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Double-strand-break repair and recombination catalyzed by a nuclear extract of *Saccharomyces cerevisiae*

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An *in vitro* system for double-strand-break repair and recombination of plasmid substrates catalyzed by extracts prepared from yeast nuclei has been developed. Recombination events that generate crossover products were detected amongst reaction products by Southern blot hybridization, or by the polymerase chain reaction (PCR). The recombination reaction was found to be stimulated by a double-strand break within homologous sequences and proceeded by a mechanism that involved branched DNA intermediates. In addition to pairing events that generate crossovers, the formation of inverted repeats (head-to-head and tail-to-tail joined products) was also detected. Two models are presented which propose that the formation of crossover products and inverted repeats occur by similar mechanisms.

Key words: DNA repair/gene conversion/recombination/yeast

Introduction

There are several lines of evidence which suggest that double-strand breaks either initiate recombination, or provide efficient substrates for recombination, in the yeast *Saccharomyces cerevisiae*. First, DNA-damaging agents, such as ionizing radiation, induce mitotic recombination (Haynes and Kunz, 1981). Mutants that are sensitive to ionizing radiation have been isolated and well characterized genetically. These mutants, classified as the *RAD52* group, generally have defects in mitotic and/or meiotic recombination (reviewed in Petes *et al.*, 1990).

Second, Orr-Weaver *et al.* (1981) have shown that non-replicating plasmids containing a cloned gene homologous to an endogenous sequence transform yeast at a high frequency when cut within the cloned DNA sequence. Transformants contain plasmid DNA integrated into the yeast genome at the homologous site. Plasmids containing gaps of several hundred nucleotides also transform with high efficiency and are repaired by recombination using chromosomal information as a template (Orr-Weaver and Szostak, 1983). These observations were, in part, responsible for the development of the double-strand-break repair model for recombination (Szostak *et al.*, 1983). An alternative pathway for processing linear plasmid DNA molecules following transformation of yeast has been reported by Kunes *et al.* (1985, 1990). When a double-strand break is made within plasmid sequences of an autonomously replicating vector, such that the break site has no homology with the yeast genome, circular plasmids are formed by head-to-head and tail-to-tail joining of linear monomer units. These inverted circular dimers are the major repair product and are proposed to arise by a homologous pairing mechanism (Kunes *et al.*, 1990).

Third, mating type interconversion is initiated by a double-strand break at the *MAT* locus (Strathern *et al.*, 1982). The HO endonuclease cleaves a specific site at *MAT* and initiates the replacement of these sequences using one of the two donor cassettes, *HML* or *HMR*, as a template for conversion (Kostriken *et al.*, 1983). It has been possible to monitor the process of mating type switching at the molecular level by placing the *HO* gene under the control of an inducible promoter (White and Haber, 1990). Following introduction of the break, exonuclease digestion generates a long 3′ tail centromere-distal to the cut site. The single-stranded end produced appears to be active in the initial invasion of the donor cassette. Double-strand breaks generated by the HO endonuclease can stimulate both mitotic and meiotic recombination when the HO endonuclease recognition sequence is placed in novel locations (Kolodkin *et al.*, 1986; Nickoloff *et al.*, 1986, 1989; Ray *et al.*, 1988; Rudin and Haber, 1988; Rudin *et al.*, 1989).

Finally, there is suggestive evidence that double-strand breaks may play a role in the initiation of meiotic recombination. A meiosis-specific double-strand break was identified within a region of the *ARG4* gene that contains an initiation site for meiotic gene conversion (Nicolas *et al.*, 1989; Sun *et al.*, 1989). There is also evidence that a strong hotspot for meiotic reciprocal exchange, created by insertion of the *LEU2* gene adjacent to the *HIS4* locus, is associated with the formation of two meiosis-specific double-strand breaks (Cao *et al.*, 1990).

Since double-strand breaks are efficiently utilized by the cellular recombination apparatus, we have devised an *in vitro* recombination system to explore how the recombination reaction is catalyzed. Previous work has shown that crude extracts prepared from yeast catalyze recombination between homologous plasmids (Symington *et al.*, 1983). The plasmid substrates used were derivatives of pBR322 that contain *Xho*I linker mutations at different positions within the gene conferring resistance to tetracycline (*Tc*). Recombination events between the Tc^s plasmids that reconstructed a Tc^r gene were detected by transformation of an *Escherichia coli recA* strain to tetracycline resistance with DNA from the reaction. However, the major problem with this system was the concern that some proportion of the recombination reaction might have proceeded after transformation into the *E.coli recA* host. Although a direct analysis of the reaction products by Southern blot hybridization revealed the presence of *Xho*I resistant molecules that could be due to either heteroduplex DNA, or corrected DNA, at one of the mutant sites, reciprocal exchange products were not detected. Branched DNA intermediates were observed by electron microscopy (Symington *et al.*, 1985) and a slight stimulatory effect on

recombination was noted when a double-strand break was introduced into one of the substrates (Symington *et al.*, 1983).

The aim of the present study was to develop an *in vitro* system for double-strand-break repair by recombination whereby the reaction could be monitored by direct physical means. Several methods were employed to detect and analyze intermediates and products of the reaction, and to examine the dependence on DNA homology and double-strand breaks.

Results

In vitro recombination substrates

The general strategy for the development of the *in vitro* system was to construct plasmid substrates that could be assayed for recombination using physical methods. The substrates are derived from the cloning vectors pBR322 and pACYC184 (Figure 1). These plasmids share 1.6 kb of homology, including the *Tc* gene, but the rest of the plasmid sequences are unique. pLS89 contains a 14 bp deletion between two *Ban*II sites within the coding region of the *Tc* gene. Since the deletion junction is a *Ban*II site, cleavage of the plasmid with this enzyme generates a 14 bp gap within homology. The substrate digested with *Ban*II prior to incubation with the yeast extract is referred to as pLS89/*Ban*II. The second plasmid, pLS96, contains a deletion of 90 bp that removes the promoter of the *Tc* gene and extends into the region of non-homology. Recombination events between these substrates can generate gene conversion or reciprocal exchange products (Figure 1).

Strategy for detection of products of recombination

To assay for crossover events, reaction products are digested with the enzyme *Eco*RI or *Pvu*II and analyzed by Southern blot hybridization. The substrates pLS89/*Ban*II and pLS96 yield fragments of 2.7 kb and 1.6 kb, and 3.5 kb and 0.4 kb, respectively, upon digestion with *Pvu*II. The reciprocal exchange-repaired product would be expected to yield fragments of 4.9 kb, 2.9 kb and 0.4 kb; thus the crossover products should be easily distinguishable from the substrate molecules. Alternatively, digestion with *Eco*RI generates fragments of 3.9 kb, and 3.8 kb and 0.5 kb from pLS96 and pLS89/*Ban*II, respectively, whereas the crossover product would yield fragments of 5.7 kb and 2.5 kb.

Recombination events should also be detectable by the polymerase chain reaction (PCR) using primers that anneal to unique sequences of each substrate adjacent to the repeats (small arrows labeled 8 and 9 in Figure 1). A reciprocal exchange between the substrates would orient the sequences to which the primers anneal so that they would be in *cis* and could amplify the intervening 1.7 kb. By using a primer with the same sequence as the 14 bp deletion (Figure 1, small arrow labeled 3) with primer 8, repair events could be detected that occurred with or without an associated exchange. The use of this primer pair would detect any event in which the 14 bp sequence were restored, thus amplifying the intervening 500 bp, but would not distinguish between crossover and non-crossover (conversion) events. However, such events could be distinguished by Southern blot analysis using an oligonucleotide with the same sequence as the 14 bp deletion (oLS1 or oLS3). Without repair of the gap, the oligonucleotide would hybridize only to DNA fragments containing the wild type *Tc* gene sequence. If repair of the break had occurred, the oligonucleotide would hybridize to DNA fragments containing the repaired region. As crossover and non-crossover events would generate restriction fragments of different size, these two events could be distinguished (Figure 1). For example, digestion with *Pvu*II would generate DNA fragments of 3.5 kb and 0.4 kb from pLS96, and fragments of 2.7 kb and 1.6 kb from pLS89/*Ban*II. Of these, only the 3.5 kb fragment from pLS96 would hybridize to oLS1. Repair of the deletion in the absence of a crossover would yield a fragment of 4.3 kb that would now hybridize to oLS1. If repair of the deletion yielded crossover products, the 4.9 kb and the 2.9 kb *Pvu*II fragments generated would both hybridize to oLS1.

Strategy for analysis of recombination intermediates

The double-strand-break repair model for recombination proposes an intermediate in the reaction in which donor and recipient duplexes are joined through two Holliday junctions (Szostak *et al.*, 1983). If such structures are generated during the *in vitro* reaction, they should be detectable by the appropriate restriction enzyme digest (Figure 2). Enzymes that cleave within the regions of non-homology would convert these intermediates into stable X-forms, whereas Holliday junctions formed between completely homologous substrates are known to be unstable following restriction enzyme digestion due to branch migration (Thompson *et al.*, 1976). The X-form intermediates formed between pLS89 and pLS96 will be larger then either substrates or products of the reaction. To identify and characterize these molecules we used a two-dimensional agarose gel electrophoresis

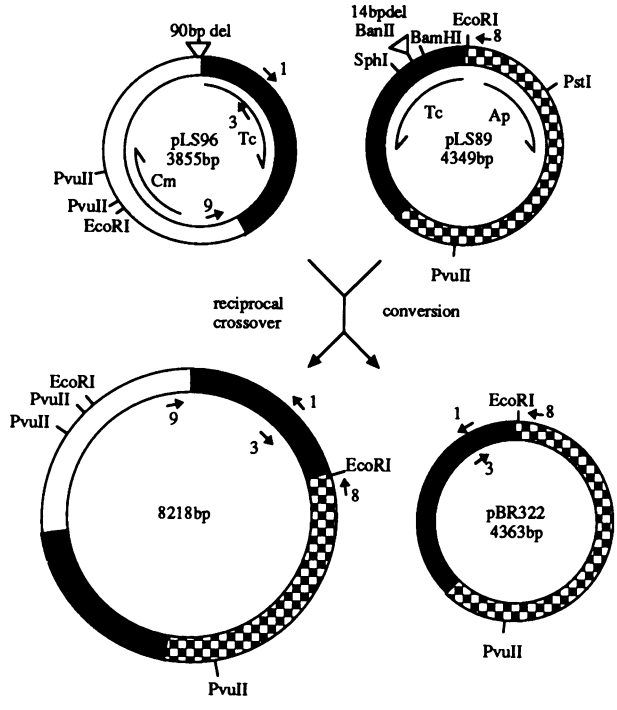

Fig. 1. Maps of the plasmid substrates and the expected recombinant products. The small arrows labeled 3, 8 and 9 correspond to the primers (oLS3, oLS8 and oLS9) used for the PCR. The arrow labeled 1 represents the oligonucleotide oLS1 used for hybridizations. Regions of homology between the plasmids are shown by solid boxes, the non-homologous parts of the plasmids are indicated by open and chequered boxes. The drug resistance genes are indicated only on the substrates pLS89 and pLS96.

system. Briefly, gels are run in the first dimension at a low agarose concentration and a low voltage gradient and in the second dimension at a high voltage gradient and a higher agarose concentration containing 0.5 μg/ml ethidium bromide. DNA molecules separate according to size and shape due to differences in conformational perturbations resulting from different electric field strengths and to differences in the frictional properties encountered during sieving in the two different agarose concentrations (McDonnell *et al.*, 1977). Linear DNA molecules form a smooth arc, whereas branched DNA molecules form characteristic arcs and spikes that migrate off the linear fragment curve (Figure 5a; Bell and Byers, 1983; Brewer and Fangman, 1987). Circular DNA used as a substrate in the reaction described would add to the complexity of the system; therefore, to simplify the interpretation of the gels,

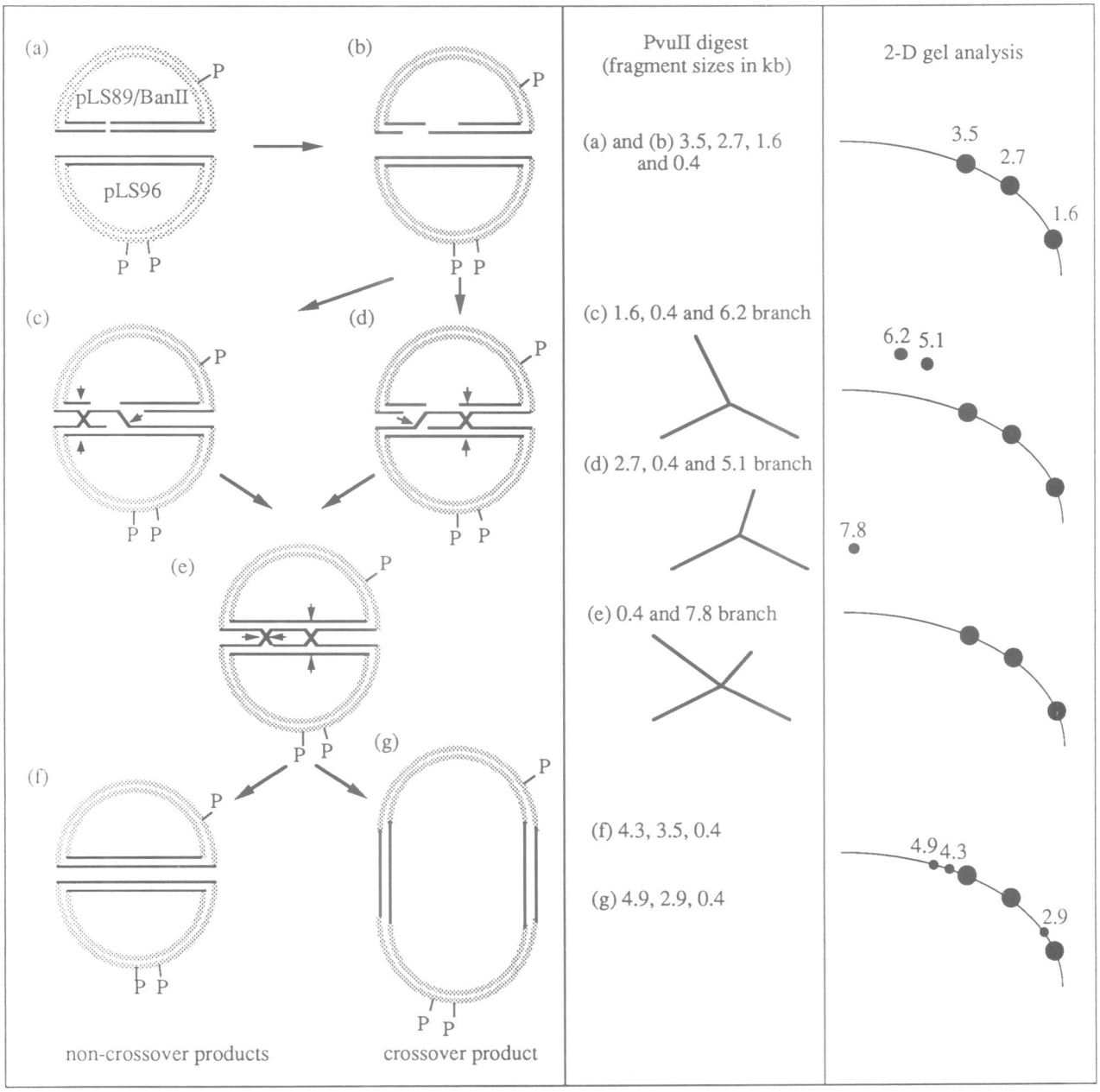

Fig. 2. The double-strand-break repair model for recombination. (**a**). The first step of the reaction is proposed to involve exonuclease degradation at the cut site to generate single-stranded tails that are active in invasion of the homologous duplex. (**b**). One of the single-stranded ends produced invades the donor duplex forming a 'one-ended' invasion intermediate as shown in (c) and (d). Note that in (c) the strands at the break site are drawn flipped to simplify the figure. (**c**), (**d**). The invading 3′ end acts as a primer for DNA repair synthesis, displacing one strand from the donor. This intermediate contains a single Holliday junction which can branch migrate to increase or decrease the length of heteroduplex. Endonucleolytic cleavage of the junction and the displaced donor strand as shown by the arrows can produce a crossover product of 4.9 kb or 2.9 kb. (**e**). The displaced donor strand anneals with the single-stranded end at the other end of the break. The 3′ end is extended by DNA synthesis thus repairing the break. Ligation of ends generates an intermediate containing two Holliday junctions. (**f**). Cleavage of both of the Holliday junctions in the same plane yields non-crossover repaired products. (**g**). Cleavage of the junctions in opposite planes, as shown by the arrows in (e), generates crossover products. The regions of homology between the substrates are shown as solid lines. The sites for cleavage of the plasmids with *Pvu*II (P) are marked. The sizes of fragments expected from the substrates, intermediates and products, following digestion with *Pvu*II, are shown to the right of the double-strand-break repair model and also their expected migration patterns by 2-D gel electrophoresis.

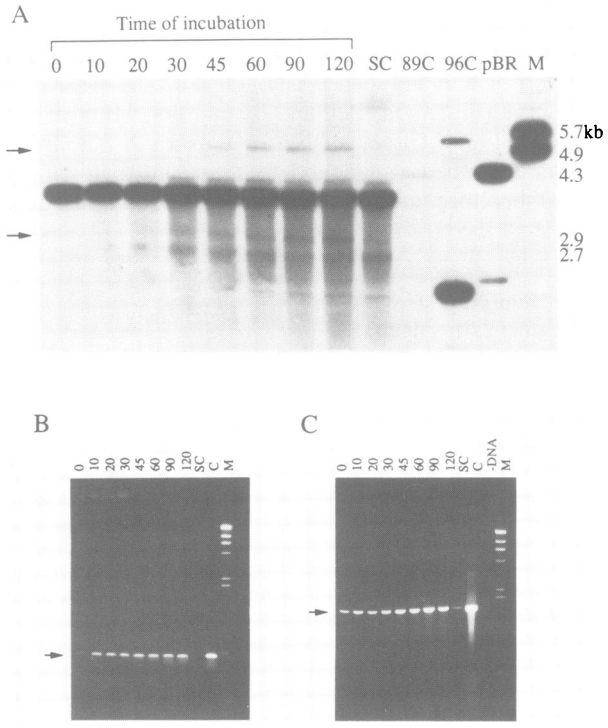

Fig. 3. The extract catalyzes the formation of crossover products. The substrates pLS89/*Ban*II and pLS96 were incubated with extract prepared from strain LS130 for the times shown. Products were digested with *Pvu*II and analyzed by Southern blotting using the oligonucleotide oLS1 as a probe (panel **A**), by PCR using primers oLS3 and oLS8 (panel **B**), and by PCR using primers oLS8 and oLS9 (panel **C**). The controls shown are as follows: SC refers to the separate incubation control; C is a pure recombinant and thus is a positive control for the PCR; 89C and 96C are the substrates prior to digestion with *Pvu*II; pBR is a pBR322 linear size marker; M is a plasmid marker that contains one of the recombinant *Pvu*II fragments (panel A), or lambda DNA digested with *Hin*dIII (panels B and C). The arrows show the position of recombinant products by Southern analysis and by PCR.

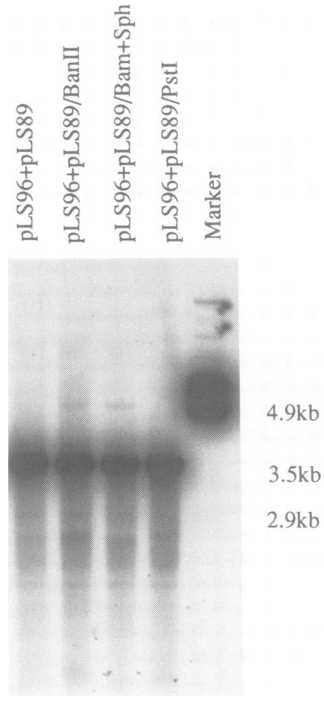

Fig. 4. The formation of crossover products is stimulated by a double-stranded break within homology. The substrates were made by digesting with the enzymes shown above each lane. Following incubation with extract from LS130 for 90 min, the products were digested with *Pvu*II and the Southern blot was probed with oLS1. The marker lane represents digestion of a plasmid containing one of the recombinant bands with *Pvu*II.

the DNA from the reaction was first digested with either *Pvu*II or *Eco*RI. This treatment cleaves all of the substrate molecules and the resulting fragments migrate on the linear fragment arc. Intermediates in the repair−recombination pathway in which one end of the break has invaded the homologous repeat will have a distinct mass and will be branched to form a three-armed structure (Figure 2c and d). If both ends of the break stably interact with the intact homologous duplex, a four-armed branched intermediate will result after restriction enzyme digestion (Figure 2e). It should be possible to detect these branched molecules if the invading strands are stabilized by covalent attachment of the ends to the donor duplex, or by extensive regions of base pairing.

Nuclear extracts catalyze the formation of reciprocal exchange products

Initially, whole cell extracts prepared from yeast using several methods were tested for their ability to catalyze reciprocal exchange between the substrates, but were not active in generating the expected diagnostic exchange fragments. However, incubation of pLS96 and pLS89/*Ban*II with a nuclear extract (Dunn and Wobbe, 1990) did yield novel *Pvu*II restriction fragments of 4.9 kb and 2.9 kb, the size expected for the reciprocal crossover products (Figure 3A). These products were apparent after a 30 min incubation with the extract (Figure 3A, lane 30) and both products appeared with approximately equal intensity. Since considerable DNA degradation occurred during the reaction, the smaller crossover fragment was more obscured by background hybridization than the larger crossover fragment, which migrates more slowly than the substrates. No diagnostic crossover fragments were detected after incubation of the substrates pLS89/*Ban*II and pLS96 independently in the extract for 1 h, followed by DNA purification and subsequent mixing of the samples for restriction analysis (Figure 3A, lane SC). Thus a direct interaction between the two plasmids is required in the extract to generate crossover products. It is unlikely that the novel restriction fragments observed were due to partial digestion of either substrate as shown by their migration in comparison with undigested substrate DNA (Figure 3A, lanes 89C and 96C).

We expected to be able to detect crossover and non-crossover products using oLS1 as a hybridization probe. However, we detected only the crossover products, not the conversion product. This result suggests that if the reaction does proceed by the mechanism proposed in the double-strand-break repair model, then resolution of the double Holliday junction-containing intermediate is biased towards crossover products. Alternatively, the *in vitro* recombination reaction might occur by a different mechanism.

The same series of reactions was also analyzed by PCR using the primer combination oLS3 and oLS8 to detect restoration of the deletion, and the combination oLS8 and oLS9 to detect crossover events (Figure 3B and C).

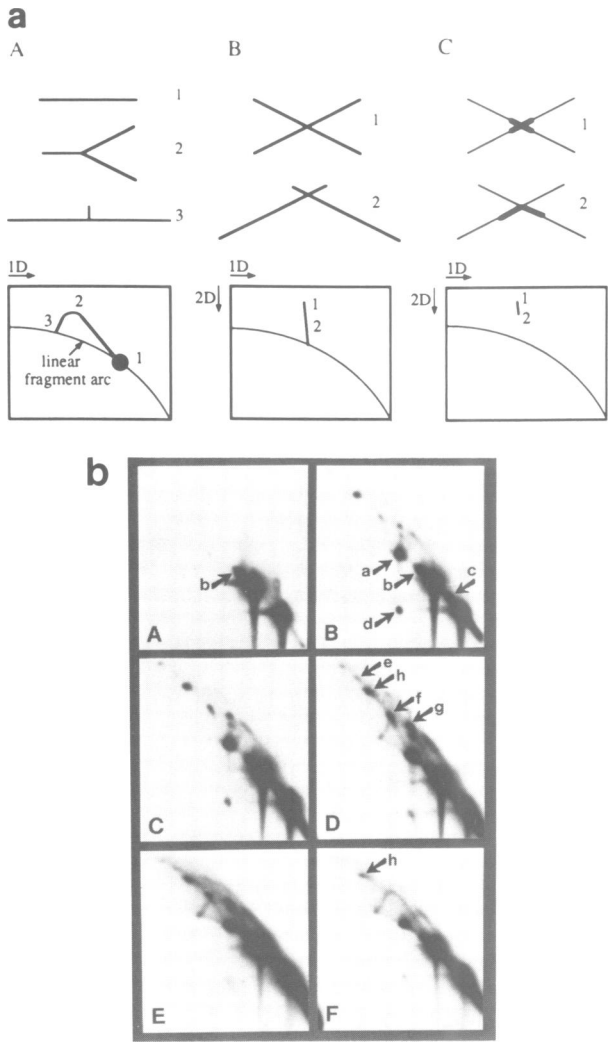

Fig. 5. Two-dimensional gel analysis of the reaction between pLS89/*Ban*II and pLS96. (**a**). Schematic representation of branched DNA molecules and their expected migration patterns by two-dimensional agarose gel electrophoresis (Brewer and Fangman, 1987). Panel **A**. A Y-shaped replication intermediate increases in mass and branching as replication proceeds through the duplex, and thus is retarded in both dimensions. When half replicated, the molecule is most branched and therefore most retarded in the second dimension. As the replication fork approaches the end of the molecule, the mass continues to increase but the molecule becomes less branched. Panel **B**. An X-shaped recombination intermediate formed between completely homologous duplexes has a constant mass, but is retarded in the second dimension. Branch migration of the junction allows the branch point to be positioned anywhere along the length of the duplex thus changing the amount of branching observed and the mobility of the X-form. Molecules in which the branch point is furthest from the ends migrate at one end of the spike, furthest from the linear arc, whereas molecules in which the branch is close to the ends migrate closer to the linear arc. Panel **C**. An X-shaped recombination intermediate generated between molecules that share only a short region of homology is restricted in the amount of branch migration by the non-homology in the arms. Thus these intermediates form a short spike in the second dimension. (**b**) 2-D gels were run as described, blotted to nylon membranes and hybridized with a plasmid that contains pLS89 and pLS96 sequences. Panels **A**–**E** represent 0, 10, 20, 60 and 90 min incubation times with extract from LS130; panel F shows the separate incubation control. The predicted identity of the labeled dots is as follows: a = head-to-head joining of pLS89/*Ban*II; b = head-to-tail joining (or partial digestion); c = tail-to-tail joining; d = 2.7 kb pLS89/*Ban*II–*Pvu*II fragment; e = 8.5 kb two-ended invasion intermediate; f = 6.5 kb one-ended invasion intermediate; g = 5.5 kb one-ended invasion intermediate; h = branched molecule containing three copies of the 2.7 kb pLS89/*Ban*II–*Pvu*II fragment.

Amplification product from both sets of reactions was observed. The difference in the amount of the amplification product between the 60 min coincubation (Figure 3B, lane 60) and 60 min separate incubation samples (Figure 3B, lane SC) was 9- to 10-fold. Unfortunately a high background was noted in control reactions that contain the two substrates and primers, but no nuclear extract. This high background is most likely due to partial elongation products generated from each primer that terminate within the region of homology. These single-stranded products could then anneal within overlapping complementary sequences and be elongated to generate an artefactual recombinant product during the next cycle; subsequent cycles would amplify this product. It should be noted that the separate incubation control has much less of the amplification product than the 0 minute time point. Presumably DNA degradation that occurs in the extract destroys potential templates for the PCR.

Recombination is stimulated by a double-strand break within homology

The role of a double-strand break or gap in the recombination reaction was investigated by using several different substrate combinations (Figure 4). Circular pLS96 was used in all of the reactions; pLS89 was either uncut, cut with *Ban*II, cut with *Bam*HI and *Sph*I to generate a 200 bp gap within homology, or cut with *Pst*I which cuts within non-homology. Reciprocal recombination products were barely detectable in the absence of a double-strand break, and were not observed if the break was made within non-homology. This result demonstrates that the reaction is stimulated by a double-strand break or gap within homologous sequences.

Analysis of recombination intermediates

To detect the formation of the intermediates predicted by the double-strand-break repair model we used 2-D gel electrophoresis (Figure 5b). A number of distinct DNA species migrating on and off the linear fragment arc were noted over a time course. Panels A–E show DNA samples incubated for 0, 10, 20, 60 and 90 min with the extract. Panel F shows the control of incubating the two substrates independently in the extract for 60 min, followed by mixing and processing as for the other samples. Digestion of the substrates with *Pvu*II yields fragments of 3.5 kb, 2.7 kb, 1.6 kb and 0.4 kb, although the two smallest fragments are not visible on these gels. Some degradation of DNA was apparent as evidenced by the smear along the linear fragment arc that increased with longer incubation times. The smears migrating down from the substrate bands were not always present and are probably an artefact of the 2-D gel system. The mobilities of the dots migrating off the linear fragment arc would correspond to those of linear molecules of 8.5 kb, 6.5 kb and 5.5 kb (Figure 5b, panel D, dots e, f and g, respectively). Of these the two smaller species are the more abundant and correspond to the mobility expected for branched DNA molecules resulting from one-ended invasions (6.2 kb and 5.1 kb). The less abundant species is more retarded in the second dimension and probably corresponds to a four-armed branched molecule (Duckett *et al.*, 1988).

The intermediates observed form discrete spots rather than spikes, suggesting that they do not undergo extensive branch migration. Artificial X-forms generated from these substrates form very short spikes and migrate in a similar position to the 8.5 kb branched molecule formed by *in vitro* recombination (unpublished data). Analysis of the reaction products

from the separate incubation control sample reveals the presence of replication forks emanating from each substrate, but the dots corresponding to branched DNA molecules are not present (Figure 5b, F). Thus the branched DNA molecules, like the crossover fragments, require a direct interaction between the two substrates in the yeast extract for their generation. The absence of the dots corresponding to branched molecules when replication forks are present (separate incubation control) confirms that they are not by-products of replication forks formed by back branch migration of nascent DNA strands.

To confirm that the branched DNA molecules predicted by mobility to be one-ended invasion intermediates contain the correct DNA sequences, triplicate filters of the 60 min coincubation reaction, and the 60 min separate incubation reaction, were hybridized with the following probes: (i) an internal fragment of pLS96 that has no homology to pLS89 (Figure 6A and B); (ii) a fragment internal to the β-lactamase gene of pLS89 (Figure 6C and D); and (iii) a DNA fragment extending from the *Pvu*II site of pLS89 to the border of homology with pLS96 (Figure 6E and F). The latter two probes distinguish the asymmetric parts of pLS89/*Ban*II obtained following *Pvu*II digestion and also have no homology to pLS96. The results presented in Figure 6 confirm that the 6.5 kb branched DNA molecule (Figure 6, dot f) contains pLS96 sequences and the larger of the two *Pvu*II fragments from pLS89/*Ban*II, whereas the smaller branched species contains pLS96 and the 1.6 kb *Pvu*II fragment from pLS89/*Ban*II (Figure 6, dot g). These species are only present in samples from coincubation of the substrates.

End-to-end joining events

In addition to the dots migrating off the arc, two novel species were found that migrated on the linear fragment arc that did not correspond in size to partial digestion, or crossover products. These molecules of 5.4 kb and 3.2 kb (Figure 5b, panel B, dots labeled a and c) correspond in size to those expected of linear pLS89/*Ban*II molecules ligated head-to-head or tail-to-tail. Since the *Pvu*II site is located asymmetrically within the substrate, the head-to-tail, tail-to-tail and head-to-head joining events can be distinguished. The head-to-tail ligation product would be expected to yield a fragment of 4.3 kb, the same size as the partial digestion product observed (Figure 5b, panel B, dot b). The 4.3 kb fragment (from partial digestion or ligation) was found to be present at the 0 min time point and did not increase in intensity through the course of the reaction. In contrast, the products of head-to-head and tail-to-tail joining were absent at 0 min, but clearly appeared as a major reaction product at 10 min, and persisted through the course of the reaction. Since this product was present in the 10 min sample, prior to the appearance of replication intermediates, it is unlikely to have resulted from replication of the linear substrate.

The 2-D gel samples from 10–90 min, also showed a discrete dot that migrated below the linear fragment arc, directly below the 5.4 kb inverted product and corresponding in mobility to the 2.7 kb substrate fragment (Figure 5b, panel B, dot d). This could be due to an intermediate in the head-to-head pairing reaction prior to ligation, which has destabilized in the second dimension. This dot decreased in intensity through the reaction suggesting that it is an intermediate. A dot migrating below the linear fragment arc,

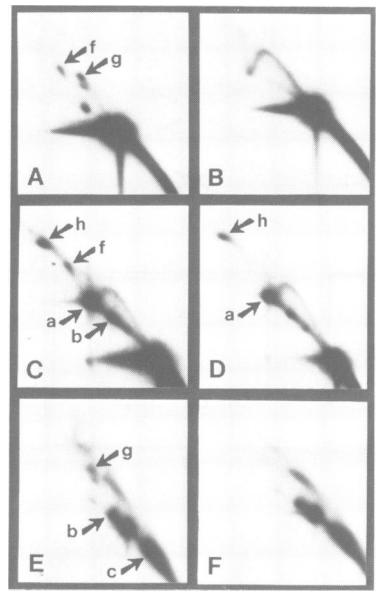

Fig. 6. Analysis of branched molecules using substrate specific probes. The 60 min coincubation (panels A, C and E), and 60 min separate incubation samples (panels B, D and F), from the pLS89/*Ban*II and pLS96 reactions were run in triplicate on 2-D gels, blotted and hybridized with the following probes: panels **A** and **B**, pLS96 specific probe; panels **C** and **D**, pLS89 probe specific for the 2.7 kb *Ban*II–*Pvu*II fragment; panels **E** and **F**, pLS89 probe specific for the 1.6 kb *Ban*II–*Pvu*II fragment. The labeled dots correspond to those shown in Figure 5.

corresponding in mobility to the 1.6 kb fragment and directly below the 3.2 kb tail-to-tail joined product has also been observed (data not shown). The instability of the end-joining products suggests that they are held together by base pairing. The DNA species corresponding to end-joining products were, as expected, present in the separate incubation control reaction (Figure 5b, panel F). An additional dot of ~8 kb, migrating off the linear fragment line as predicted for a branched molecule, was observed in both coincubation and separate incubation reactions (compare dot h, Figure 5b, panels D and F). Directly below this dot, minor spots were observed on long exposure autoradiograms corresponding in size to the 5.4 kb end-joined species and the 2.7 kb substrate fragment. Thus, this species appears to consist of three molecules of pLS89/*Ban*II.

The head-to-head joined product of 5.4 kb, and the branched species predicted to contain three copies of pLS89/*Ban*II, both hybridized specifically with the probe unique for the 2.7 kb fragment of pLS89 (Figure 6C and D, dot a). The tail-to-tail inverted product was detected using the other pLS89 probe, specific for the 1.6 kb *Ban*II–*Pvu*II fragment (Figure 6E and F, dot c). Both probes also detected a dot corresponding to either the head-to-tail ligation product of 4.3 kb or to the partial digestion product.

Surprisingly, the head-to-head and tail-to-tail joining reactions appeared to occur with greater efficiency than head-to-tail joining (Figures 5 and 6). This reaction has been shown to occur with high efficiency in nuclear extracts derived from mammalian cells (H.Young and R.Fishel, personal communication), and is also consistent with the observation that inverted circular dimers are the major repair product when linear DNA is transformed into yeast (Kunes *et al.*, 1985, 1990).

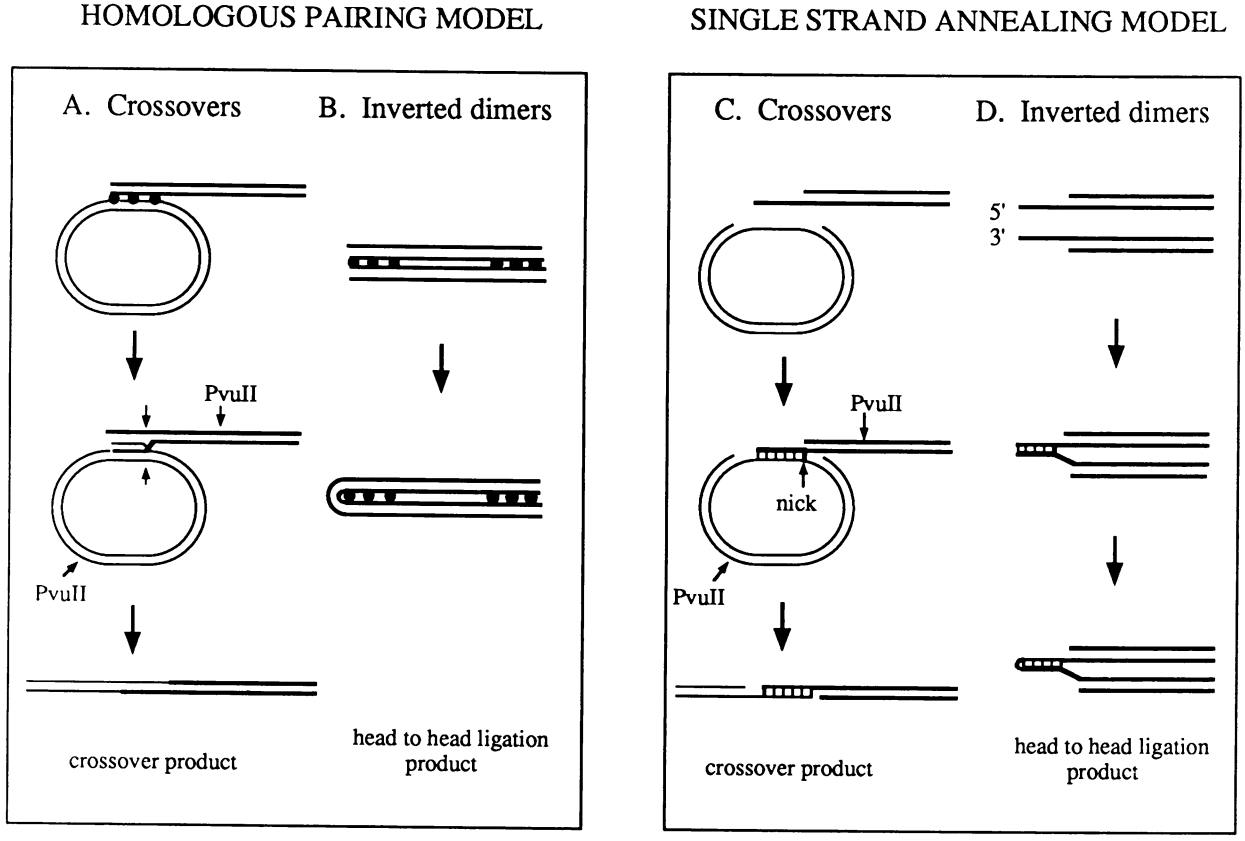

Fig. 7. Models for the formation of crossover fragments and inverted dimers. **A**. The homologous pairing model predicts that synapsis occurs between homologous DNA substrates. Pairing between pLS89 and pLS96 would be followed by strand invasion to form a branched DNA molecule containing a Holliday junction. Resolution of the junction can yield crossover or non-crossover products. **B**. Synapsis of two linear molecules aligns the broken ends. The ends are joined by ligation to form the inverted product. **C**. By the single-strand annealing pathway degradation of both substrates is required to expose complementary regions of DNA. The single-stranded regions anneal to form a heteroduplex joint. To form crossover products, pLS96 could be nicked adjacent to the paired region as shown. The resulting recombinant linear product would contain gaps that could be filled and sealed by the combined activities of DNA polymerase and ligase. Cleavage with either *Pvu*II or *Eco*RI generates the expected crossover fragment. **D**. Pairing between single-stranded regions of two pLS89 linear molecules could result in the formation of inverted dimers by ligation of the two ends. The 3′ end present on one of the degraded strands could be used as a primer for DNA repair synthesis repairing the gapped region on the unligated strand. The proposed homologous pairing protein(s) are shown by solid circles between duplex molecules.

Discussion

The double-strand-break repair model (DSBR) proposes that a DNA duplex containing a break invades an intact homologous duplex generating an intermediate that contains two Holliday junctions (Szostak *et al.*, 1983; Figure 2). Resolution of this intermediate can yield either crossover or non-crossover products. The reaction is considered to be conservative as there is no loss of information. In contrast, some extrachromosomal recombination events that are observed in higher eukaryotes appear to occur by a non-conservative mechanism (Lin *et al.*, 1984, 1990; Carroll *et al.*, 1986; Chakrabarti and Seidman, 1986). The substrates for these reactions generally contain direct repeats, and the repair event results in loss of one of the repeats. The reaction is dependent on a break within one of the repeats, or in the DNA between them. It has been proposed that these events occur by exonuclease degradation, or by unwinding (Wake *et al.*, 1985) of one strand from each side of the break until regions of complementary sequence are exposed. Annealing of these sequences can then result in restoration of one of the repeats and a net loss of information. This model for recombination–repair has been called the single-strand annealing (SSA) model (Lin *et al.*, 1984, 1990).

The repair products generated in our *in vitro* system are predominantly crossovers. These products could occur either by strand invasion, or by degradation of DNA strands and reannealing (Figure 7). The one-ended invasion intermediates, and the two-ended invasion intermediate predicted by the DSBR model could be resolved by the appropriate endonucleolytic cleavages to yield crossover products. However, only the two-ended invasion intermediate can yield repaired non-crossover products. To generate crossovers by the SSA model, extensive degradation of both substrates would be required (Figure 7). The uncut plasmid (pLS96) would have to undergo nicking and exonuclease degradation (or unwinding) within the region of DNA sequence homology with pLS89. The linear substrate would also be processed to form a single-stranded tail. The exposed complementary single-stranded regions from both substrates could anneal. Nicking, gap filling and ligation of several strands would then produce a recombinant product. Our results argue against the SSA model since a direct interaction between the substrates in the extract is required to generate both intermediates and crossover products. The nuclease activities predicted by the SSA model should act on each substrate independently. Subsequent mixing of these DNAs during restriction enzyme cleavage should allow sufficient time for annealing to occur if complementary regions were present. It is also predicted

from the SSA model that a double-strand break made outside of the region of homology would allow access to a nuclease that could then degrade into the repeats and eventually expose complementary sequence. Thus a break outside of homology may slow the reaction but would not be inhibitory. The results presented in Figure 4 strongly suggest that a break or gap is required within homology for crossover products to be detected. Finally, the repair activities predicted by the SSA model to generate mature recombinants would have to occur in the extract to generate an intact strand to be detected using the PCR. If the recombinant products contained nicks they would be lost during the denaturation step involved in the PCR procedure.

Although the results presented support a strand invasion model, the major reaction products appear to be 'half-reciprocal exchanges' rather than the 'full-reciprocal exchanges' predicted by the double-strand-break repair model. This conclusion is drawn from the analysis of branched DNA intermediates observed on two-dimensional gels. The major reaction products observed using this gel system are one-ended invasion intermediates (Figures 5 and 6). Such structures can be resolved to generate a crossover product in the absence of the reciprocal end invasion event (Figure 2). The two alternative intermediates are recovered in equal amounts suggesting that there is no bias favoring one end over the other. These results imply that recovery of both crossover fragments by restriction enzyme analysis, either *in vivo* or *in vitro*, is not evidence for reciprocal recombination in the classical sense. Independent one-ended invasions without end bias produce the same result. If meiotic recombination occurred by one-ended invasions, then reciprocal crossover events would be rare, and spore viability would be extremely low. As this is not the case, meiotic events that are initiated by double-strand breaks may occur by a mechanism that ensures double (two-ended) invasion events, for example, by maintaining pairing between chromosomes.

For most mitotic recombination events, unlike meiotic events, all of the products of a recombination event are not recovered. Thus the evidence that mitotic events occur by a reciprocal mechanism is weak. The experiments of Rudin *et al.* (1989) in which the *HO* system is used to force double-strand-break repair between directly repeated *lacZ* genes on a plasmid have shown that reciprocal events are rare. Loss of one of the repeats occurs in 87% of the repair events. This could occur by two-ended invasions that are resolved preferentially in the crossover configuration, by biased one-ended invasion events or by single-strand annealing between degraded ends. Analysis of the DNA products formed after HO induction has shown that the reciprocal deletion product corresponding to the region between the repeats is not formed, suggesting that this repair event occurs by a non-conservative mechanism. The plasmid substrate containing repeats in inverted orientation cannot undergo deletion formation. Kinetic analysis of the reaction products from this substrate has shown that the first product observed following introduction of the break represents a one-ended invasion crossover product. There is a lag before the DNA fragments corresponding to both crossover products are detected. In their system there was a strong bias for one end invading before the other. An increase in events where plasmid DNA was lost was also noted. This could be due to one-ended invasions or due to extensive DNA degradation from the break site. DSBR between inverted repeats, unlike repair events between direct repeats, has to be reciprocal to generate a viable product. Thus the results obtained from our *in vitro* system, which have shown that one-ended invasions are a primary repair product, are consistent with those obtained using plasmid substrates *in vivo*.

During the course of these experiments we have noted a highly efficient end-joining activity with a strong bias towards formation of inverted repeats. This bias suggests that a homologous pairing mechanism may be involved (Figure 7; Kunes *et al.*, 1990). The two reactions described, formation of crossover products and head-to-head ligation, could conceivably be alternative outcomes of a similar pairing reaction. Proteins that promote pairing of homologous duplexes could pair pLS89/*Ban*II with itself or with pLS96 (Figure 7A and B). Pairing between two pLS96 molecules would have no consequence without a reactive end. Once paired the free end(s) of the linear substrate may invade the homologous duplex to generate a branched structure, or participate in ligation if two duplex ends are present. The strand exchange proteins previously described could potentially play a role in these pairing reactions (Kolodner *et al.*, 1987; Sugino *et al.*, 1988). Alternatively, inverted repeats could arise through the SSA model (Figure 7D). Exonucleases that degrade the 5′ end from one duplex and the 3′ end from a second duplex would expose complementary sequences. The annealed product could undergo a ligation event to generate a head-to-head molecule; DNA repair synthesis from the degraded 3′ end would then fill in the gap to generate an intact duplex.

In summary, we have demonstrated that nuclear extracts prepared from yeast catalyze a recombination reaction to yield crossover products. The reaction is stimulated by a double-stranded break or gap within homologous DNA sequences. The substrate DNAs must interact directly in the yeast extract to form recombinant products, and branched DNA molecules are formed during the reaction. The data presented are more consistent with a strand invasion model rather than single-strand annealing, but determination of the precise mechanism involved awaits the purification of the reaction components.

Materials and methods

Media and growth conditions

Media for yeast growth were prepared as described by Sherman *et al.* (1986). Yeast strains derived from LBL1N were grown at 25°C as the *cly8* mutation causes cell lysis at 28°C; other yeast strains were propagated at 30°C. Bacterial strains were grown at 37°C in LB medium supplemented with ampicillin (50 μg/ml), or chloramphenicol (25 μg/ml) when necessary.

Bacterial and yeast strains

E.coli strain DH5α was used for transformation and propagation of plasmids. The yeast strains LS130 and LS151 are *pep4* derivatives of LBL1N (α, *ade5, met13-c, cyh*^{r}, *trp5, ade6, cly8, ade2-1, ura3-1*) and W303-1A (α, *leu2-3,12, his3-11,15, ura3-1, can1-100, ade2-1*), respectively. The *pep4::URA3* disruption was constructed by transformation (Ito *et al.*, 1983) of strains LBL1N and W303-1A with an *Eco*RI−*Xho*I fragment from pTS15 (Rothman *et al.*, 1986) using the one step transplacement method (Rothstein, 1983). Ura$^+$ transformants that contained the *pep4* disruption were confirmed by Southern analysis.

DNA isolation and manipulations

Small and large scale plasmid DNA isolations were by the alkali lysis method (Birnboim and Doly, 1979). Substrates for *in vitro* reactions were purified by two cycles of equilibrium centrifugation in CsCl−ethidium bromide

density gradients. The substrate DNAs were further purified by phenol extraction and ethanol precipitation. Substrate DNA was made linear by digestion with the appropriate restriction enzyme, then purified by phenol extraction and ethanol precipitation. Standard cloning procedures were used for the construction of plasmids (Maniatis *et al.*, 1982). Restriction enzymes, the Klenow fragment of *E.coli* DNA polymerase, T4 DNA ligase and *Xba*I linkers were purchased from New England Biolabs. Plasmid pLS89 was constructed by digestion of pBR322 with *Ban*II followed by ligation at low DNA concentration; the resulting plasmid was Tc^s and contained a single *Ban*II site. Plasmid pLS96 was constructed by digesting pACYC184 with *Cla*I and *Xba*I, repairing the 3′ recessed ends with Klenow enzyme, and then reclosing by ligation.

Isolation of yeast nuclei and preparation of nuclear extracts

The method used for the isolation of yeast nuclei (Dunn and Wobbe, 1990) is a modification of the procedure used by Nelson and Fangman (1979). Two litre cultures of yeast were grown in YPD with shaking until they reached a cell density of 5×10^7/ml. Cultures were harvested by centrifugation at 5000 r.p.m. for 5 min at room temperature in a Sorvall GSA rotor. The cells were washed with 200 ml of room temperature 1 M sorbitol, then resuspended in 50 ml S buffer (1.1 M sorbitol, 20 mM KPO_4 (pH 7.0), 0.5 mM $CaCl_2$). Zymolyase 100T (Seikagaku America Inc.) was added at 0.5 mg/ml and protease inhibitors at the following final concentrations: PMSF (phenyl methylsulfonylfluoride, 0.5 mM), pepstatin A (1 mg/l) and leupeptin (0.5 mg/l). Protease inhibitors were added to all solutions used for nuclei isolation and preparation of extracts unless otherwise stated. Cells were spheroplasted at 25–30°C (depending on strain background) with gentle agitation until > 95% spheroplasts were obtained. The spheroplasts were collected by centrifugation at 1000 *g* for 5 min at 4°C, washed two times with 40 ml of ice-cold SPC buffer [1 M sorbitol, 20 mM PIPES (sodium piperazine-*N*-*N*′-bis (2-ethane sulfonate), pH 6.3), 0.1 mM $CaCl_2$], then resuspended in 1 ml of SPC. The resuspended spheroplasts were slowly pipetted into 100 ml of ice-cold FL buffer (18% (w/w) Ficoll-400 (Pharmacia), 20 mM PIPES (pH 6.3), 0.5 mM $CaCl_2$) in an ice bath with continuous stirring. Stirring was continued for 20 min to allow lysis to occur. Unlysed cells and debris were removed from the lysate by centrifugation at 5500 r.p.m. in a Sorvall SS34 rotor for 15 min at 4°C. Nuclei were collected from the resulting supernatant by centrifugation at 11 500 r.p.m. in a Sorvall SS34 rotor for 20 min at 4°C. The nuclei were resuspended in 15 ml SPC buffer, centrifuged at 8500 r.p.m. (SS34 rotor) for 10 min, 4°C, and then resuspended in 5 ml L buffer (50 mM Tris–HCl (pH 7.5), 10 mM $MgSO_4$, 1 mM EDTA, 1 mM DTT, 2 mM spermidine, 0.5 M $(NH_4)_2SO_4$). The nuclei were incubated on ice for 20 min with occasional mixing by tube inversion. The lysate was centrifuged at 40 000 r.p.m. in a Beckman 50Ti rotor for 40 min at 4°C. Proteins were precipitated from the resulting supernatant by the addition of solid $(NH_4)_2SO_4$ to 55% saturation. The protein precipitate was collected by centrifugation at 15 000 r.p.m. for 10 min. The protein pellets thus obtained could be stored for several weeks at −75°C with no apparent loss of activity. The protein pellet was suspended in 50 mM HEPES (potassium *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonate, pH 7.8), 1 mM EDTA, 1 mM DTT, 10% (w/v) glycerol at a concentration of 5–10 mg/ml prior to use in the recombination assays.

Recombination assays

Reactions (50 μl) contained 35 mM HEPES (pH 7.8), 10 mM $MgCl_2$, 5 mM ATP, 2 mM spermidine, 2 mM DTT, 0.5 mM NAD, 50 μM each CTP, GTP and UTP, 20 μM dNTPs, 100 μg/ml BSA and 2 μg total substrate DNA. The nuclear extract was added to 0.5–0.8 mg/ml protein concentration (the optimal concentration was determined for each batch of extract) and incubation carried out at 30°C for the specified time. Reactions were stopped by the addition of EDTA to 20 mM and the DNA was purified by two extractions with phenol:chloroform:isoamylalcohol (25:24:1) followed by ethanol precipitation. The plasmid DNA was suspended in 30 μl 10 mM Tris–HCl (pH 8.0), 1 mM EDTA; 5 μl was generally used for restriction analysis with each enzyme.

Electrophoresis

Agarose gels were prepared and run in TBE (89 mM Tris–borate, 89 mM boric acid, 2 mM EDTA). Two-dimensional agarose gels were prepared and run essentially as described by Brewer and Fangman (1987). 0.4% agarose was used for the first dimension and electrophoresis carried out at 1.5 V/cm for 16 h; the second dimension was 1% agarose plus 0.5 μg/ml ethidium bromide with electrophoresis at 6 V/cm for 5 h. Size standards were either bacteriophage lambda DNA digested with *Hin*dIII, or substrate DNAs in the parental and recombined configurations. DNA fragments to be used for hybridization probes by random prime labelling were isolated from low melting temperature agarose gels.

Southern analysis

After electrophoresis DNA was transferred to nylon membrane ('Hybond', Amersham Corp.) and hybridized with probes prepared using the random primer method (Feinberg and Vogelstein, 1983) or else with [γ-^{32}P]ATP and T4 polynucleotide kinase (van de Sande *et al.*, 1973).

PCR

The PCR was carried out according to Saiki *et al.* (1985) using the *Taq* DNA polymerase (Perkin Elmer Cetus). To reduce variation due to pipetting errors, master mixes containing all components except DNA were prepared. Reactions of 50 μl contained 50–100 pg DNA and were carried out for 30 cycles (each of: 94°C, 1 min; 60°C, 2 min; 72°C, 3 min). After cooling to room temperature, 20 μl from the aqueous phase was removed and analyzed by electrophoresis. Negatives of photographed ethidium bromide stained gels were scanned using a Molecular Dynamics Laser Densitometer.

Oligonucleotides used for DNA hybridizations, or for primers for the PCR were synthesized using the Applied Biosystems 391 DNA Synthesizer. The oligonucleotides used have the following base composition: oLS1 5′TCGGGCTCGCCACTTCGGGCTCAT; oLS3 5′GAGCCCGAAGTGGCGAGCCC; oLS8 5′GGCGTATCACGAGGCCCTTTCG; oLS9 5′GCAACTTCAGCAGCACGTAGGGG.

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