the displaced DNA tail. However, we have not observed these latter types of structures. Resolution of this structure could only occur by DNA replication to generate monomeric products. This is inconsistent with our results, which show that reciprocal exchanges also occur, and suggests that nonspecific annealing of gapped circular molecules does not occur in our system.

A mechanism for the generation of a figure-eight intermediate between circular and linear substrates is provided by the double-strand break repair model (28). The kinetics of the formation of the band containing figure-eight molecules in reactions with circular substrates is inconsistent with this mechanism because figure-eight molecules were formed before the formation of the required linear intermediates. However, the observation of a figure-eight band enriched in Tc^r molecules formed in reactions between pRDK35-*Bam*HI and pRDK39 suggests that recombination between these substrates in vitro can occur by a mechanism that is similar to that proposed by the double-strand break repair model (28). This observation further suggests that this material contains two types of figure-eight molecules, i.e., those formed by recombination between circular molecules and those formed by recombination between circular and linear molecules.

The other major intermediate bands formed during the reaction contain DNA molecules with α or σ configurations. These appear to be the product of a reaction between circular and linear substrates. This is suggested by the observation that the bands containing these structures appear early during the reaction between circular and linear substrates but not until later during the reaction between circular substrates coincident with the conversion of circular to linear DNA. These structures may be formed by invasion of the circular molecules by one end of the linear DNA. The ends of the linear molecule could be partially single stranded due to either the action of exonucleases or localized melting of the DNA helix. These ends may be catalyzed to invade a homologous region of a duplex molecule to generate a D loop at the fusion point. If the displaced strand was degraded by the action of a nuclease specific for single-stranded DNA, then molecules like the ones observed would be formed. These molecules could then branch migrate to produce α forms containing regions of heteroduplex DNA and subsequently could be processed to monomer circles. This final processing step can occur in the yeast extract, as circular monomer recombination products are formed during reactions between circular and linear substrates catalyzed by yeast cell extracts (26) as well as after transformation of α and σ forms into *E. coli* where they yield almost exclusively circular monomers. A similar reaction is catalyzed by *E. coli recA* protein (17). Alternately, some of the σ forms could be intermediates in double-strand break repair recombination; however, the σ forms that are converted to α forms are unlikely to be intermediates in double-strand break repair (28). Although our data suggest that α and σ structures arise by a strand exchange mechanism, it is conceivable that they are produced by the processing of figure-eight forms. It seems unlikely that the α and σ forms are produced by the combined action of nucleases and nonspecific annealing because this type of mechanism would yield variable-length tails instead of the specific-length tails observed here.

The experiments presented here have identified several recombinant DNA species that are formed during a recombination reaction catalyzed by yeast cell extracts. The structures of some of these species are consistent with their being recombination intermediates and suggest several mechanisms by which recombination events are catalyzed in vitro. The elucidation of the exact mechanisms will require the purification of sufficient amounts of these molecules in pure form to permit additional structural studies, as well as the determination of how they are further processed during the recombination reaction. In addition, the formation and processing of these structures predicts the existence of several enzymatic activities that might be involved in recombination in *S. cerevisiae* and could be purified. Toward this end, we have recently demonstrated the existence of a yeast activity that cleaves Holliday junctions (27; L. S. Symington and R. Kolodner, Proc. Natl. Acad. Sci. USA, in press) and appears to be similar to T4 endonuclease VII and T7 endonuclease I (8, 19). We have also demonstrated that the *S. cerevisiae* system catalyzes mismatch repair events (C. Muster-Nassal and R. Kolodner, unpublished data).

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