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Disruptions of the *Ustilago maydis REC2* gene identify a protein domain important in directing recombinational repair of DNA

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Summary

The *REC2* gene of *Ustilago maydis* encodes a homologue of the *Escherichia coli* RecA protein and was first identified in a screen for UV-sensitive mutants. The original isolate, *rec2-1*, was found to be deficient in repair of DNA damage, genetic recombination and meiosis. We report here that the *rec2-197* allele, which was constructed by gene disruption, retains some biological activity and is partially dominant with respect to *REC2*. The basis for the residual activity is probably as a result of expression of a diffusible product from the *rec2-197* allele that augments or interferes with *REC2* functions. This product appears to be a polypeptide expressed from a remnant of the 5′ end of the open reading frame that was not removed in creating the gene disruption. The mutator activity and disturbed meiosis of *rec2-197* suggest that the Rec2 protein functions in a process that avoids spontaneous mutation and insures faithful meiotic chromosome segregation. A prediction based on the phenotype of *rec2-197* is that Rec2 protein interacts with one or more other proteins in directing these functions. To identify interacting proteins we performed a yeast two-hybrid screen and found Rad51 as a candidate. Rec2-197 and Rad51 appear to interact to a similar degree.

Introduction

DNA repair and recombination proficiency require homologous pairing and DNA strand exchange functions. In prokaryotes this activity is provided by the RecA protein and, similarly, in eukaryotes it is provided by RecA homologues, which may be elaborated in multiple forms (Shinohara and Ogawa, 1999). In *Saccharomyces cerevisiae*, four different RecA homologues have been identified (Kans and Mortimer, 1991; Aboussekhra *et al*., 1992; Basile *et al*., 1992; Bishop *et al*., 1992; Shinohara *et al*., 1992; Lovett, 1994). While structurally related, the genes are not functionally redundant. Inactivation of any one of them results in a double-strand-break repair deficiency (Rattray and Symington, 1995). Multiple homologues have also been identified in *Schizosaccharomyces pombe* and *Homo sapiens*, and evidence is also accumulating in these systems showing that the genes may function independently in a non-redundant manner (Thacker, 1999).

Ustilago maydis is an extremely radiation-resistant fungus that is as evolutionarily distant from *S. cerevisiae* and *S. pombe* as from *H. sapiens*. Two RecA homologues have been identified in *U. maydis*. One is orthologous to Rad51 and is so named (Ferguson *et al*., 1997). The other, encoded by the *REC2* gene (Rubin *et al*., 1994), is much more divergent and more than twice the size of Rad51. Both genes are expressed in mitotic cells. There is no redundancy in their functions as mutation in either results in loss of DNA repair and recombination proficiency. Genetic analysis indicates epistatic interaction between the two genes in certain recombination and repair functions, but some measure of independent activity in other functions (Ferguson *et al*., 1997). Biochemical analysis indicates that Rec2 catalyses certain homologous pairing and strand exchange reactions more proficiently than Rad51 (Bennett and Holloman, 2001), adding to the speculation that the proteins might be specialized for separate processes or dedicated to different functions.

Rec2 was first identified by genetic analysis through isolation of the *rec2-1* allele in a screen for DNA repair mutants (Holliday, 1965). The mutant is extremely sensitive to UV, ionizing radiation and alkylating agents, is defective in recombination and is blocked in meiosis (Holliday, 1967; Fotheringham and Holloman, 1991). The gene was cloned by complementation (Bauchwitz and Holloman, 1990) and was found to contain a single uninterrupted open reading frame (ORF) of 2342 bp, which is transcribed to yield an unspliced message of 2.7 kb (Rubin *et al*., 1994). Using DNA sequence analysis it was determined that the *rec2-1* allele results from a 0.8 kbp

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deletion spanning the 5′ end of the gene that removes promoter elements and the proximal part of the coding sequence (Rubin *et al*., 1994). No other *rec2* allele was isolated in screens for DNA repair mutants, but the *rec2-197* allele was created at the time the *REC2* gene was cloned to provide proof that the putative gene isolated was indeed the structural gene and not a suppressor (Bauchwitz and Holloman, 1990). The allele was constructed using a one-step gene disruption procedure in which a DNA fragment within the *REC2* ORF was removed and replaced by an antibiotic resistance cassette expressing drug resistance (see Fig. 1). As the sequence deleted from the *REC2* ORF encompassed the region of sequence similarity with RecA and included the nucleotide binding P-loop, it was presumed that the *rec2-197* allele was a complete loss-of-function mutation.

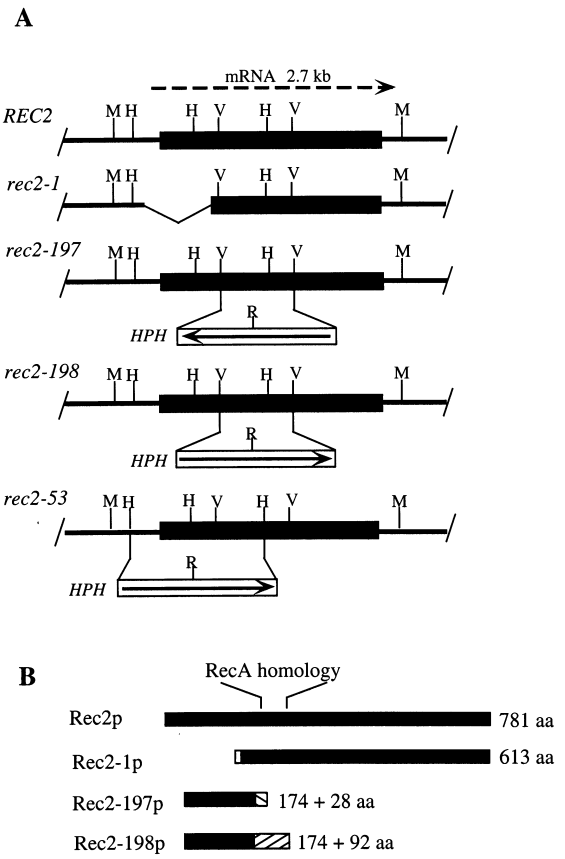


Fig. 1. *rec2* alleles and predicted gene product structures.
A. Genomic sequences are shown schematically for the *rec2* alleles with open reading frames indicated by the black boxes. The boundaries of the wild-type *REC2* mRNA are drawn approximately to scale in relation to the restriction map, as determined by S1 mapping. The drug resistance cassette containing the hygromycin phosphotransferase gene (*HPH*) is indicated by the open box. The directions of transcription are indicated by arrows. Restriction sites are abbreviated as follows: M, *Mse*I; H, *Hin*dIII; V, *Eco*RV; R, EcoRI.
B. Protein sequences and predicted chimeric proteins formed by the fusions of sequences in the *HPH* cassette are shown schematically with the residues encoded by the *REC2* open reading frame indicated by the black boxes. The small open box on the left in Rec2-1 is a stretch of 19 residues brought into frame by conjunction of the DNA sequences flanking the deletion underlying the *rec2-1* allele. Diagonal hatches schematically indicate the additional sequences completing the open reading frame in the fusions with the *HPH* cassette. The predicted sequences of the chimeric Rec2-197 and Rec2-198 proteins shown schematically have the following strings of amino acids fused to the 174 N-terminal residues of Rec2: Rec2-197, STNSQSSIVNVNHEYFTQLASRRFGGIQ; Rec2–198, SSGGRCESCATKRESTRAGRNQKKPPPRCQVDKSTH LFQVQIGKSKIRARSAIRNGNRVTEVAKSSXKWKKN VVTTSEFCKLQKKNLGTDEX.

However, additional considerations raised the question whether either allele was a true null. More careful Northern blot analysis revealed that a truncated message was present in *rec2-1,* presumably through enlistment of a cryptic promoter (Rubin *et al*., 1994). Inspection of the *rec2-1* DNA sequence on both sides of the deletion indicated that a novel ORF could be generated through conjunction of the flanking sequences. This ORF would be predicted to encode a 613 amino acid Rec2 protein variant with a novel 19-residue leader sequence derived from upstream of the deletion in lieu of the N-terminal 187-residue sequence of the wild-type Rec2 protein. As the deletion would remove the putative nuclear localization signal but would not impinge upon the region of RecA homology including the ATP binding loop, it seemed possible that an active protein might be produced, but would perhaps be crippled in its function by diminished nuclear import. Efforts were made using Western blot analysis to detect a Rec2 variant in *rec2-1* using antibodies raised to recombinant Rec2 protein, but no signal was detected. Nevertheless, we could not rule out the possibility that a variant protein was produced below the level of detection. Furthermore, it became clear in the studies described below that the *rec2-197* allele retained some biological activity in spite of its deletion of the region related to RecA and is partially dominant, unlike the *rec2-1* allele.

Therefore, we analysed the *rec2* locus in more detail, characterizing additional deletion mutants in an effort to establish the complete loss-of-function phenotype. Through our analysis it has become clear that, apart from DNA repair and recombination, there are unappreciated roles for the *REC2* gene in chromosome segregation and mutation avoidance. These results establish that *REC2* is at the intersection of several cellular processes that serve to repair and maintain the integrity of the genome.

Results

Response of rec2-197 *to DNA clastogens differs from* rec2-1

rec2-197 (Table 1) was confirmed as sensitive to UV and was killed with kinetics almost identical to *rec2-1* (Fig. 2A).

Table 1. *Ustilago maydis* strains.

	rec2 allele	genotype[a]
Haploids		
UCM5	*REC2*	*ade1-1 leu1-1 a2b2*
UCM54	*rec2-1*	*rec2-1 pan1-1 nar1-1 a1b1*
UCM156	*rec2-198*	*rec2-198::HPH ade1-1 leu1-1 a2b2*
UCM164	*rec2-197*	*rec2-197::HPH ade1-1 leu1-1 a2b2*
UCM342	*REC2*	*rad51-1::HPH ade1-1 leu1-1 a2b2*
UCM350	*REC2*	*pan1-1 nar1-6 a1b1*
UCM482	*rec2-53*	*rec2-53::HPH pan1-1 nar1-6 a1b1*
UCM513	*rec2-197*	*rec2−197::HPH pan1-1 nar1-6 a1b1*
Diploids		
UCM33	*REC2/REC2*	*ade1-1/+ pan1-1/+ nar1-1/+ met1-2/+*
UCM110	*rec2-1/rec2-1*	*rec2-1/rec2-1 pan1-1/+ nic1-1/+ ade1-1/+ met1-2/+ ino1-4/ino1-5*
UCM147	*REC2/rec2-1*	*rec2-1/+ ade1-1/+ leu1-1/+ pan1-1/+ nar1-1/+*
UCM479	*REC2/rec2-197*	*rec2-197::HPH/+ ade1-1/+ leu1-1/+ pan1-1/+ nar1-6/+*
UCM480	*rec2-1/rec2-197*	*rec2-197::HPH/rec2-1 ade1-1/+ leu1-1/+ pan1-1/+ nar1-1/+*
UCM515	*rec2-197/rec2-197*	*rec2-197::HPH/rec2-197::HPH ade1-1/+ leu1-1/+ pan1-1/+ nar1-1/+*

a. Terminology for disruptions is indicated by the *::* for insertion of the *HPH* gene cassette.

As the damage produced in DNA by UV irradiation with 254 nm light is limited mainly to cyclobutane pyrimidine dimers and pyrimidine–pyrimidone (6–4) lesions (Friedberg *et al*., 1995), we were interested in comparing the response of *rec2-197* and *rec2-1* with other types of clastogenic agents to learn if there was any difference in the damage-processing pathways (Sancar, 1996). We measured survival after treatment using the alkylating agent methyl methanesulphonate (MMS), which produces DNA strand breaks following excision of alkylated bases (Singer and Kusmierek, 1982), and bleomycin, a free radical-generating agent that induces both single-strand and double-strand DNA breaks by reactive oxygen through Fenton reaction chemistry (Takeshita *et al*., 1978). Survival kinetics of *rec2-197* and *rec2-1* were similar when treated with MMS (Fig. 2B). However, their sensitivities to bleomycin were markedly different; *rec2-1* was extremely sensitive while *rec2-197* was resistant at a level almost approaching a wild-type control (Fig. 2C). These results suggest that Rec2 could function in different pathways for repair of DNA damage depending on the type of damage, but that its role in each pathway might not necessarily be equivalent.

Rec2-197 *has elevated spontaneous mutator activity*

In *S. cerevisiae*, inactivation of the recombinational repair pathway by mutation in *RAD51* or *RAD52* results in an elevated spontaneous mutator activity (Morrison and Hastings, 1979; Prakash *et al*., 1980; Malone and Esposito, 1981). Presumably, lesions arising in DNA that are normally processed by recombinational repair are shunted into an error-prone repair pathway in the absence of recombination. Similarly, we have noted a very modest elevation in spontaneous mutation rate in both the *U. maydis rad51-1* and *rec2-1* mutants, easily quantified by measuring forward mutation to drug resistance using any one of a number of metabolic inhibitors or antibiotics. We utilized the drug, nourseothricin, a streptothricin-related polyamine antibiotic that interferes with translation via direct binding to ribosomes (Haupt *et al*., 1978; Cundliffe, 1989). Measuring spontaneous mutation by forward resistance to this compound is convenient as backgrounds are clean and identification of resistant mutants is unambiguous.

As determined by fluctuation analysis, the rate of mutation to nourseothricin resistance (Nst^R) was 1.25×10^{-8} per cell per generation in the wild type (Table 2). In *rec2-1* the rate was elevated almost 10-fold, but in *rec2-197* it was higher by more than two orders of magnitude. As *rec2-197* expresses resistance to hygromycin, we were concerned that the Nst^R phenotype was artefactual and was simply a consequence of overlapping resistance to hygromycin. Therefore, as a control we measured mutation to Nst^R in the *rad51-1* mutant, which was created by the same gene disruption procedure used to generate *rec2-197*, i.e. by replacing a fragment within the ORF with the identical *HPH* (for hygromycin phosphotransferase) cassette expressing hygromycin resistance. In this case, the mutation rate was also elevated over the wild type, but only to the modest 10-fold extent observed in *rec2-1*. Therefore, it appears that resistance to hygromycin does not concomitantly confer, overlap or cross-feed resistance to nourseothricin.

To learn whether the mutator activity was indeed associated with the *rec2-197* allele and not as a result of some other adventitious, unrelated mutation introduced at the time the gene disruption was constructed, we measured mutator activity in *rec2-198*, an allele identical to *rec2-197* except with the *HPH* cassette expressing

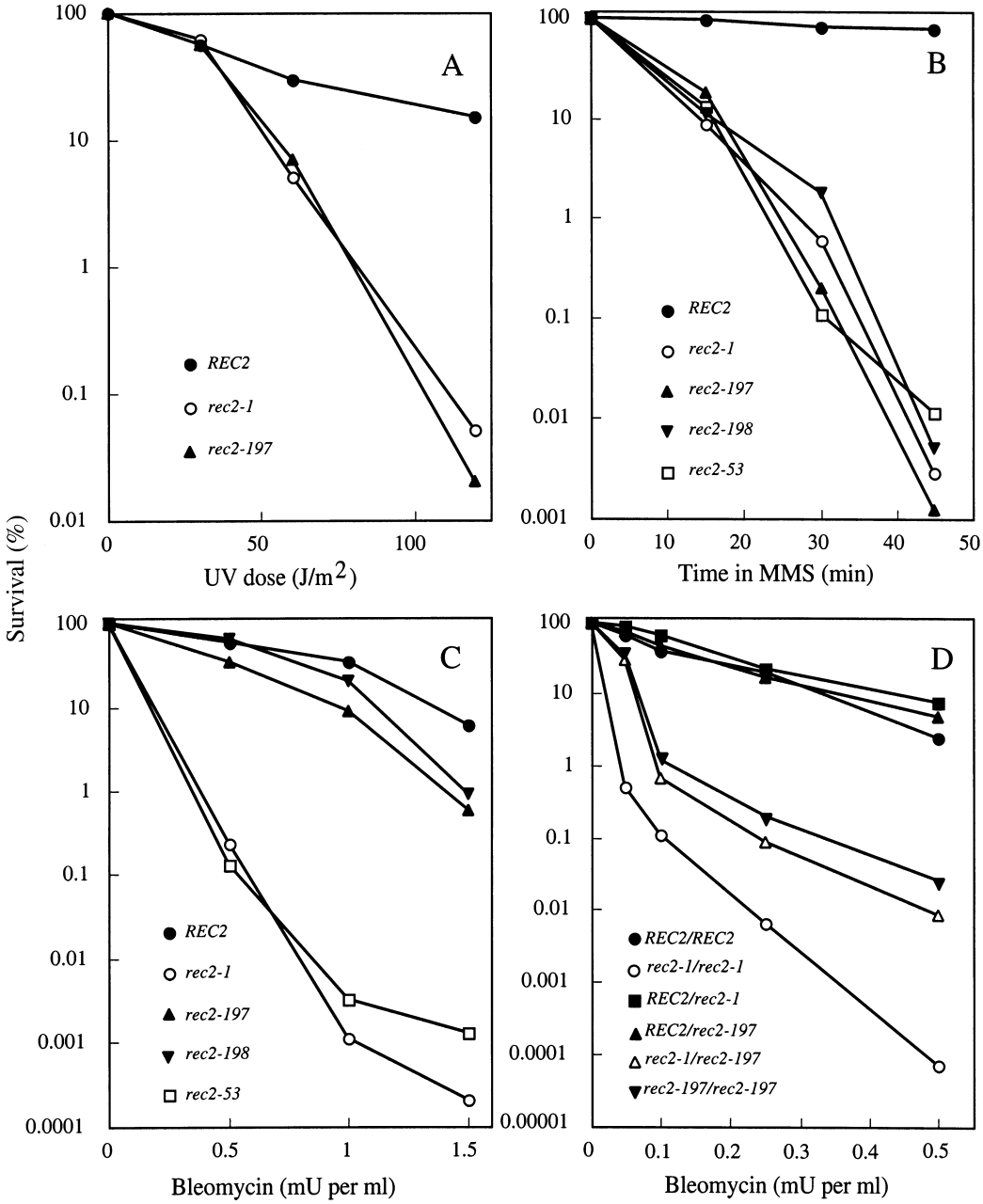


Fig. 2. Survival of *rec2* mutants after treatment with DNA clastogens.
A. Logarithmic cell cultures were serially diluted and plated on YEPS medium and then irradiated with UV light.
B. Or cell cultures were held in 0.02% MMS for the indicated times, then plated.
C. and D. Or cell cultures were plated on medium containing the indicated amounts of bleomycin.

resistance to hygromycin inserted the opposite orientation (Fig. 1). As in *rec2-197*, the spontaneous mutation rate to Nst^R in *rec2-198* was correspondingly high (Table 2). These results indicate that the elevated Nst^R mutator phenotype of *rec2-197* is a particular property of the disrupted gene structure.

As an additional control to test the generality of the mutator effects noted, we measured mutation to 5-fluoro-orotic acid resistance ($5\text{-}FOA^R$). This drug is an analogue of orotic acid, a precursor in the biosynthetic pathway leading to uracil. It inhibits the growth of wild-type cells and permits the growth of cells with a mutation in the gene for orotidine monophosphate decarboxylase or orotidine monophosphate pyrophosphatase (Boeke *et al.*, 1984). Thus, it has a different pharmacological basis of action compared with nourseothricin. In haploids, rates of $5\text{-}FOA^R$ followed the same general pattern as with Nst^R, although they were not as extremely elevated in *rec2-197*

Table 2. Spontaneous forward mutation rates.[a]

Strain	Nourseothricin resistance		5-fluoroorotic acid resistance	
	Rate $\times 10^{-8}$	Fold increase	Rate $\times 10^{-9}$	Fold increase
Haploids				
REC2	1.3 ± 0.1	1.0	4.5 ± 0.7	1.0
rec2-1	11.3 ± 2.6	9.4	28.7 ± 7.6	6.4
rec2-197	195 ± 25	198	58.2 ± 20	13
rec2-198	290 ± 30	232	180 ± 30	40
rec2-53	16.5 ± 6.5	13.2	23.0 ± 2.5	5.1
rad51-1	7.0 ± 3.6	11.4	29.7 ± 9.5	6.6
Diploids				
REC2/REC2	1.6 ± 1.1	1.0	< 0.1	1.0
rec2-1/rec2-1	14.0 ± 3.0	8.8	ND[b]	–
REC2/rec2-1	2.7 ± 0.9	1.7	ND	–
REC2/rec2-197	91.5 ± 34	57	ND	–
rec2-1/rec2-197	1105 ± 475	690	1.0	> 10
rec2-197/rec2-197	4500 ± 500	2800	0.8	> 10

a. Results of at least two independent determinations are presented.
b. ND, not determined

and *rec2-198*. As the two drugs have different molecular targets it is not surprising that the rates of resistance were different. The very high levels of Nst^R compared with 5-FOA^R could be as a result of a greater number of targets and, possibly, an amplification effect resulting from increased misreading of message (Haupt *et al*., 1978; Cundliffe, 1989). Resistance to 5-FOA is recessive (Boeke *et al*., 1984) and in *REC2* homozygous diploids was at a rate lower than the sensitivity of detection by our assay ($< 10^{-10}$ per cell per generation). We note, however, that in the heteroallelic diploid *rec2-1/rec2-197* and the homozygous diploid *rec2-197/rec2-197,* 5-FOA-resistant mutants were formed at a rate within the detectable range, 1×10^{-9} per cell per generation. This is probably several orders of magnitude higher than in the *REC2* homozygous diploid, which might reasonably be guessed as $\approx 10^{-13}$ to 10^{-14} per cell per generation, the product of the mutation rate (1×10^{-9}) times an estimated mitotic recombination rate ($\approx 10^{-4}$ to 10^{-5}) (Esposito and Bruschi, 1993).

Meiotic chromosome segregation is disturbed by rec2-197

Intending to establish a firmer relationship between the strong mutator activity and DNA repair deficiency, we performed crosses with *rec2-197 and* wild-type tester strains to determine whether these traits were indeed linked. We also intended to obtain *rec2-197* in the opposite mating-type background so that homozygous crosses could be performed. However, it became immediately clear in test crosses that meiosis was disturbed. In a control (Table 3, Cross 1) the *rec2-1* allele segregated 1:1 when the MMS sensitivity of meiotic progeny was monitored. However, in the *rec2-197* cross (Table 3, Cross 2), there was a strong bias towards recovery of only MMS^R progeny and a high frequency of appearance of MMS^R Hyg^R segregants, an illegitimate class. These observations indicate that the *rec2-197* allele did not segregate independently and imply that, in the majority of progeny, the *rec2* locus was heterozygous. This finding

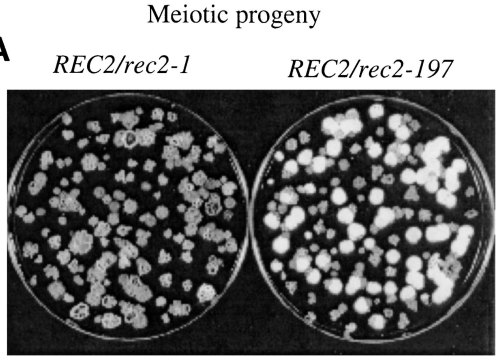
Table 3. Segregation during meiosis.

Cross 1 UCM5 × UCM54 ($MMS^R \times MMS^S$)	*REC2* × *rec2-1*	
$n = 102$	MMS^R	MMS^S
Expected	51	51
Observed	49	53

Cross 2 UCM350 × UCM164 (MMS^R $Hyg^S \times MMS^S$ Hyg^R)	*REC2* × *rec2-197*		
$n = 148$	MMS^R Hyg^S	MMS^S Hyg^R	MMS^R Hyg^R
Expected	74	74	0
Observed	64	9	75

suggested that *rec2-197* causes a chromosome segregation defect.

To measure the generality of this effect, we plated meiotic progeny on non-selective medium containing charcoal. Typically, this black medium can be used in a quick test to identify diploids that are easily seen as mycelial colonies which appear white and fuzzy (Banuett and Herskowitz, 1989), while haploids look smooth and translucent (Fig. 3A). In a control cross with *rec2-1* mated to the wild-type tester strain, 0.51% of the colonies appeared positive in this fuzz reaction indicating that haploids issued normally from this cross. However, in the *rec2-197* cross, 38.5% of the isolated progeny were fuzz positive (Fig. 3B). Strictly speaking, the fuzz reaction is not a true test for diploidy, but is positive when the *b* mating type loci are present as a pair of heteroalleles (Kronstad and Leong, 1989). Thus, it does not measure the global chromosomal state of ploidy, but it is probable that the fuzz-positive progeny were at least disomic for the chromosome containing the *b* locus. Among the non-fuzz progeny, 67% were of the illegitimate MMS^R Hyg^R class, indicating heterozygosity at the *rec2* locus. These results reinforce the notion that chromosome segregation in *rec2-197* crosses is abnormal and the appearance of true haploids among the progeny is rare.



Fuzz reaction

B

Pairwise alleles	Strain	Fuzz positive (%)
REC2/REC2	UCM33	1.1
REC2/REC2	UCM350 x UCM5	3.6
REC2/rec2-1	UCM350 x UCM54	0.5
REC2/rec2-197	UCM350 x UCM164	38.5
REC2/rec2-53	UCM350 x UCM482	3.2

Fig. 3. Fuzz reaction in meiotic progeny. Teliospores were obtained from gall tissue of plants infected with diploids constructed by mating the indicated strains. After isolation, teliospores were treated with ether and germinated on YEPS. When microcolonies became visible to the eye after incubation for 24 h, plates were scraped, colonies resuspended in water and dispersed to single cells by vigorous vortexing, then spread for isolation onto plates containing charcoal medium. Plates were incubated at room temperature under fluorescent lights for 3 days.
A. Positive fuzz reaction of meiotic products from the indicated crosses is evident by the white colonies.
B. The yields of fuzz-positive meiotic products from the indicated matings are indicated quantitatively.

As a final method to assess the influence of *rec2-197* on the fidelity of chromosome segregation, we scored prototrophs among the meiotic progeny from the heterozygous cross UCM350 × UCM164 (Table 3, Cross 2) as a simple measure for assortment of auxotrophic markers. As there were four segregating auxotrophic markers in the cross, *pan1, nar1, ade1* and *leu1*, the frequency of prototrophy above that predicted for randomly segregating markers, 0.5^4 or 6.2%, could be taken as a measure of chromosome missegregation. However, as *ade1-1* and *leu1-1* are loosely linked (Holliday, 1961), the fraction of prototrophs among the progeny would be expected to be between 0.5^4 and 0.5^3, or between 6.2% and 12.5%. In the control mating with *rec2-1* (UCM5 × UCM54, Table 3, Cross 1), we found 13% of prototrophs among the meiotic progeny, a frequency close to that expected. But from the heterozygous cross with *rec2-197*, the frequency of prototrophs was 60%. Thus, approximately half the meiotic progeny were heterozygous for auxotrophic markers according to this method. In conclusion, it appears that in heterozygous crosses with the *rec2-197* allele there is a global disturbance in chromosome segregation with aneuploids probably representing the majority of meiotic products.

Teliospores homozygous for rec2-197 *fail to germinate*

Teliospores form as end-products of a differentiation process following the systemic spread of the infectious dikaryotic hyphae through maize plants (Banuett and Herskowitz, 1996). In *U. maydis*, little information is available about the timing of meiosis with respect to teliospore development, but it is known that teliospores germinate at late prophase I (O'Donnell and McLaughlin, 1984). By inference, this event would place the initiation of recombination at a temporal point earlier in the teliospore maturation process and would suggest that teliospores are arrested in meiosis I. In crosses homozygous for *rec2-1*, production of teliospores appears normal. However, these teliospores fail to germinate and, thus, do not give rise to haploid meiotic products (Holliday, 1967). This abortive meiosis is presumably as a result of failure in repairing the DNA strand breaks initiating recombination and concomitant activation of a DNA-damage checkpoint (Grushcow *et al*., 1999) barring completion of meiosis I. As noted above, in crosses heterozygous for *REC2* and *rec2-197*, we observed that teliospore production and germination appeared normal, but that chromosome segregation was aberrant. However, similar to the case with *rec2-1* homozygous crosses, teliospores were formed but no

germination was observed in *rec2-1/rec2-197* heterozygous crosses or *rec2-197* homozygous crosses.

Analysis of diploids indicates dominant negative interactions with rec2-197

Certain features of the *rec2-197* phenotype were revealed in the heterozygous situation. Spontaneous mutator activity to Nst^R of the *rec2-197/REC2* heterozygous diploid was elevated about 60-fold higher than in the *REC2* homozygous diploid (Table 2). This indicates that *rec2-197* exerts a dominant negative effect in the presence of the wild-type allele. In the *rec2-197/rec2-1* heteroallelic diploid and the *rec2-197/rec2-197* homozygous diploid the rate was 10–40 fold higher still. Thus, the activity in the heterozygous situation with respect to *REC2* is only partially dominant compared with the situation in which no *REC2* gene product is present. Attenuation of bleomycin sensitivity was also evident in the *rec2-197/rec2-1* and *rec2-197/rec2-197* diploids compared with the *rec2-1* homozygous diploid, although not to the extent as observed with the *rec2-197* allele in the haploid (Fig. 2D). This might be as a result of some diploid-specific effect in which the overall threshold of resistance to bleomycin was lowered because all diploids tested, including the *REC2* homozygote, were found to be more sensitive to bleomycin than haploids. In any event, the dominant interactions suggest that a diffusible product is probably made from the *rec2-197* allele and this product interferes with certain functions promoted by *REC2*.

Chimaeric Rec2 fusion proteins are created by disruption of REC2

The weight of the evidence demonstrating significant phenotypic differences between *rec2-197* and *rec2-1* in DNA repair proficiency, mutator activity and meiotic chromosome segregation made it clear that *rec2-197* was not devoid of genetic activity as was assumed earlier. Given the nature of the *rec2-197* gene structure, a probable basis for the apparent genetic activity is that the allele manifests through expression of an interacting domain present on the N-terminal peptide fragment from the remnant *REC2* ORF upstream of *HPH*, the hygromycin resistance gene cassette. The deletion created to generate *rec2-197* leaves enough coding sequence at the 5′ end of the *REC2* ORF to encode a polypeptide of 174 N-terminal amino acids of Rec2. This region includes the putative nuclear localization motif and a run of acidic residues. As the reading frame is incomplete, we determined the DNA sequence across the site of the junction at which the disruption was formed to define the boundary of the ORF and establish the structure of the translational fusion. Sequence analysis of the 1.6 kbp *Hin*dIII–*Eco*RI fragment spanning the junction (Fig. 1) indicated that an additional 28 amino acids were added before a stop codon was brought into frame, but no sequence motifs that might provide insight into function were present.

We considered whether the *rec2-197* phenotype was a specific consequence of this particular chimaeric fusion or perhaps the more general condition resulting from expression of a fusion polypeptide containing the 174 N-terminal residues. The latter possibility seemed more probable in view of the elevated mutator activity associated with the *rec2-198* allele noted above (Table 2), and in light of the similarity of *rec2-198* to *rec2-197* in resistance to bleomycin and sensitivity to MMS (Fig. 2). As mentioned above, *rec2-198* was constructed in the identical way to *rec2-197* but with the *HPH* cassette in the opposite orientation. This construction leaves the coding information for the 174 N-terminal Rec2 residues intact but brings into register an altogether different sequence from the complementary strand of the *HPH* cassette to complete the ORF (Fig. 1). The DNA sequence of this junction region indicated that *rec2-198* could encode a chimaeric Rec2 fusion protein with a heterologous tail of 92 amino acids. BLAST analysis indicated that there was no similarity between the 28- and 92-residue polypeptide tails or any recognizable conserved domain present in either polypeptide sequence, as determined by RPS (reverse position-specific) BLAST. Thus, the virtually identical phenotypes of *rec2-197* and *rec2-198* appear to result from expression of the 174 N-terminal Rec2 residues in fusion with an apparently random C-terminal sequence and not from a specific chimaeric protein created by completing the ORF with a particular heterologous sequence.

rec2 *deletion lacking the N-terminal sequence and nucleotide binding P-loop*

As it was uncertain whether the *rec2-1* allele retained some biological activity, we constructed a new deletion designed to represent a true null allele. Ideally, we had wanted to remove the entire *REC2* ORF, but this proved to be problematical using the traditional one-step gene disruption procedure owing to a paucity of appropriate restriction enzyme sites at the 3′ end of the ORF and the close presence of an adjacent essential gene. We have attempted alternative knockout procedures such as polymerase chain reaction (PCR)-mediated disruption techniques reported to be effective in yeasts (e.g. Baudin *et al*., 1993; Kaur *et al*., 1997), but unfortunately have not yet been successful adapting such methods in *U. maydis*. Therefore, we used the traditional one-step disruption procedure to make a large deletion, removing a segment of the upstream untranslated region containing putative

promoter elements identified previously (Rubin *et al*., 1994) and approximately half the ORF encompassing the translational start site, the sequence encoding N-terminal protein region present in *rec2-197* and the region of homology with RecA protein, including the Walker A box known to be essential for recombination and repair proficiency (Rubin *et al*., 1994). The construction of the deletion was accomplished by replacing a sequence spanning these elements with the *HPH* cassette expressing Hyg^R (Fig. 1). The resulting allele, *rec2-53*, was very sensitive to killing by MMS and bleomycin (Fig. 2C), displayed only a slightly elevated mutation rate to Nst^R (Table 2) and appeared normal in meiotic chromosome segregation in a heterozygous cross with *REC2*, as determined by the fuzz assay (Fig. 3B). In these regards, the phenotypes of *rec2-53* and *rec2-1* were virtually identical, leading us to conclude that *rec2-1* is probably a null allele.

Yeast two-hybrid analysis suggests rec2-197 *encodes a protein interacting domain*

As the dominant negative phenotype of *rec2-197* was suggestive of interaction between Rec2 and an additional partner or partners, we initiated efforts to identify putative interactors by yeast two-hybrid screening. Our strategy was to express the Rec2-197 polypeptide as a fusion with the Gal4 DNA-binding domain to capture interacting proteins. Unfortunately, this fusion by itself had a low level of transactivator activity that was significant enough to interfere with the planned screen and, as such, was considered impracticable. Alternatively, the full-length Rec2 protein fused to the Gal4 DNA-binding domain had an acceptably low level of transactivator activity. Therefore, we decided to express the full-length Rec2 protein as bait, capture potential interactors and test promising candidates with the Rec2-197 fusion. A level of transactivation higher than the basal level exhibited by expression of Rec2-197 alone could then be interpreted as an indication of a specific interaction.

We performed two-hybrid screening using a cDNA library prepared from DNA-damaged induced cells. Approximately 2×10^6 independent co-transformants containing both bait and prey plasmids were screened, yielding 184 colonies with robust and reproducible growth when tested on medium selective for interaction. Interactor plasmids were rescued from 108 individual isolates and the DNA sequence was determined from 46 of these. Of known DNA repair genes, we identified *RAD51*, which had been previously cloned and determined by genetic analysis to interact in an epistatic fashion with *REC2* in repair of DNA damage induced by ionizing radiation and chemical alkylation (Ferguson *et al*., 1997). As we had previously characterized *RAD51* in some detail genetically, we decided it would be an informative candidate to investigate for interactions with *rec2-197*. Therefore, we performed quantifiable measurements of β-galactosidase in extracts of yeast strains co-transformed with plasmids containing Rad51 fused to the Gal4-transactivator domain and Rec2 or Rec2-197 fused to the Gal4-DNA binding domain (Table 4). Assays indicated that interaction between Rad51 and the Rec2-197 protein was as strong as with the full-length Rec2 protein. These results further support the genetic evidence that a specific protein-interaction domain is present within the N-terminal Rec2 sequence encoded by the *rec2-197* allele.

Discussion

In this study, we have established through several lines of investigation that there is some residual biological activity associated with the *rec2-197* allele, a deletion created by replacing 0.8 kbp of the *REC2* ORF with a 2.0 kbp cassette expressing antibiotic resistance. Evidence supporting this conclusion includes the resistance of *rec2-197* to killing by bleomycin, the high spontaneous mutator activity associated with the allele and the aberrant meiosis observed in genetic crosses. The dominant features of the *rec2-197* allele in disturbing meiotic chromosome segregation, elevating spontaneous mutation and attenuating bleomycin sensitivity in the heterozygous diploid suggest a diffusible factor is expressed that interferes with the normal processes governed by *REC2*. The probable identity of this factor is a chimaeric polypeptide composed of the N-terminal 174 residues of Rec2 protein and a C-terminal run of from 28 to 92 amino acids of apparently random sequence.

The Rec2 protein is a polypeptide of 781 amino acids with an internal stretch of about 50 residues encompassing the nucleotide-binding P-loop highly similar in

Table 4. Yeast two-hybrid analysis of Rad51 interaction.

DNA binding domain	–	–	Rec2	Rec2-197	Rec2	Rec2-197	p53
Activation domain	–	Rad51	–	–	Rad51	Rad51	T antigen
Specific activity[a]	0.9 ± 0.05	0.9 ± 0.2	1.5 ± 0.2	6.8 ± 3.4	26 ± 5.4	33 ± 3.2	66 ± 12

a. β-galactosidase activity, nmol hydrolysed min^{-1} per 10^7 cells. Values represent the means of five to eight separate determinations on individual co-transformants. Fusions with p53 and SV40 T antigen were included as a positive control.

sequence to a corresponding region in RecA and Rad51 protein family members. The N-terminal sequence of 174 amino acids that remains in the *rec2-197* allele shares no significant sequence similarity with any other sequence in the databases. However, it does contain a nuclear localization sequence motif and an acidic stretch in which 11 of 12 residues are aspartate. The *rec2-1* allele is missing the coding information for this N-terminal stretch plus only an additional 13 residues. The phenotypic differences between *rec2-1* and *rec2-197* appear to reflect the consequence of expressing the protein domain contained in this short polypeptide stretch.

The pleiotrophic nature of the *rec2-197* phenotype probably represents manifestations of aberrant *REC2* gene function in several pathways dedicated to the repair, transmission and maintenance of genome stability. These pathways appear to intersect at a junction with *REC2*, providing an important role in governing how DNA lesions are channelled. Processing could depend on the nature of the lesion and the operational capacity of the pathways available. A simple model to account for the residual activity and dominant negative features of *rec2-197* holds that Rec2 protein has different functional domains, including one contained in the N-terminal remnant present in *rec2-197,* and that its role in recombinational repair can be directed by additional protein partners interacting through the different domains. In certain instances, interaction of a partner protein with the N-terminal Rec2 domain alone might be sufficient to enable a recombinational repair event or else disable the event through a negative interaction. In other instances, Rec2 might be envisioned to act sequentially with a partner in a pathway but independent of any intermolecular interaction.

In light of the current findings with the yeast two-hybrid system and in view of the previous genetic analysis (Ferguson *et al*., 1997), it appears that Rad51 is an interacting partner of Rec2. Such a scenario as that outlined above featuring interaction of Rec2 with Rad51 as a partner might account for the differential sensitivities to DNA clastogens. Rec2 and Rad51 are both required for survival after cellular DNA has received heavy damage from radiation or reactive chemicals, and epistasis analysis (Ferguson *et al*., 1997) indicates that the genes function in a common pathway in repairing damage. One could imagine that repair takes place by a mechanism in which Rec2 and Rad51 act in procession during the channelling of DNA lesions through a pathway dedicated to survival. In the case of repairing UV-induced photoproducts or damage resulting from chemical alkylation, repair requires Rad51 and Rec2 acting independently. But in the case of bleomycin damage that results from free radical damage, requirements for repair can be satisfied by Rad51 alone as long as there is a residual Rec2 domain to interact with and possibly activate Rad51.

A similar mechanism, but with opposite effect, could explain the observed meiotic chromosome segregation defect. Faithful segregation of homologues at meiosis I is dependent on recombination (Baker and Carpenter, 1972; Malone and Esposito, 1981). In *S. cerevisiae*, meiosis-specific double-strand DNA breaks generated by the action of Spo11 are processed by Rad51 and Dmc1 to yield strand exchange intermediates (Shinohara and Ogawa, 1999). Cross-overs associated with reciprocal exchange of chromosome arms are needed for faithful segregation of homologues (Kleckner, 1996; Roeder, 1997). Even minor disturbances in the level of crossing over can have massive effects on chromosome disjunction. For instance, in *msh4* or *msh5* crossing over is reduced only about twofold, but chromosome non-disjunction is greatly elevated (Ross-Macdonald and Roeder, 1994; Hollingsworth *et al*., 1995). In mutants with more substantial effects on recombination, such as *rad51* or *dmc1*, meiosis is not completed owing to activation of a meiotic cell cycle checkpoint induced by failure to repair double-strand DNA breaks (Grushcow *et al*., 1999). Similarly, in *U. maydis* teliospores are formed in the *rec2-1* mutant but these do not complete meiosis to yield haploid products because they are deficient in recombination (Holliday, 1967). In the case of the *rec2-197* heterozygous cross, the high frequency of aneuploidy among the meiotic progeny probably indicates that crossing over can proceed to some extent through the action of Rec2 produced from the wild-type allele, but is partially disabled by interference from the Rec2-197 polypeptide. Rad51 could well be an interacting partner poisoned by the domain present in the Rec2-197 protein.

The effect of *rec2-197* on spontaneous mutation is probably a reflection of an overlap between recombinational repair and error-prone repair processes. While recombination is traditionally regarded as being a high-fidelity process, it has been recognized for a number of years that mitotic recombination is associated with elevated mutation (Esposito and Bruschi, 1993). In *S. cerevisiae*, repair of double-strand DNA breaks during mitosis is associated with elevated mutation in the vicinity of the break (Strathern *et al*., 1995). The *RAD52* epistasis group of genes is needed for recombinational repair of the break, and the *trans* lesion bypass gene *REV3*, which encodes a subunit of an error-prone DNA polymerase, is required for the observed induced mutation (Holbeck and Strathern, 1997). The elevated spontaneous mutation we observe associated with the *rec2-197* allele probably reflects a disturbance in the interaction between the *REC2*-recombinational repair pathway and the lesion bypass machinery. Lesions arising during mitotic growth,

such as those that might be associated with DNA replication fork collapse or regression, could be channelled through a recombinational pathway that utilizes the error-prone polymerase system for completing repair. Direct interaction between the Rec2-197 domain and a component of the polymerase holoenzyme, as envisioned in the model above, or an alteration in the level or nature of the lesions feeding into the *trans* lesion system by disabling the recombination repair machinery could result in the elevated mutation level.

We conclude that the N-terminal region of Rec2 contains an interactive domain that harnesses the recombinational activity of the protein to several biological pathways. The interactive nature of this domain suggests an explanation for the genesis of the unusual UV-induced mutation responsible for the *rec2-1* allele. Namely, removal of the entire coding sequence for that domain has fewer deleterious biological consequences than a mutation that would lead to expression of an intact protein, but with the potential for dominant negative interactions. We hope to define the Rec2-directed pathways in more detail using the *rec2-197* allele as a tool to probe some of the novel interacting genes identified in the two-hybrid screen.

Experimental procedures

Plasmids

pCM158 has a 9.5 kbp genomic DNA fragment containing the *REC2* gene inserted into the *Bam*HI site of the shuttle vector pCM54 (Bauchwitz and Holloman, 1990). pCM166 is a derivative of pBluescript II (Stratagene) with a 5.0 kbp *Ssp*I–*Bam*HI fragment containing the *REC2* gene from pCM158 inserted into the *Eco*RI site after blunting the ends. pCM197 and pCM198 were prepared by inserting a 2.0 kbp cassette containing *HPH,* an *hsp70*-hygromycin phosphotransferase gene fusion expressing Hyg^R derived from pHL1 (Wang *et al*., 1988), in place of the 0.8 kbp *Eco*RV fragment of pCM166. The orientation of the *HPH* gene in pCM197 is in the opposite direction to *REC2* and in pCM198 is in the same direction to *REC2*. pCM610 is a derivative of pUC19 with a 2.0 kbp *Eco*RI–*Sma*I fragment containing a carboxin resistance gene derived from pAHB5 (Bottin *et al*., 1996). pCM709 is a derivative of pCM610 with a 9.0 kbp fragment containing the *REC2* gene from pCM158. pCM808 is a derivative of pCM709 with the 2.0 kbp *HPH* cassette inserted in place of the 1.5 kbp sequence of two contiguous *Hin*dIII fragments overlapping the 5′ end of the *REC2* open reading frame (ORF). The *REC2* ORF was moved as a 2.4 kbp *Nde*I–*Bam*HI fragment derived from pCM349 (Kmiec *et al*., 1994) into pACT2 (Clontech Laboratories) to form pCM596, and into pAS2-1 (Clontech Laboratories) to form pCM597. This latter construction fuses the entire *REC2* ORF with the *GAL4* DNA binding domain forming the bait plasmid in the yeast two-hybrid screen. For domain mapping of Rec2-197 by two-hybrid analysis, the *rec2-197* allele was introduced into the bait plasmid pAS2-1. This was performed in two steps. Initially, the 2.4 kbp *Nde*I–*Bam*HI sequence containing the *REC2* ORF in pCM596 was excised in a 2.4 kbp *Bgl*II fragment (thus preserving the *Nde*I and *Bam*HI sites) and inserted into the *Bam*HI site of pUC19 to yield pCM706. The 1.4 kbp *Msc*I–*Mlu*I fragment was removed from within the *REC2* sequence of pCM706 and replaced with a 2.6 kbp *Msc*I–*Mlu*I fragment spanning the sequence of the *rec2-197* allele disrupted by the *HPH* gene from pCM197 to yield pCM856. The *rec2-197* allele was then moved from pCM856 as a 3.6 kbp *Nde*I–*Bam*HI fragment into pAS2-1, fusing the allele in frame with the *GAL4* binding domain to form pCM860. Plasmid pCM580, which is a pBluescript II SK^+ derivative containing the *RAD51* gene on a 1.1 kbp DNA fragment (Ferguson *et al*., 1997), was used as a template for modifying the gene using a polymerase chain reaction (PCR) procedure to introduce an *Nde*I site at the start of the ORF. The *RAD51* gene was then excised as a 1.1 kbp *Nde*I–*Xma*I fragment and inserted into pACT2 to form an in frame fusion with the *GAL4* activator domain, yielding pCM598.

U. maydis *strains and methods*

Preparation of media and procedures for diploid construction, meiotic analyses, gene disruptions and other manipulations were carried out as described previously (Holliday, 1974; Fotheringham and Holloman, 1989; 1990; Kojic and Holloman, 2000). Genotypes and *rec2* alleles of strains used in this study are listed in Table 1. Haploid strain UCM164 was constructed by one-step disruption using plasmid pCM197 as described previously (Bauchwitz and Holloman, 1990), such that the *REC2* allele in UCM5 was replaced by *rec2-197*. UCM156 containing the *rec2-198* allele was constructed in the same way using pCM198. UCM342 contains the *rad51-1* allele constructed by one-step gene disruption as described previously (Ferguson *et al*., 1997). UCM482 contains the *rec2-53* allele and was constructed by one-step gene disruption of UCM350 using pCM808 by selecting for Hyg^R transformants and screening among these for carboxin sensitivity. UCM513 contains the *rec2-197* allele and was constructed in a similar way to UCM164, but by disrupting the *REC2* gene in UCM350, a host strain of different mating type. Diploid strain UCM147 *(REC2/rec2-1)* was constructed by mating UCM5 *(REC2 ade1-1 leu1-1 a1b1)* with UCM54 *(rec2-1 pan1-1 a2b2)*. Diploid strains UCM479 (*REC2/rec2-197*), UCM480 (*rec2-1/rec2-197*) and UCM515 (*rec2-197/rec2-197*) were constructed by mating UCM164 with UCM350, UCM54 and UCM513 respectively. *ade, ino, leu, met, nic, pan, nar, a* and *b* refer to requirements for adenine, inositol, leucine, methionine, nicotinic acid, pantothenic acid, inability to utilize nitrate and mating type loci respectively.

DNA repair proficiency

Cultures were grown to cell densities of $\approx 2 \times 10^7$ ml^{-1}, washed in water then spread on solid medium. Plates were irradiated under a 30 watt germicidal lamp delivering a fluence of 0.67 J m^{-2} s^{-1}. For determining sensitivity to methyl methanesulphonate (MMS), washed cells were resuspended at 1×10^8 per ml in 0.02% MMS. After incubation at room temperature for appropriate lengths of time, aliquots were

removed, brought to 1% in sodium thiosulphate, diluted and plated (Ferguson *et al*., 1997). For determining sensitivity to bleomycin, cells were diluted and spread on plates containing bleomycin at the appropriate concentrations. Plates were used within 3 days after pouring. In all cases, survival was assessed by counting colonies visible to the eye after incubating plates for 5 days at 30°C.

Mutator activity

Mutation rates were determined by fluctuation analysis using the method of the median (Lea and Coulson, 1949). Nine independent cultures of each strain to be tested were started by inoculating 2 ml of YEPS medium with an entire isolated colony. After approximately 20 generations of expansion, cultures were spread onto YEPS plates containing nourseothricin (30 $\mu g\,ml^{-1}$) or 5-fluroorotic acid (1 $mg\,ml^{-1}$) plus uracil (25 $\mu g\,ml^{-1}$). Resistant colonies were determined after incubation of the plates for 5 days. Cell densities were determined by counting viable colonies after appropriate dilution onto non-selective medium. Nourseothricin (clonNAT) was obtained from the Hans-Knöll Institut für Naturstoff-Forschung and 5-fluoroorotic acid (5-FOA) was from U.S. Biologicals.

Yeast two-hybrid analysis

A *U. maydis* cDNA library for two-hybrid screening was prepared from RNA extracted from DNA-damaged induced cells. Exponentially growing cells were harvested 4 h after addition of 0.02% MMS to the culture. Total RNA was extracted and enriched for the polyA$^+$ fraction by chromatography on oligo(dT) cellulose. The cDNA library was constructed as described (Gubler and Hoffman, 1983). After introduction of *Eco*RI linkers, the library was inserted into plasmid pGAD10 (Clontech Laboratories). The library was composed of approximately 2.5×10^6 individual inserts in the range of 0.3–4 kbp with an average size of 1.2 kbp. Screening (Chien *et al*., 1991) was performed after lithium acetate-mediated co-transformation of the Rec2-bait plasmid pCM597 and the pGAD10 library into *Saccharomyces cerevisiae* strain PJ69-4A (*MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) (James *et al*., 1996), according to Clontech Laboratories yeast protocols handbook PT3024-1. Co-transformants were selected on synthetic drop-out medium lacking tryptophan and leucine, and interactor candidates were selected on synthetic medium lacking adenine. For quantitative measurement of interactions between Rec2 and Rad51, extracts were prepared from cells fractured by freezing in liquid nitrogen. Cell cultures were grown under selective conditions as above for co-transformation when testing controls. As there appeared to be strong selection pressure against the Rad51-containing plasmid, measurements for interaction with Rad51 was performed with cells cultured under selection for interaction. β-galactosidase activity was assayed using o-nitrophenyl-β-D-galactopyranoside (ONPG) according to the Clontech protocols and is expressed in units per 10^7 cells in which one unit represents the hydrolysis of 1 nmol of ONPG min^{-1} at 37°C.

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