Extrachromosomal Recombination Is Deranged in the *rec2* Mutant of *Ustilago maydis*

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ABSTRACT

Transformation of a *leu1* auxotroph of *Ustilago maydis* to prototrophy with an autonomously replicating plasmid containing the selectable *LEU1* gene was found to be efficient regardless of whether the transforming DNA was circular or linear. When pairs of autonomously replicating plasmids bearing noncomplementing *leu1* alleles were used to cotransform strains deleted entirely for the genomic copy of the *LEU1* gene, Leu$^+$ transformants were observed to arise by extrachromosomal recombination. The frequency of recombination increased severalfold when one plasmid of the pair was made linear by cleavage at one end of the *leu1* gene, but increased 10–100-fold when both plasmids were first made linear. The increase in recombination noted in wild-type and *rec1* strains was not apparent in the *rec2* mutant unless the members of the pair of plasmids were cut at opposite ends of the *leu1* gene to yield linear molecules offset in only one of the two possible configurations. Use of a pair of plasmid substrates designed to measure nonreciprocal and multiple exchange events revealed only a minor fraction of the total events arise through these modes, and further that no stimulation occurred when the plasmid DNA was linear. It is unlikely that the defect in *rec2* lies in a mismatch correction step since a high yield of Leu$^+$ recombinants was obtained from the *rec2* mutant when it was transformed with heteroduplex DNA constructed from plasmids with the two different *leu1* alleles.

GENETIC recombination and DNA repair are intimately related, interconnected processes. A widely held view is that recombination evolved as part of an overall DNA repair strategy providing means for maintaining the integrity of the genome (*e.g.*, BERNSTEIN, HOPF and MICHOD 1987; SMITH 1985). It is thought that in the case of life-threatening events such as radiation-induced double-strand breaks in the cellular DNA the recombination machinery normally present in the cell could act to repair the break precisely (RESNICK 1976). The isolation of mutants simultaneously altered in both recombination and repair has provided a compelling argument for overlap in these processes.

Analysis of plasmid recombination in *Saccharomyces cerevisiae* has led to a firm conceptual understanding of how genetic recombination might be coupled with repair of double-strand breaks in DNA (SZOSTAK *et al.* 1983) and has provided insight into the function of genes controlling recombinational repair (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981; EMBRETSON and LIVINGSTON 1984; SYMINGTON, MORRISON and KOLODNER 1984). By contrast in eukaryotic organisms other than *S. cerevisiae* the relationship between recombination and repair remains poorly understood. Although it has been observed in a number of systems that plasmid molecules containing double-strand breaks are recombinogenic (BRENNER, SMIGOCKI and CAMERINI-OTERO 1986; FOLGER, THOMAS and CAPECCHI 1985; LIN, SPERLE and STERNBERG 1984, 1990; MARYON and CARROLL 1991; SEIDMAN 1987; SONG *et al.* 1985; WAKE, VERNALEONE and WILSON 1985), there has been slow progress in establishing the mechanistic basis of the observations. Intractable genetics in the higher eukaryotic systems studied has precluded a more penetrating analysis of the mechanism.

The interest of this laboratory is in understanding mechanisms of recombination in the fungus *Ustilago maydis*. A genetic system for analyzing recombination in *U. maydis* was established by HOLLIDAY (1965, 1967) a number of years ago with the isolation of two mutants simultaneously defective in recombination and repair. Both mutants were found to have complex phenotypes. The *rec1* mutant was found to be sensitive to ultraviolet light, ionizing radiation, and alkylating chemicals. Mitotic allelic recombination was slightly elevated, but was no longer inducible by DNA-damaging agents. Meiosis was aberrant in crosses homozygous for *rec1* yielding a large proportion of inviable products. In addition there were pleiotropic effects on mitotic chromosome segregation, cell division and mutagenesis (HOLLIDAY *et al.* 1976). The *rec2* mutant was also found to be sensitive to the same spectrum of DNA-damaging agents as *rec1*. There was no radiation-inducible allelic recombination in the *rec2* homozygous diploid. More strikingly, meiosis was completely blocked (HOLLIDAY 1967).

We have extended Holliday's studies to the molecular level by isolating the *REC1* and the *REC2* genes (TSUKUDA, BAUCHWITZ and HOLLOMAN 1989; BAUCHWITZ and HOLLOMAN 1990) and have begun to analyze recombination in *U. maydis* using plasmid-based systems (FOTHERINGHAM and HOLLOMAN 1990). The aim of the present study was to establish an extrachromosomal recombination system utilizing a pair of autonomously replicating plasmids bearing different alleles of a selectable gene. Our interest was to examine the genetic control of intermolecular recombination during double-strand break repair of the plasmid molecules.

MATERIALS AND METHODS

Plasmid construction: The *LEU1* gene of *U. maydis* is contained on a 3.0-kb *Eco*RI-*Hin*dIII DNA fragment (FOTHERINGHAM and HOLLOMAN 1989). pCM216 (see Figure 1) contains this 3.0-kb fragment inserted into the polylinker region of pCM163, a pBluescript II SK derivative (Stratagene) containing the 383-bp *U. maydis ARS* (TSUKUDA *et al.* 1988) in place of the 127-bp *Ssp*I fragment. Plasmid pCM245 is a derivative of pCM216 in which the *Nco*I site proximal to the *Eco*RI site has been destroyed by filling in with Klenow DNA polymerase. This 4-bp insertion renders the *LEU1* gene inactive. In pCM246 the distal *Nco*I site has been filled in, thereby inactivating the *LEU1* gene as above. Plasmid pCM259 was derived from pCM216 by removing essential sequences from the 5′ and 3′ ends of the *LEU1* gene. An essential 1.5-kb *Pvu*I-*Hin*dIII fragment was deleted from one end of the *LEU1*-containing fragment along with an essential 100-bp *Eco*RI fragment from the other end of the fragment. To ablate the 3.0-kb *Eco*RI-*Hin*dIII *LEU1*-containing sequence from the genome of the host strains of interest, the one-step gene disruption vector pCM162 was constructed. The original *LEU1* clone isolated from a library screen in the *leu1-1* mutant was 6.5 kb in length (FOTHERINGHAM and HOLLOMAN 1989). This was subcloned to yield the 3.0-kb fragment that contained the *LEU1* gene. pCM159 contains two fragments from the original cloned fragment that flank the 3.0-kb *LEU1* gene fragment, but does not include any *LEU1* sequences. The two fragments are 0.7 and 0.8 kb in length and are fused together to form a *Xho*I site. A 2.0-kb *Xho*I-*Sal*I fragment from pHL1 (WANG, HOLDEN and LEONG 1988) containing a *U. maydis hsp*70-hygromycin phosphotransferase gene fusion (*HPH*) conferring resistance to hygromycin (Hyg^R)was inserted into the *Xho*I site of pCM159 to yield pCM162.

Strains: *U. maydis* strains UCM128, UCM132 and UCM186 (Table 1) were derived from UCM3, UCM20 and UCM54, respectively, by a transplacement procedure (ROTHSTEIN 1983) using the vector pCM162 made linear by digestion with *Hin*dIII. Southern blot hybridization analysis of Hyg^R Leu$^-$ transformants obtained revealed the 3.0-kb *LEU1* gene fragment had been ablated from these strains. The only homology between the recombination substrate plasmids pCM245, pCM246, pCM259 and the genome is the 383-bp *ARS*. Use of the term "wild-type" in the text denotes the recombination genotype to be *REC1 REC2* and has no bearing on the presence of auxotrophic markers.

Transformation and recombination analysis: *U. maydis* strains were transformed to leucine prototrophy essentially as described before (FOTHERINGHAM and HOLLOMAN 1990) except that 2 μg/ml pantothenic acid was added to the medium and ammonium sulfate was used in place of potassium nitrate as the nitrogen source. Cotransformation was performed using equal amounts of plasmid DNA; in general this was 0.2 μg each, an amount found empirically to be within the linear region of the dose-response curve. Frequencies were normalized to a control using pCM216 alone so that variations in competency and strain differences were checked. This was accomplished by dividing the number of transformants per μg of test DNA by the number of transformants per μg of pCM216 DNA. The transformation efficiency of pCM216 varied from 1 to 5 × 10^4 per μg during the course of these experiments. The results of the experiments in each of Figures 3 through 6 are not averages, but are typical individual experiments. Each experiment was repeated 3 to 5 times with similar results.

Heteroduplex formation. Heteroduplex DNA was made by denaturing and reannealing linear forms of pCM245 and pCM246. Equimolar mixtures of pCM245 and pCM246 made linear by digestion with *Bam*HI or *Cla*I were brought to 0.2 M with NaOH. After 10 min at room temperature Tris-HCl, pH7.5, was added to 0.2 M followed by HCl to 0.2 M to reneutralize the solution. DNA was renatured by thermal annealing at 65° for 15 min, precipitated by addition of 2 M ammonium acetate and 1 volume of isopropanol, and redissolved in 10 mM Tris-Cl, pH 7.5, 1 mM EDTA. Analysis by gel electrophoresis after digestion with *Nco*I revealed that about half of the product was homoduplex and half was heteroduplex DNA.

TABLE 1

***Ustilago maydis* strains and plasmids**

Strain	Relevant genotype	Source
UCM3	*REC1 REC2*	HOLLIDAY 518
UCM20	*rec1-1 REC2*	HOLLIDAY 539
UCM54	*REC1 rec2-1*	HOLLIDAY 218
UCM128	*REC1 REC2 Δleu1::HPH*	This work
UCM132	*rec1-1REC2 Δleu1::HPH*	This work
UCM186	*REC1 rec2-1 Δleu1::HPH*	This work
Plasmids		
pCM159	*Δleu1 ARS*	
pCM162	*Δleu::HPH ARS*	
pCM216	*LEU1 ARS*	
pCM245	*leu1 ARS*	
pCM246	*leu1 ARS*	
pCM259	*leu1 ARS*	

RESULTS

Recombination substrates: The experimental approach chosen was to measure recombination between two autonomously replicating plasmids bearing different noncomplementing alleles of the cloned *U. maydis LEU1* gene. Genetic control of extrachromosomal recombination was studied by transforming the different *rec*$^-$ strains which had been deleted for the entire *LEU1* gene. Reconstruction of a functional *LEU1* gene by interchromosomal plasmid recombination thereby permitted growth of transformants on leucine-free medium. In one set of substrates, pCM245 and pCM246, *leu1* alleles were generated by directed mutagenesis in which small insertion mutations were created at two different restriction endonuclease sites in

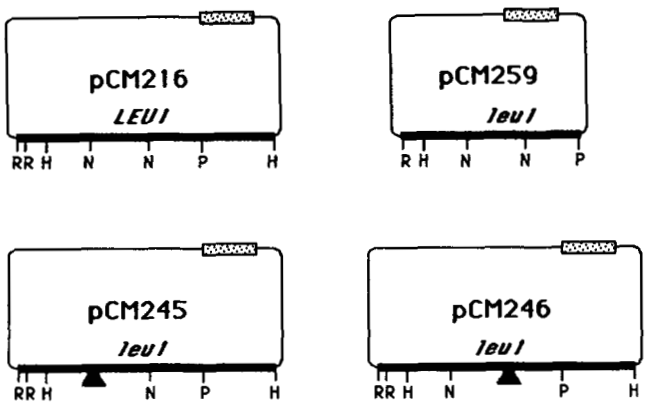


FIGURE 1.—Recombination substrates. Plasmids pCM245 and pCM246 were derived from pCM216 and contain *leu1* alleles with 4-bp insertions (carets) at either *Nco*I site within the *LEU1* gene. pCM259 lacks essential sequences at both ends of the *LEU1* gene. Restriction sites: R (*Eco*RI); N (*Nco*I), P (*Pvu*I); H (*Hin*dIII). The solid bar represents *leu1* alleles. The stippled bar represents the 383-bp *ARS*.

the gene (Figure 1). Recombinants arising would be expected to result from the sum of reciprocal and nonreciprocal events. To assess the relative contribution of each of these two events, a second pair of plasmid substrates was utilized in which the recombination events allowed were limited to the nonreciprocal or multiple exchange class only. pCM259 containing one of the *leu1* alleles in this pair of substrates was deleted for essential 5′ and 3′ sequences. The other *leu1* allele from pCM245, as in the first set, contained a small insertion mutation spanned by the truncated *leu1* sequence present in pCM259. With this pair of substrates recombinants would be expected to arise from nonreciprocal and multiple exchanges, events other than single crossovers.

Transformation is independent of DNA conformation: When wild-type, *rec1-1* and *rec2-1* strains were transformed with the *LEU1*-containing autonomously replicating plasmid pCM216, the yield of Leu$^+$ transformants increased linearly with the dose of input DNA over at least a 100-fold range (Figure 2). There was little variation in yield of transformants regardless of the *rec* genotype. Similarly, when the transforming DNA was made linear by digestion with a restriction endonuclease, the dose response curves in all three genetic backgrounds increased linearly and were virtually superimposable upon the curves obtained using uncut plasmid DNA. When the plasmid DNA was bisected by digestion with a restriction endonuclease that cleaved at two different sites to separate the *ARS* from the *LEU1*-containing fragment, transformation became second order with respect to the input DNA concentration as expected for a bimolecular reaction.

Plasmid recombination is stimulated by double-strand breaks: Cotransformation of *U. maydis* with the recombination substrates pCM245 and pCM246 yielded Leu$^+$ transformants with the square of the dose of total input DNA (Figure 2), indicative of a

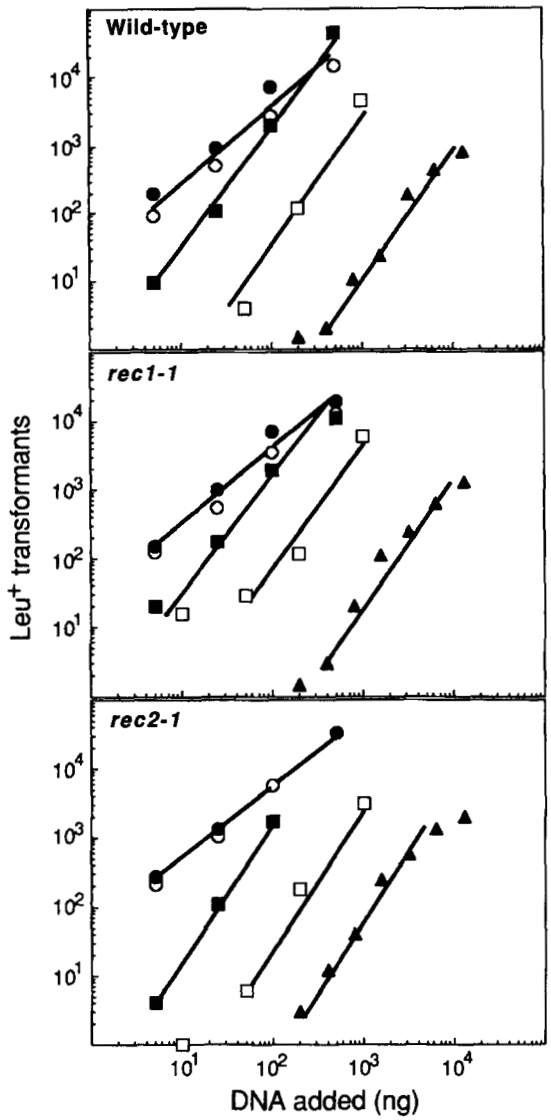
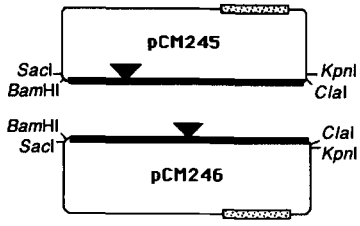


FIGURE 2.—Transformation order during intramolecular and intermolecular recombination. Wild-type (UCM128), *rec1-1* (UCM132) and *rec2-1* (UCM186) strains were transformed to Leu$^+$ with the indicated amounts of the following DNAs: pCM216 (*closed circles*); pCM216 cut to the linear form with *Bam*HI (*open circles*); pCM216 bisected with *Hin*dIII (*closed squares*). Intermolecular recombination was measured after cotransformation with equal amounts of circular pCM245 and pCM246 (*closed triangles*) or with *Bam*HI-cut pCM245 and *Cla*I-cut pCM246 (*open squares*).

bimolecular reaction. Some variability in transformation efficiency from experiment to experiment and from strain to strain was noted, but was controlled by parallel transformation using the parental *LEU1*-vector pCM216. Recombination frequencies were then calculated after normalization based on the transformation frequency obtained with pCM216. There was little significant difference in the frequency obtained after cotransformation of wild-type, *rec1-1* and *rec2-1* strains with intact pCM245 and pCM246. When one of the two plasmid substrates was made linear by cutting at one end of the *leu1* sequence or the other, there was a severalfold increase in transformation frequency (Figure 3). The increase was in general



Expt.	Input DNA pCM245	Input DNA pCM246	Wild-type Total	Wild-type Freq. (x10³)	*rec1-1* Total	*rec1-1* Freq. (x10³)	*rec2-1* Total	*rec2-1* Freq. (x10³)
1	-	-	2	0.4	3	0.2	12	1.2
	*Bam*HI	-	35	6.3	84	6.8	46	4.7
	-	*Bam*HI	10	1.8	20	1.6	15	1.5
	*Bam*HI	*Bam*HI	201	36.2	265	21.4	35	3.6
2	-	-	1	0.1	2	0.3	0	<0.1
	*Sac*I	-	6	0.7	6	0.8	3	0.4
	-	*Sac*I	2	0.2	3	0.4	2	0.3
	*Sac*I	*Sac*I	57	6.6	22	2.8	6	0.8
3	-	-	0	<0.3	1	0.3	15	0.8
	*Cla*I	-	9	3.0	6	1.5	10	0.5
	-	*Cla*I	7	2.4	7	1.8	30	1.6
	*Cla*I	*Cla*I	57	19.3	44	11.0	50	2.7
4	-	-	1	0.2	1	0.2	4	0.4
	*Kpn*I	-	13	2.2	2	0.4	3	0.3
	-	*Kpn*I	8	1.4	4	0.8	7	0.8
	*Kpn*I	*Kpn*I	82	13.8	18	3.7	19	2.1

FIGURE 3.—Influence of DNA topology on recombination. *U. maydis* strains were transformed with 0.2 μg of the indicated pairs of plasmids and Leu⁺ recombinants were selected. Recombination frequencies were calculated by dividing the transformation frequency obtained from a pair of substrates by the transformation frequency obtained with the *LEU1* control vector pCM216.

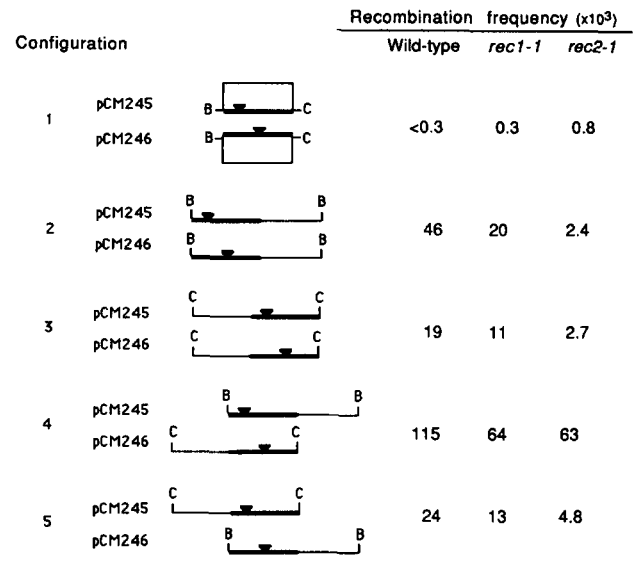


FIGURE 4.—Suppression of the *rec2* recombination deficiency by DNA configuration. *U. maydis* strains were transformed with 0.2 μg of the pairs of plasmids made linear with the restriction enzymes as indicated. Restriction sites: B(*Bam*HI); C (*Cla*I).

more pronounced when the plasmid was cut with restriction endonucleases yielding recessed 3′ ends, rather than protruding 3′ ends. When both plasmid substrates were made linear, the increase in transformation frequency was substantially higher in the wild-type and *rec1-1* strains, but not in the *rec2-1* strain (Figure 3). The greater than 100-fold increase in recombination frequency noted when wild-type cells were transformed with linear molecules suggests a role for double-strand breaks in *U. maydis* recombination and argues that the preferential topological substrate for extrachromosomal recombination is linear DNA.

Recombination in *rec2-1* is governed by configuration of the marker and the double-strand break: The influence of allele configuration on recombination was examined by testing pairs of plasmid substrates made linear by cutting with different combinations of restriction endonucleases. With linear plasmids in the offset configuration 5 (Figure 4), the frequencies of recombination in wild-type, *rec1-1*. and *rec2-1* were about the same as what were observed with aligned linear molecules in configuration 2 or 3. There was a 30–150 fold increase in recombination frequency in wild-type and *rec1-1*, but little stimulation in *rec2-1*. On the other hand, when the linear molecules were in offset configuration 4, there was a great increase in recombination frequency in all three strains, although the frequency seen in wild-type was still about twofold higher than in *rec1-1* or *rec2-1*. The dose response curve with the linear molecules in offset configuration 4 in all three genetic backgrounds increased with the square of the DNA concentration indicating there is no change from the bimolecular mode of reaction seen with uncut molecules (Figure 2). Thus, recombination in *rec2-1* is stimulated by double-strand breaks, but is dependent on the placement of the alleles and the strand breaks.

A minor fraction of events is accounted for by nonreciprocal and multiple exhanges, but is not configuration dependent: The fraction of recombination events arising from nonreciprocal and multiple exchanges was measured using pCM245 and pCM259 (Figure 1). With this pair of substrates generation of Leu⁺ recombinants is limited to events other than single reciprocal crossovers. The percentage of recombination due to nonreciprocal and multiple exchanges was calculated as the ratio of the frequency of events between pCM245 and pCM259 to that of pCM245 and pCM246. This number was doubled since only exchange of the *leu1* insertion mutation in pCM245 to *LEU1* could be selected in cotransformation with pCM259. In wild type the frequency of recombination using these substrates was greatly reduced (Figure 5). These exchanges accounted for only 24% of all events when the substrates were circular and 34% when linear. In *rec1-1* and *rec2-1* the frequencies were also reduced to roughly one quarter of all events. No stimulation was observed in any strain when substrates with the linear offset configuration were used.

Mismatch correction is efficient in *rec2-1*: It is possible that recombinants arise from the aligned linear molecules through correction of mismatched bases in heteroduplex DNA formed after pairing of complementary strands. The defect in *rec2-1* could lie anywhere along this path. To test proficiency in cor-

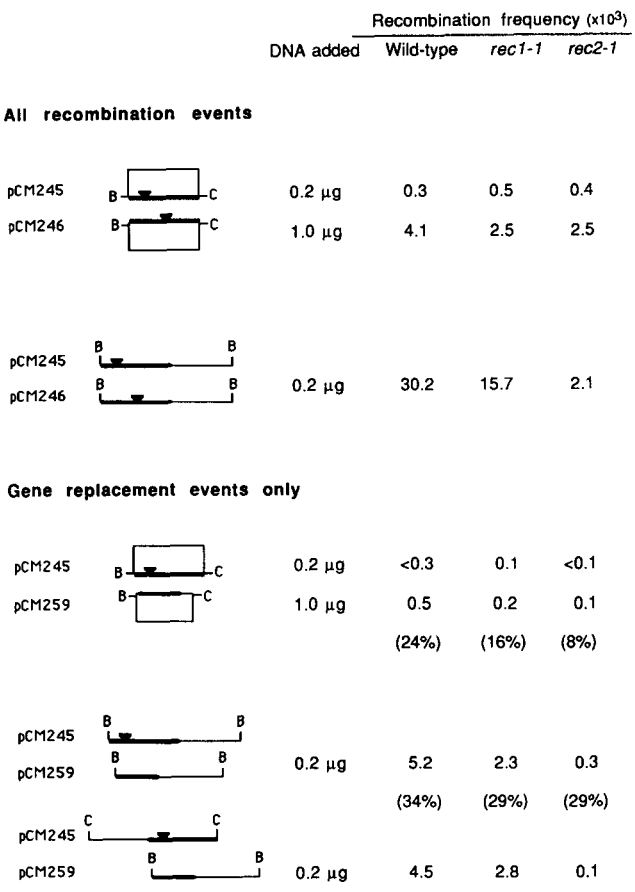


FIGURE 5.—Gene replacement with circular and linear plasmid DNA substrates. *U. maydis* strains were transformed to Leu$^+$ with the given amounts of plasmid DNA substrates. Percentage indicated is the fraction of nonreciprocal and multiple crossover events (collectively referred to here as gene replacements) to the total recombination events obtained with pCM245 and pCM246 in an equivalent configuration. Restriction endonuclease sites: B (*Bam*HI); C (*Cla*I).

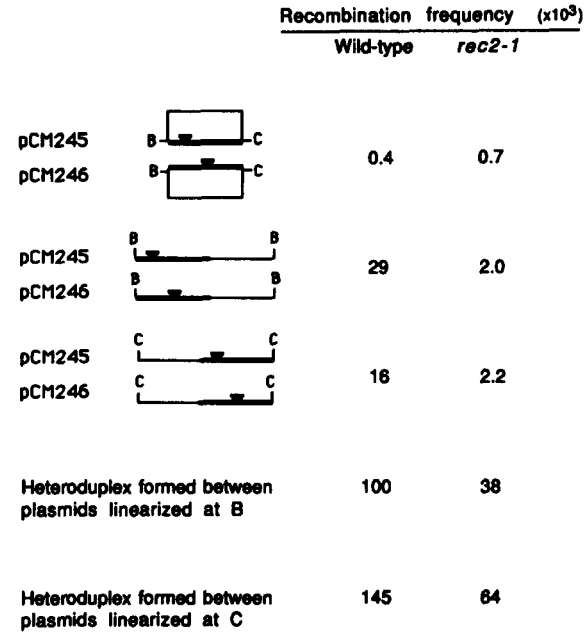


FIGURE 6.—Transformation with heteroduplex DNA. *U. maydis* strains were transformed to Leu$^+$ with 0.2 μg of plasmid DNA as indicated and with heteroduplex mixtures prepared by denaturation and reannealing pairs of linear molecules as described in MATERIALS AND METHODS.

recting mismatches, *U. maydis* strains were transformed with heteroduplex DNA made *in vitro* by melting and reannealing mixtures of linear pCM245 and pCM246. DNA thus prepared consists of a mixture of homoduplexes and heteroduplexes due to random association of denatured single strands. Using this DNA we observed the frequency of transformation to Leu$^+$ prototrophy to be greater than the frequency of recombination using pairs of aligned linear molecules indicating that correction of these heteroduplexes was even more efficient than recombination (Figure 6). In *rec2-1* the frequency of correction to Leu$^+$ was reduced slightly, to 40% of the level observed in wild-type, but was by no means reduced to the extent observed for recombination of linear molecules.

DISCUSSION

In bacterial systems transformation with closed circular DNA has been found to be much more efficient than transformation with the linear form of the DNA (CONLEY and SAUNDERS 1984). One explanation for this observation has been that DNA ends provide access for degradation by intracellular nucleases. Support for this notion in *Escherichia coli* comes from analysis of transformation in *recBC* mutants where it has been inferred that linear DNA with unprotected ends is degraded through the exonucleolytic activity encoded of the recBCD enzyme (*e.g.*, AMUNDSEN *et al.* 1990). In *Saccharomyces cerevisiae* transformation of protoplasted cells with autonomously replicating plasmids has also been found much more efficient when the DNA is in the closed circular rather than linear form (ORR-WEAVER and SZOSTAK 1983), although there has been disagreement over this point when transformation is done using LiCl (ITO *et al.* 1983; WHITE and SEDGWICK 1985). Exonucleolytic degradation is less rampant in yeast but has been observed at double-strand breaks formed at the *MAT* locus during mating type switching (WHITE and HABER 1989) and in meiosis at the *ARG4* locus (SUN, TRECO and SZOSTAK 1991). Exonucleolytic degradation, deletion formation and peculiar rearrangements are also apparent when transforming plasmid DNA is cut within sequences nonhomologous to the yeast genome (ORR-WEAVER and SZOSTAK 1983; KUNES, BOTSTEIN and FOX 1985).

The high efficiency of transformation of *U. maydis* using linear DNA stands in contrast to the findings with *E. coli* and *S. cerevisiae* protoplasts, but is similar to what has been observed in mammalian systems where circular and linear DNAs are nearly equivalent in transformation efficiency (WAKE *et al.* 1984). *U. maydis* is extremely resistant to killing by ionizing radiation presumably because it has a powerful DNA

repair system and can protect and rejoin DNA ends formed after strand breakage. The equivalent efficiency of transformation by circular and linear DNA molecules must be a manifestation of potent end-joining activity or else the absence of degradative activities. The former seems likely in view of the abundance of deoxyribonucleases in *U. maydis* (RUSCHE, ROWE and HOLLOMAN 1980; HOLLOMAN, ROWE and RUSCHE 1981; YARNALL, ROWE and HOLLOMAN 1984) and in light of our previous studies on transformation demonstrating that linear DNA molecules are readily joined together end-to-end to form large concatemers (FOTHERINGHAM and HOLLOMAN 1990). Thus, the remarkable joining mechanisms established in mammalian cells (*e.g.*, ROTH and WILSON 1988) may not be limited to higher eukaryotes.

In view of the strong stimulation of plasmid recombination in *U. maydis* by double-strand breaks, it seems likely that the preferred topology of plasmid DNA molecules destined for recombination is linear, a conclusion also reached in studies on recombination in mammalian cells (WAKE, VERNALEONE and WILSON 1985). The requirement for linear substrates would appear to put recombination in competition with the much greater ability of the cell to recircularize linear molecules. It could well be that the DNA molecules entering the pool destined for recombination are those that have escaped end-joining. Alternatively, the putative end-joining mechanism might be responsible for the formation of a precursor of recombination whose resolution would yield recombinant molecules.

Evidence has been obtained that double-strand breaks in the cellular DNA of *U. maydis* are repairable by a damage-inducible recombinational repair system (LEAPER, RESNICK and HOLLIDAY 1980; LEE and YARRANTON 1982). The frequency of allelic recombination has been found to be greatly enhanced after irradiation of mitotically growing cells, although no such increase is observed in the radiation-sensitive *rec1* or *rec2* mutant. It is possible that recombinational repair in *U. maydis* could take place through a pathway akin to the double-strand-break repair model (SZOSTAK *et al.* 1983) or by a mechanism similar to the single-strand annealing model (LIN, SPERLE and STERNBERG 1984; WAKE, VERNALEONE and WILSON 1985) which is proposed to proceed via annealing of complementary sequences in molecules made partially single-stranded through the action of exonucleases or unwinding activities. While both models envisage processing of broken DNA ends to expose single-stranded tracts as initiating events, the outcome of the models is different in that the double-strand-break repair model is conservative, but the single-strand annealing model is not. It is possible that both conservative and nonconservative pathways are in operation during plasmid recombination in *U. maydis* (Figure 7). Aligned linear DNA molecules and offset

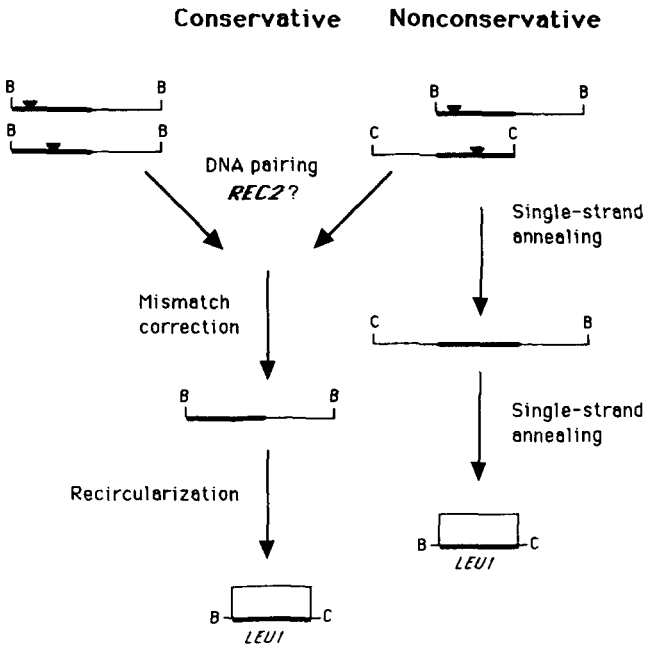


FIGURE 7.—Extrachromosomal recombination mechanisms. Diagrammed on the left is a conservative recombination pathway. It proceeds by DNA pairing steps, including synapsis, strand exchange, and resolution of a recombination intermediate, arbitrarily followed by mismatch correction steps to yield a *LEU1*-containing linear plasmid able to recircularize and replicate. The pathway is conservative in that DNA is not irrevocably destroyed in the process. We postulate that the *REC2* gene product functions in DNA pairing. Diagrammed on the right are offset linear DNA molecules. These can enter the conservative pathway, but in addition may enter the nonconservative pathway to recombination by single-strand annealing. Through this mechanism strand-specific partial degradation of homologous DNA molecules exposes complementary single-stranded regions by which the molecules anneal yielding a partial dimer. Repetition of this process on the flanking direct repeats would yield a circular *LEU1*-containing plasmid able to replicate as above. This pathway is imagined to operate independently of the *REC2* gene product.

linear DNA molecules could recombine by gene conversion or by homologous pairing and crossing over, followed by recircularization of the linear recombinant molecules. An additional process opened up to offset linear DNA molecules would require free DNA ends and overlapping homology as shown. It is the latter process that might be driven by the single-strand annealing reaction, although it is also plausible that strand exchange could be initiating at one end by a double-strand-break mechanism but then is constrained by the second double-strand break. By either mechanism the location of the mutant alleles with respect to the double-strand breaks restricts the production of the reconstructed *LEU1* gene.

Recombination substrates in the most efficient configuration for recombination are linear molecules with offset homology such that the insertion mutations disrupting the marker are near the ends of the *leu1* gene. The differences in recombination frequencies as a function of configuration could be explained in terms of the single-strand annealing model which

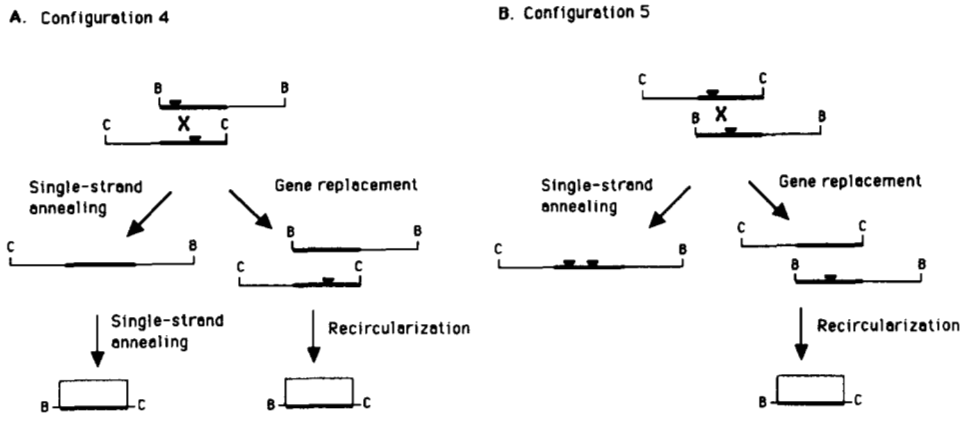


FIGURE 8.—Configuration dependence of plasmid recombination. **A**, Recombination between two plasmids made linear by cutting at opposite ends of the *leu1* gene such that the insertion mutations lie proximal to DNA termini (configuration 4). These plasmids could undergo nonreciprocal and multiple crossovers (referred to here as gene replacements) to yield monomer length linear molecules which could then recircularize and replicate. Alternatively, a single-strand annealing event between the two mutant loci could yield a partial dimer with vector sequences directly repeated. Intramolecular recombination between these repeats would generate monomer plasmid which could replicate as above. **B**, Recombination between linear plasmids such that the insertion mutations lie distal with respect to the DNA termini (configuration 5). These plasmids could recombine by the two modes described in **A**. However, a single-strand annealing event between the two mutant loci in this case would be unproductive, yielding a *LEU1*-containing fragment unlinked to vector sequences and unable to replicate.

could yield a circular plasmid with an intact *LEU1* gene only when the offset homology was in the preferred configuration (Figure 8, configuration 4). The other offset configuration (Figure 8, configuration 5) would give rise to a circular plasmid containing both of the mutations in the *leu1* gene. Therefore, this pathway is productive only for molecules in configuration 4. It should be noted that while the models shown picture the recombination products as unit length molecules we have found that with each pair of plasmid substrates the products of recombination are concatemers composed of tandem arrays of inverted and direct repeats of monomer units similar to what we have noted before in our previous studies on the structure of transforming DNA in *U. maydis* (FOTHERINGHAM and HOLLOMAN 1990). Concatemerization could occur before or after the pathways as we have drawn them, but this does not bear on the essential reaction mechanism shown.

The defect in extrachromosomal recombination manifest in *rec2-1* provides evidence in support of alternative recombination pathways and clues to the function of *REC2*. The defect in recombination noted in our studies does not extend to all configurations of linear molecules, only to a subset, suggesting the operation of multiple recombination pathways in *U. maydis*. A *REC2*-dependent pathway would appear to function in recombination of aligned linear molecules and linear molecules in one offset configuration, while a *REC2*-independent pathway would appear to function in recombination of offset linear molecules whose allelic partners are oriented in an opposite spatial sense.

The *rec2* mutant is extremely sensitive to ionizing radiation (HOLLIDAY 1965; LEAPER, RESNICK and HOLLIDAY 1980) which is known to induce double-strand breaks in addition to other types of lesions and DNA adducts (FRIEDBERG 1985). The defect in *rec2* is unlikely to involve a step in removal of damaged DNA residues as excision repair is probably normal (UNRAU 1975). Neither is the defect likely to involve joining of free DNA ends. The transformation frequencies of wild-type and *rec2-1* strains with the *LEU1*-containing plasmid were almost identical when the plasmid was introduced cut into two fragments. Our speculation is that the *REC2*-dependent pathway involves homologous pairing and strand exchange whereas the *REC2*-independent pathway proceeds by single-strand annealing. In our view the defect in *rec2-1* is likely to involve some step in pairing of DNA duplexes.

Only a minor fraction of the total extrachromosomal recombination events observed was accounted for by nonreciprocal and multiple exchanges. This is surprising given that most mitotic recombination of chromosomal alleles in *U. maydis* is due to gene conversion (HOLLIDAY 1966) and raises the notion that allelic recombination proceeds via different pathways depending on the nuclear location or genomic state of the alleles in question. It could well be that the set of genes governing control of allelic recombination is dependent on chromatin structure which in turn is responsive to the transcriptional and replicative metabolic activity of the cell.

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