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Structure of *REC2*, a Recombinational Repair Gene of *Ustilago maydis*, and Its Function in Homologous Recombination between Plasmid and Chromosomal Sequences

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Mutation in the *REC2* gene of *Ustilago maydis* leads to defects in DNA repair, recombination, and meiosis. Analysis of the primary sequence of the Rec2 protein reveals a region with significant homology to bacterial RecA protein and to the yeast recombination proteins Dmc1, Rad51, and Rad57. This homologous region in the *U. maydis* Rec2 protein was found to be functionally sensitive to mutation, lending support to the hypothesis that Rec2 has a functional RecA-like domain essential for activity in recombination and repair. Homologous recombination between plasmid and chromosomal DNA sequences is reduced substantially in the *rec2* mutant following transformation. The frequency can be restored to a level approaching, but not exceeding, that observed in the wild-type strain if transformation is performed with cells containing multiple copies of *REC2*.

It has become increasingly clear that the cellular response to DNA damage encompasses a complex set of processes geared not only to preserve the integrity of the genome but also, in a broader sense, to contribute to survival of the organism. In well-researched model systems such as *Saccharomyces cerevisiae*, studies on DNA repair have revealed that mutation in any one of several dozen genes can lead to radiation sensitivity. These genes can be grouped into three general categories based loosely on epistatic relationships and genetic properties (11). They are referred to as the excision repair group, the error-prone repair group, and the recombinational-repair group, the last group so named because the pathway of repair disabled is governed by functions dedicated to genetic recombination. Mutation in the genes defining this group leads to defects in induced recombination, extreme sensitivity to ionizing radiation, lethality upon mating-type switching in homothallic strains, sporulation deficiency, and inability to repair double-strand gaps in plasmid DNA during transformation (for a review, see reference 25). All of these defects can be accounted for by loss of a basic recombination function. Discovery that at least two genes in the recombinational repair group, namely *RAD51* and *RAD57*, have extensive homology with the *Escherichia coli recA* gene (1, 3, 18, 33) underscores the notion that this group of genes is directly involved in genetic recombination.

Such an extensive inventory of radiation-sensitive mutants is unrivaled in any other eukaryotic experimental system. Nevertheless, pathways of DNA repair as exemplified in *S. cerevisiae* provide a paradigm for thinking about DNA repair in other organisms and a platform for generalizing about the mechanisms. Radiation-sensitive mutants obtained in other less-mainstream experimental systems can also be categorized according to the three classes of yeast DNA repair genes. For instance, mutants of *Ustilago maydis* that appear to be representative of all three of these groups have been isolated (15, 21). The *uvs3* mutant is recombination proficient, extremely sensitive to UV rather than ionizing radiation, and defective in excision of pyrimidine photodimers (42). This mutant would be appropriately categorized in the excision repair group. The *rec1* mutant is sensitive to both UV and ionizing radiation and hyperactive for mitotic allelic recombination and exhibits a mutator phenotype reminiscent of that of the error-prone repair group (17, 37). The *rec2* mutant is extremely sensitive to ionizing radiation and defective in mitotic crossing over and induced gene conversion and fails to complete meiosis (16). Thus, *rec2* corresponds to the recombinational-repair class.

In a study designed to continue exploration of the mechanism of recombination in *U. maydis*, pairs of autonomously replicating plasmids bearing noncomplementing alleles of a selectable marker were used to cotransform the DNA repair-deficient strains mentioned above that were deleted entirely for the genomic copy of the marker (10). Generation of prototrophy proceeded through extrachromosomal recombination. Introduction of double-strand breaks into the plasmid DNA greatly stimulated recombination, but no such stimulation was apparent in the *rec2* mutant unless the genetic markers on the plasmids were oriented in such a way as to circumvent recombination by a conservative pathway. It was deemed unlikely that the defect in *rec2* was in a mismatch correction step, since artificially formed heteroduplex DNA containing the two allelic markers was highly active in transformation to prototrophy. These results were interpreted to mean that the *REC2*-dependent pathway involved homologous pairing and strand exchange and that the defect in the *rec2* mutant was likely to involve a step in pairing of DNA duplexes. This intriguing possibility aroused our interest in investigating the *REC2* gene further.

To learn more about the role of *REC2* in recombination, we cloned and characterized the gene and continued efforts to analyze recombination of plasmid DNA substrates in the *rec2* mutant. In the study presented in this paper, the aims were to determine the nature of the *REC2* gene product, to test the significance of structural motifs found in the sequence, and to measure recombination between a cloned gene on a plasmid and the homologous sequence in the genome.

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TABLE 1. Strains of *U. maydis* used in these studies

Strain	Genotype
UCM5	*REC2 ade1-1 leu1-1 a2b2*
UCM54	*rec2-1 pan1-1 nar1-1 a1b1*
UCM164	*Δrec2::HPH ade1-1 leu1-1 a2b2*
UCM288	*Δrec2::HPH ADE1/ade1-1 leu1-1 a2b2*
UCM289	*Δrec2::HPH ADE1-REC2/ade1-1 leu1-1 a2b2*
UCM302	*Δrec2::HPH ADE1-rec2-2/ade1-1 leu1-1 a2b2*
UCM303	*Δrec2::HPH ADE1-rec2-3/ade1-1 leu1-1 a2b2*
UCM304	*Δrec2::HPH ADE1-rec2-5/ade1-1 leu1-1 a2b2*
UCM305	*Δrec2::HPH ADE1-rec2-7/ade1-1 leu1-1 a2b2*
UCM306	*Δrec2::HPH ADE1-rec2-4/ade1-1 leu1-1 a2b2*
UCM320	*Δrec2::HPH ADE1-rec2-6/ade1-1 leu1-1 a2b2*
UCM321	*Δrec2::HPH ADE1-rec2-8/ade1-1 leu1-1 a2b2*

MATERIALS AND METHODS

***Ustilago maydis* strains and methods.** All strains (Table 1) used were from laboratory stocks or were derived by standard molecular genetic methods for *U. maydis* (see references 8 and 41). The designations *ade*, *leu*, *pan*, and *nar* refer to requirements for adenine, leucine, and pantothenic acid and to inability to metabolize nitrate, respectively. *a1b1* and *a2b2* refer to mating-type alleles. Radiation sensitivity was determined as described previously (40). In general, cultures were grown to a density of 2×10^7 cells per ml and the cells were collected by centrifugation, washed in water, and plated by spreading from 10^3 to 10^6 cells on yeast extract-peptone-sucrose (YEPS) medium. Cells were irradiated with UV with a 30-W General Electric germicidal lamp delivering 0.2 J/m^2/s. Survival was determined by counting of colonies visible after incubation of plates for 3 days at 32°C. Transformation to leucine prototrophy was performed with *U. maydis* protoplasts as previously described (10). Homologous recombination was measured by transforming *leu1-1* strains to Leu$^+$ with pCM381 over a range of input DNA (1 to 10 μg of DNA) at which the response was approximately linear. Some variation in transformation frequency resulted from differences in protoplast competence and was controlled for by use of the autonomously replicating plasmid pCM216 as a standard (usually 30 ng of DNA). Recombination frequency was calculated as the number of Leu$^+$ transformants obtained with pCM381 divided by the number of Leu$^+$ transformants obtained with pCM216 per microgram of DNA. Specific recombination frequency was calculated as the number of Leu$^+$ transformants obtained with pCM381 per microgram of DNA divided by the number of Leu$^+$ transformants obtained with pCM216 per microgram of DNA.

Plasmids. pCM54 is a pUC12 derivative containing the hygromycin phosphotransferase-*hsp70* promoter gene fusion (*HPH*) used in selection for hygromycin resistance (44) and the 383-bp *Ssp*I active subfragment from the autonomously replicating sequence of *U. maydis* inserted into the *Ssp*I site. pCM230 is a shuttle vector based on pBluescript II SK$^+$ and contains the *U. maydis ARS* (41) and the *HPH* gene. pCM158 is pCM54 containing a 9-kbp fragment from a *Sau*3AI partial genomic digest of DNA from UCM3 which is inserted at the *Bam*HI site in multiple cloning sequence (4). This fragment contains the entire *REC2* gene. The almost complete *LEU1* gene of *U. maydis* is contained on a 3.0-kbp *Eco*RI-*Hin*dIII DNA fragment (8, 31). pCM216 (10) has this 3.0-kbp fragment inserted into a pBluescript II SK$^+$ derivative (Stratagene) containing the 383-bp *U. maydis* autonomously replicating sequence (41). pCM216 fully complements the auxotrophy of the *U. maydis leu1-1* mutant. pCM381 is pBluescript II SK$^+$ containing a 2.7-kb *Hin*dIII portion of the *LEU1* gene which lacks a 300-bp segment essential for complementation. It does not replicate autonomously. pCM425 was generated by inserting an 823-bp *Hin*dIII fragment (+233 to +1057 with respect to the initiation methionine of the putative Rec2 open reading frame [ORF]) from the *REC2* gene into pBluescript II SK$^+$. This plasmid was used as a template for construction of site-directed mutants within the span of this portion of *REC2*. pCM427 was made by inserting the 3.2-kbp *Mse*I fragment, which contains the entire *REC2* gene, into the *Hin*cII site of pCM325 (an adaptor plasmid with a duplicated multiple cloning site [39]). This enables excision of the *REC2* gene on a 3.2-kbp *Bam*HI fragment. pCM430 was constructed by ablating the 5′-most *Hin*dIII site in the noncoding region upstream from the *Ssp*I site of the *REC2* gene in pCM427. This plasmid was used as the *REC2* backbone for construction of all of the *REC2* site-directed mutants. The *ADE1* gene of *U. maydis* is contained on a 5.0-kbp *Bam*HI-*Xba*I fragment that was isolated by complementation of the adenine auxotrophy of the *ade1-1* mutant. pCM441 is a derivative of pBluescript II SK$^+$ carrying this *Bam*HI-*Xba*I fragment modified by removal of an essential 100-bp *Nco*I fragment from within the coding region of the gene (6a). Cleavage of pCM441 with *Nco*I creates a gap within the *ADE1* gene that does not overlap the *ade1-1* lesion and yet enables transformation of *ade1-1* strains to adenine prototrophy upon transformation. pCM463 was constructed by insertion of a 3.2-kbp *Bam*HI fragment containing the *REC2* gene from pCM430 into the *Bam*HI site of pCM441. pCM474 was constructed by insertion of a 3.2-kbp *Bam*HI fragment containing the *rec2-2* gene into the *Bam*HI site of pCM441. Similarly, pCM476 contained the *rec2-3* gene, pCM478 contained *rec2-4*, pCM479 contained *rec2-5*, pCM480 contained *rec2-6*, pCM481 contained *rec2-7*, and pCM500 contained *rec2-8*. All plasmids were amplified in *E. coli* XL-1 Blue (Stratagene) *endA1 hsdR17 supE44 thi-1* λ^- *recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q} *lacZ* Δ*M15* Tn*10*{*tet*^{R}}].

Nucleic acid techniques. Southern blot hybridization was carried out as described previously (9). For Northern (RNA) blot hybridization, RNA was prepared by LiCl precipitation of nucleic acids extracted from cells sheared open by violent agitation with glass beads in a solution containing phenol and sodium dodecyl sulfate (SDS) and was separated on agarose gels run in formaldehyde as described before (40). Both RNA and DNA samples were transferred to Zeta-Probe membranes (Bio-Rad Laboratories) in 0.4 M NaOH, and hybridizations were carried out in 7% SDS–0.5 M sodium phosphate (pH 7.2)–1 mM EDTA at 65°C. DNA probes were labeled by random priming with the Klenow fragment of *E. coli* DNA polymerase I (6). Radiolabeled bands in blots were visualized by autoradiography and quantitated by scanning with a Molecular Dynamics Series 400 Phosphor Imager. Autoradiograph images were digitized with a scanner and Photoshop software (Adobe Systems, Inc.).

***REC2* DNA sequence determination.** The *REC2* gene was isolated originally on a 9-kbp genomic DNA fragment (4). A 5.0-kbp *Kpn*I-*Ssp*I fragment encompassing the *REC2* gene was subcloned from the original cloned fragment, and overlapping deletions were prepared by controlled digestion with *E. coli* exonuclease III and mung bean nuclease (13). Plasmid deletions obtained were tested for the ability to complement the UV sensitivity of the *U. maydis rec2-1* strain UCM54 after irradiation with 40 J of 254-nm UV light per m^2. The boundaries of the complementing fragments were found to correspond approximately to the 3.5-kbp *Mlu*I-*Eco*RI fragment depicted in Fig. 1. DNA sequence was determined by the enzymatic chain termination method with Sequenase 2.0

TABLE 2. Oligonucleotide primers synthesized for site-directed mutagenesis

Primer	Sequence
rec2-2 A	5'GAC GAC CTG TTC GGC GGT GGG
rec2-2 B	5'CCC ACC GCC AAG CAG GTC GTC
rec2-3 A	5'GGC TCT GGT GCG ACC CAG ATG
rec2-3 B	5'CAT CTG TGG CGC CCA AGA CCG
rec2-4 A	5'GTA TTC TCA GCC GGC TCC CGA
rec2-4 B	5'TCG GGA GCC GGC TGA GAA TAC
rec2-5 A	5'CGA GAG CTC GCC GAC CTG CTA
rec2-5 B	5'TAG CAG GTC GGC GAG CTC TCG
rec2-6 A	5'CTG CTA GGC GCT GGG GTG CGT
rec2-6 B	5'ACG CAC CCC AGC GCC TAG CAG
rec2-7 A	5'CTA GGC GGT GCG GTG CGT TCC
rec2-7 B	5'GGA ACG CAC CGC ACC GCC TAG
rec2-8 A	5'GGC TCT GGT AGG ACC CAG ATG
rec2-8 B	5'CAT CTG GGT CCT ACC AGA GCC

(United States Biochemical Corp., Cleveland, Ohio) and α-^{35}S-dATP as described by Tabor and Richardson (36). Denatured double-stranded DNA was sequenced directly (38) on 6% polyacrylamide gels containing 7.7 M urea with oligonucleotide primers synthesized as needed (Oligos, Etc., Wilsonville, Oreg.). Sequence homology searches and alignments were carried out with the FASTA algorithm at the Rockefeller University Computer Center, and the RDF2 program was used for statistical verification of homologies (24). Certain motifs were identified with the PROSEARCH software and PROSITE database (2). Restriction endonuclease mapping, DNA sequence project management, and ORF analysis were carried out with DNASTAR (DNASTAR, Inc., Madison, Wis.).

Isolation of the *rec2-1* allele. By restriction endonuclease mapping of genomic DNA extracted from the *rec2-1* mutant, it was determined that a 0.8-kbp stretch spanning a landmark *Hin*dIII site in the wild type was deleted. The DNA fragment encompassing the region of deletion was isolated from a size-selected library prepared from UCM54 genomic DNA after digestion with *Hin*dIII and *Pst*I. DNA fragments in the size range 0.3 to 1.5 kbp were eluted from a preparative agarose gel after electrophoresis and ligated together with pBluescript II SK$^+$ DNA previously cut with *Hin*dIII and *Pst*I. After transformation into *E. coli* XL-1 Blue, colonies were replica plated onto nitrocellulose membranes (BA-85S; Schleicher & Schuell, Keene, N.H.), prepared for hybridization (12), and screened with a 0.3-kbp *Hin*cII-*Pst*I fragment as probe. From approximately 400 colonies, 1 positive was found. Its identity was confirmed by restriction enzyme mapping, and its DNA sequence was determined.

Construction of site-directed mutations. Using plasmid pCM425, which contains a fragment of the *REC2* gene that spans the region of homology with RecA, as template, we performed site-directed mutagenesis using the two-stage overlap extension method with PCR (14). The different mutant subfragments were inserted into the backbone of plasmid pCM430, which contains the entire *REC2* gene flanked by *Bam*HI sites. The oligonucleotide primers synthesized for site-directed mutagenesis were as shown in Table 2. The oligonucleotides which flanked the mutant fragments and which were used in all of the PCRs were the M13 universal primer 5'GTA AAA CGA CGG CCA GT and the reverse sequencing primer 5'AAC AGC TAT GAC CAT G.

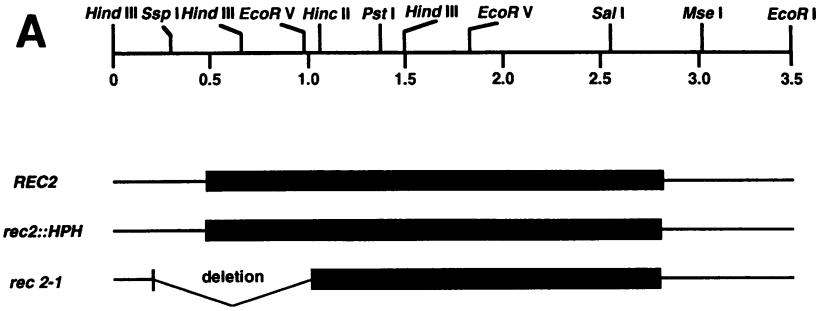
Nucleotide sequence accession number. The GenBank accession number for the sequence reported in Fig. 1B is L18882.

RESULTS

Molecular characterization of the *REC2* gene. The *REC2* gene was subcloned from the original isolate (4) and localized on a 3.2-kbp *Ssp*I-*Eco*RI fragment that fully complemented the radiation-sensitive phenotype of the *rec2-1* mutant. DNA sequence analysis revealed that this fragment contained a single uninterrupted ORF of 2,343 bp (Fig. 1A). Several lines of evidence establish that this ORF encodes the *REC2* gene. First, the ATG codon lies in an acceptable sequence context for initiation of translation (20). Second, the 0.8-kbp *Eco*RV fragment that was replaced by a hygromycin phosphotransferase cassette (44) during construction of a *rec2* disruption allele (4) lies within this ORF. Third, the *rec2-1* allele deletes 0.8 kbp extending 0.6 kbp into the ORF from the first ATG codon and includes 0.2 kbp of upstream sequence. Fourth, a 2.7-kb mRNA detected by use as a probe of a restriction fragment internal to the ORF is not detected in the *rec2-1* mutant (see Fig. 7). The boundaries of this RNA as determined by S1 nuclease protection place the termini approximately at positions −150 and +2500 with respect to the ORF, with no indication of splicing (not shown).

The *REC2* gene encodes a protein of 781 amino acids with a calculated mass of 83,935 Da (Fig. 1B). The sequence K-R-I-K, spanning residues 14 to 17, is similar to the motif K-X-X-K/R, which appears in many nuclear localization signals described elsewhere (29). The acidic stretch from amino acids 93 to 104, in which 11 of 12 residues are aspartate, is reminiscent of acidic regions found in proteins known to interact with chromatin (45) as well as in transcription factors in which such regions serve to mediate protein-protein interactions (26). Residues 251 to 258 (G-E-S-G-S-G-K-T) and 438 to 442 (V-V-V-D) correspond to consensus Walker A (G/A-X-X-X-X-G-K-T/S)- and B (four hydrophobic residues followed by D)-type purine nucleotide-binding site motifs (43). The sequence T-P-R-K spanning residues 697 to 700 is similar to the Cdc2 protein kinase phosphorylation site motif (S/T-P-X-R/K [32]).

A search for structural relationships turned up no overall homology between Rec2 and any other protein. However, the search revealed that a 47-amino-acid stretch encompassing the nucleotide-binding A motif exhibits a remarkable degree of conservation in primary structure with a region of *E. coli* RecA protein that is highly conserved among all bacterial species known to date (see Fig. 2 and reference 30). This same region is also conserved among three RecA-related proteins identified in *S. cerevisiae*, namely Dmc1 (5), Rad51 (1, 3, 33), and Rad57 (18). Comparison of the sequences of these proteins reveals 42% (Dmc1), 36% (Rad57), 40% (Rad51), and 39% (*E. coli* RecA) amino acid identities within this 47-residue stretch. It should be noted that the relationship between Rec2 and RecA as well as the eukaryotic RecA homologs does not result solely from the presence of the Walker A-type nucleotide-binding motif. Other proteins containing this motif were not identified in the homology search. The region of homology has been analyzed extensively in *E. coli* RecA and has been shown to be important for interaction with ATP. Crystallographic analysis indicates that the region immediately preceding the Walker A-type nucleotide-binding motif is structurally unusual and consists of a helix and β-sheet separated by a loop (34, 35). This region has been implicated in mediation of a structural transition that is coupled to ATP binding (19).



B

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-175                                                AATATTCACGATTCTGATGTGGAAGCGTAAGGAGAAGCAGATTAGGTGCTGGTAGGAGCACCTCAACA
                                                        SspI
-107 AGCTAGCCGCCTTGTCGTGCTCATCCCAGTCTTCCACAGCCCCAACCATCGTAGCGGCTGCGCATCGCCACGAATGGTTGCGACTCACAGCTTTGCACGTGCTAAATC

   1 ATGACTGGCATCGCGATCGCCGATGTTGGCTGCATTTCGAAACGCATCAAGGCGTGCTGTCGTCGAGCAAAGCTCTTCAGTACCGACGAGATCCTCCTCAGCCCACCG
   1 M  T  G  I  A  I  A  D  V  G  C  I  S  K  R  I  K  A  C  C  R  R  A  K  L  F  S  T  D  E  I  L  L  S  P  P
                                                        NLS
 109 CAGCAATTGGCACACGTGTTGCGCATATCCCAAGCAGATGCCGATCTGCTTCTTCTCCAAGTGGCCACGGCATCTGCTCCACCTCCCATCTCGGTACTCGATGCGCTC
  37 Q  Q  L  A  H  V  L  R  I  S  Q  A  D  A  D  L  L  L  L  Q  V  A  T  A  S  A  P  P  P  I  S  V  L  D  A  L

 217 AATGGCAAGCTTCCTGCTACCAACCTGGACCAGAACTTCTTTGACGCCGTCGCAGCTGCTGACGATGACGACGACGACAATGATGATGACGATGACAAAGCCGATTCC
  73 N  G  K  L  P  A  T  N  L  D  Q  N  F  F  D  A  V  A  A  A  A  D  D  D  D  D  D  N  D  D  D  D  D  K  A  D  S
                                                                    Chromatin Binding Motif
 325 GGTTCGGCCGACGCTTCAGACACGAGCGATGCGGATGATCAACATCTCAACGACGCAAGGTTTGCATCGTCTTGCATCGTGCCCCCAACACAGGGGTACGATGGCAAC
 109 G  S  A  D  A  S  D  T  S  D  A  D  D  Q  H  L  N  D  A  R  F  A  S  S  C  I  V  P  P  T  Q  G  Y  D  G  N

 433 TTTCCCGGCGCACAATGCTTTGTCTACGATTCCGATGCCGGCTCGGACAGTGATGCACGCAGTAGCATCGACGCTGTGATGCACGAAGATATCGAGCTACCGTCCACC
 145 F  P  G  A  Q  C  F  V  Y  D  S  D  A  G  S  D  S  D  A  R  S  S  I  D  A  V  M  H  E  D  I  E  L  P  S  T

 541 TTTTGCCGTCCACAAACACCACAAACCCACGATGTTGCCCGTGACGAGCATCATGATGGGTATCTTTGCGATCCCAAAGTTGACCACGCCTCGGTCGCCAGAGACGTC
 181 F  C  R  P  Q  T  P  Q  T  H  D  V  A  R  D  E  H  H  D  G  Y  L  C  D  P  K  V  D  H  A  S  V  A  R  D  V

 649 TTATCGCTCGGACGCCAACGACATGTATTCTCAAGCGGCTCCCGAGAGCTCGACGACCTGCTAGGCGGTGGGGTGCGTTCCGCTGTGCTCACCGAGCTCGTCGGTGAA
 217 L  S  L  G  R  Q  R  H  V  F  S  S  G  S  R  E  L  D  D  L  L  G  G  G  V  R  S  A  V  L  T  E  L  V  G  E

 757 AGCGGCTCTGGTAAGACCCAGATGGCTATCCAAGTTTGCACTTATGCCGCTCTCGGCTTGGTTCCGCTGAGCCAAGCTGACGATCACGACAAGGGCAACAACACATTT
 253 S  G  S  G  K  T  Q  M  A  I  Q  V  C  T  Y  A  A  L  G  L  V  P  L  S  Q  A  D  D  H  D  K  G  N  N  T  F
        Motif A
 865 CAATCCAGGACTTTCGTACGAGACCCGATACACGCTTCGACCAAAGACGACACACTAAGCGACATTCTGCAGAGCTACGGCATGGAGCCCTCGATTGGATCTCACCGC
 289 Q  S  R  T  F  V  R  D  P  I  H  A  S  T  K  D  D  T  L  S  D  I  L  Q  S  Y  G  M  E  P  S  I  G  S  H  R

 973 GGTATGGGCGCGTGCTACATCACATCTGGTGGCGAGCGCGCAGCGCATTCGATCGTGAACCGAGCTCTGGAACTTGCAAGCTTTGCTATCAACGAACGCTTTGATCGC
 325 G  M  G  A  C  Y  I  T  S  G  G  E  R  A  A  H  S  I  V  N  R  A  L  E  L  A  S  F  A  I  N  E  R  F  D  R

1081 GTCTATCCGGTCTGCGATCCTACACAAAGCTCGCAGGACGCCGATGGGCGCCGCGACGCATTGCTGGCCAAGGCACAGCAGCTTGGTCGTCGACAAGCGCTTGCCAAC
 361 V  Y  P  V  C  D  P  T  Q  S  S  Q  D  A  D  G  R  R  D  A  L  L  A  K  A  Q  Q  L  G  R  R  Q  A  L  A  N

1189 TTGCACATAGCCTGCGTCGCTGATGTCGAGGCATTGGAGCATGCTCTCAAGTACAGTTTGCCTGGCTTGATTCGTCGATTGTGGTCGAGTAAGCGTCAGTCGGGCGTA
 397 L  H  I  A  C  V  A  D  V  E  A  L  E  H  A  L  K  Y  S  L  P  G  L  I  R  R  L  W  S  S  K  R  Q  S  G  V

1297 TCGCGCGAGATTGGCGTTGTGGTGGTAGACAATCTTCCAGCGCTTTTCCAGCAAGATCAAGCGGCAGCGAGCGATATCGACTCGCTCTTCCAACGCTCAAAGATGCTA
 433 S  R  E  I  G  V  V  V  V  D  N  L  P  A  L  F  Q  Q  D  Q  A  A  A  S  D  I  D  S  L  F  Q  R  S  K  M  L
                    Motif B
1405 GTCGAGATCGCGGATGCGCTCAAGCGTATCAGTGCTGTACAATGGCGTGGCGCTTCAGATTGTGGTTCCTCTGCAGGTAGAGCGGTGCTGGTGCTGAACCACGTCAGC
 469 V  E  I  A  D  A  L  K  R  I  S  A  V  Q  W  R  G  A  S  D  C  G  S  S  A  G  R  A  V  L  V  L  N  H  V  S

1513 GATGCGTTTGGAATCGACAAGCAGATTGCACGGCGCTTCGTATTCGACTCGGCGCACCGCATCCGAACGCGTCGGTCTCATTTTGCACGCAACGATCCTGGCACATCA
 505 D  A  F  G  I  D  K  Q  I  A  R  R  F  V  F  D  S  A  H  R  I  R  T  R  R  S  H  F  A  R  N  D  P  G  T  S

1621 AGTCAAGCGCCAACCTCGGCATTCAGCGGTGGCACTGGATCGGCGTTACCCGACCAGCCGCTAGCGATGGATGTGGCTAGCCAGACTGCGTTCACCAGCGGGCTGCTC
 541 S  Q  A  P  T  S  A  F  S  G  G  T  G  S  A  L  P  D  Q  P  L  A  M  D  V  A  S  Q  T  A  F  T  S  G  L  L

1729 GCCTCGATCGCGCCTACGCTGGCGGAAGCGGTTGGCGCACGCGAGCTCGACTCGGCGTGCGCTTCCAACGATGTGCCGCTCCGCACACTTGAAGCACGCACTGCACAG
 577 A  S  I  A  P  T  L  A  E  A  V  G  A  R  E  L  D  S  A  C  A  S  N  D  V  P  L  R  T  L  E  A  R  T  A  Q

1837 CTCGGTCAGACCTGGAGCAACCTGATCAATGTGCGCGTGTTTCTGTCCAAAACGCGCGCCAGGATATGCATGCGCGACGATCAGGCACCAGCATGCGAGCCAGTGCGC
 613 L  G  Q  T  W  S  N  L  I  N  V  R  V  F  L  S  K  T  R  A  R  I  C  M  R  D  D  Q  A  P  A  C  E  P  V  R

1945 CAAAACACCAATCAACGTGGTACGGCGAGCAAGTCGCTCATGAATACGGTGCGCAAAGCGGCGGTGGTCATCAATCCATTTGGCGCAACCATGTTAGACGTCGGCGTC
 649 Q  N  T  N  Q  R  G  T  A  S  K  S  L  M  N  T  V  R  K  A  A  V  V  I  N  P  F  G  A  T  M  L  D  V  G  V

2053 GACAAGAGCGCGCTGAGACAGCTACGGTTTGTCATTACGCCGCGCAAAGCGGTGCATGTGCTGAATGCGTATCCATCGACAGTGATGCATGCCATGCATGCGACCGCT
 685 D  K  S  A  L  R  Q  L  R  F  V  I  T  P  R  K  A  V  H  V  L  N  A  Y  P  S  T  V  M  H  A  M  H  A  T  A
                                        Cdc 2 Kinase
2161 GACAGCACGCCCCGCTCCAGAGTCACAGCAGCAACAGCGCGCAGCAGAGCGCCACCCAGCGGAGCAAGAGGACGCCGATCAAGACCTCTTCGGAGAAGCGCTGCAAGAG
 721 D  S  T  P  A  P  E  S  Q  Q  Q  Q  R  A  A  E  R  H  P  A  E  Q  E  D  A  D  Q  D  L  F  G  E  A  L  Q  E

2269 CATCACTGGCTAGCCATCGACGAGCTCCAATCGCACACCACCGCGCGTCCGACTTCCCGAGCCGCCCAAGCTGGCTGAGTGAAAGATTGACTGAGTCATCTCACGTCT
 757 H  H  W  L  A  I  D  E  L  Q  S  H  T  T  A  R  P  T  S  R  A  A  Q  A  G

2377 GCGATCCAGAATCCTTCGTATTTCTACACACATCACAGGATCGTGTTCGTATTCGCGATCATATCGTACACAACTCAAGTTATTGACGTTGAAATGCATTCGTGATTC

2485 ACGCTTGTAGCATGCTAGACGCGAGGCAAGTCTCTTTGGCGCTCATGTTTAAGCTGGCACAGGCACGAGCGTCGATTCGGGAAAATGGAAAAAAGGAAGAACGGCACC

2593 AAGATTGACTGTGTTTAAGTTGAGAGCAAATCGACAACAGTGAAGCATGCTACAAGTTGTCGAGCTAGGCGCCGATCTGCGCGTCCCATGATGCTCTCAGCTGCGGTT

2701 CGACGGCGTTCCAGATGTGCGACCATGTGTCGTCGCCCACCTGTGCTCTGAATTGGTCGAGCGCGGATTTGAACCAGACCTTGACTTGTGCGCCGTGGAGGATGTGCT

2809 TGGTAGCGTCCGATTTGATCGTTTCGCTGGCGGCCAATTTGGTGAAGCCGGTCTGGAAGCCTGCAGCATGGTCTTCGTCGGCGAACAGCAGATCCACGTCTTGCGTCT

2917 GCGTCGCCGAGCTGGGCGTGAGCAGCAACCGCAACAGCGCTGCGAGCAATGTTGGCAACACGCTCACATTCGGCGCTCGACGCATGGCCGATGAATTCACCAACAAGC

3025 TCGCAA
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FIG. 1. Sequences of the *U. maydis REC2* gene and the deduced protein product. (A) The restriction endonuclease map of the 3.2-kbp *Ssp*I-*Eco*RI fragment that complements the radiation sensitivity of the *rec2-1* mutant is shown. Schematic representation of the large uninterrupted ORF found to reside in the genomic fragment from *REC2* and the truncated version found to be present in the *rec2-1* allele is presented. A mutant Δ*rec2*::*HPH* gene constructed in vitro by disrupting the *REC2* gene with a hygromycin phosphotransferase cassette (stippled) is also indicated. (B) The DNA sequence of the 3.2-kbp fragment is shown. The numbering is given with respect to the putative initiation methionine. The putative nuclear localization signal (residues 14 to 17), chromatin-binding acidic motif (93 to 103), nucleotide-binding motif A (251 to 258), nucleotide-binding motif B (438 to 442), and Cdc2 protein kinase phosphorylation site (697 to 700) are underlined. Four stop codons are underlined.

Targeted mutagenesis of the *REC2* gene and construction of *rec2* mutant strains. To address the biological importance of the region with homology to the RecA protein, a series of single-amino-acid substitutions was engineered by site-directed mutagenesis (Fig. 2). These included changes at residues within the A nucleotide-binding motif and at residues highly conserved in the loop structure and an identical change at a residue that is responsible for the well-known *recA13* allele of *E. coli* that lies within this region. The resulting *REC2* alleles were then introduced into a *rec2* null mutant so that biological activity of the alleles could be assessed. Two strategies were taken. One was to introduce the alleles as single-copy integrants in the genome. The second was to introduce the alleles on a multicopy autonomously replicating vector. The latter course was obviously expeditious, but the former course was followed because of concern that evaluation of phenotypic effects might be complicated by the presence of multiple copies of a crippled, but partially active, gene.

Lack of a direct selection scheme that could signal successful integration of mutated *REC2* alleles into the homologous site in the genome necessitated an alternative integration strategy based upon ectopic recombination at a selectable locus. This was accomplished by targeting integration to *ade1-1* enabled by recombination mediated through a cloned but inactivated *ADE1* marker on a plasmid vector (Fig. 3). This vector, pCM441, contains an allele of the *ADE1* gene deleted for an essential 100-bp *Nco*I fragment located within the coding region and does not complement the auxotrophy of *ade1-1* strains. However, the plasmid efficiently transforms *ade1-1* mutants to adenine prototrophy through recombination with

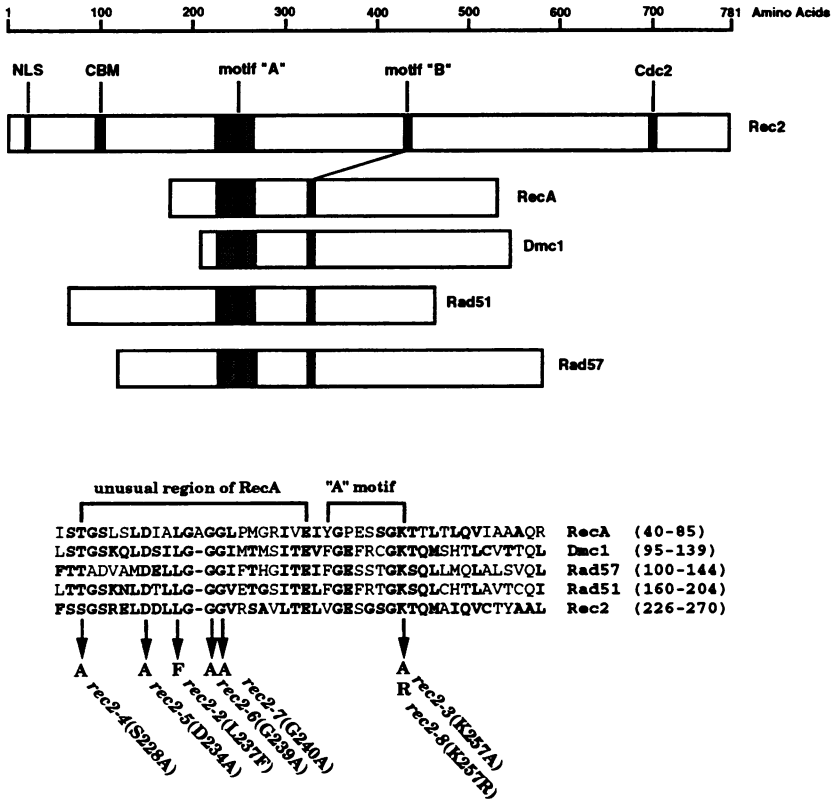

FIG. 2. The Rec2 protein has homology with the RecA protein. A schematic representation (upper panel) of Rec2 with RecA, Dmc1, Rad51, and Rad57 is shown for comparison of relative sizes and the locations of homologies and motifs. A search of the NR database consisting of 83,838 sequences (NR includes all sequences from the SwissProt, PIR, GenPept and GPUpdate databases) was performed. The results of this search show a region of homology of 47 amino acids between amino acids 224 and 270 of Rec2 in common with Dmc1 (FASTA score, 117), Rad57 (FASTA score, 99), Rad51 (FASTA score, 94), and RecA (FASTA score, 68). The statistical significance of these alignments was verified by the RDF2 computer program with standard deviations as indicated: Dmc1, 17.4; Rad57, 13.4; Rad51, 14.1; and RecA, 10.1 (24). Amino acid identities and similarities with Rec2 are indicated with boldface type. The wild-type Rec2 sequence is shown below, with arrows indicating the different missense mutations that were constructed, as described in Materials and Methods. The nuclear localization signal (NLS), chromatin-binding acidic motif (CBM), nucleotide-binding motifs A and B, and Cdc2 phosphorylation site are indicated by solid bars. The region of RecA homology is indicated by the stippled region.

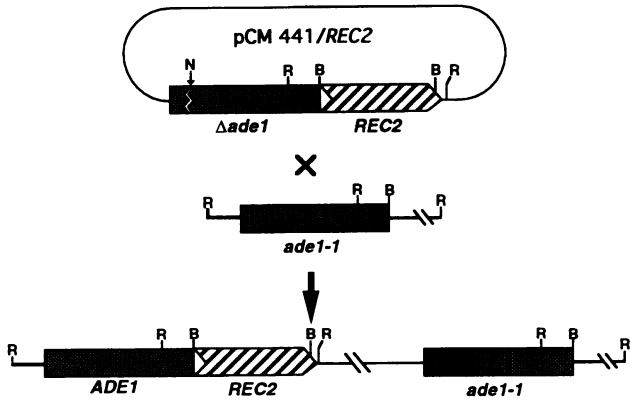

FIG. 3. Strategy for ectopic integration of *REC2* alleles at *ade1-1*. Plasmid pCM463 (pCM441/*REC2*) contains a gap in the *U. maydis ADE1* gene and a 3.2-kbp *Bam*HI fragment which contains the *REC2* gene. Recombination by a single crossover event at the *ade1* locus will result in ectopic integration of the *REC2* allele. This is verified by Southern analysis after digestion of genomic DNA with *Eco*RI. The *ade1-1* lesion is represented by the solid bar, although its precise location is not known. Restriction endonuclease recognition sites are N (*Nco*I), R (*Eco*RI), and B (*Bam*HI).

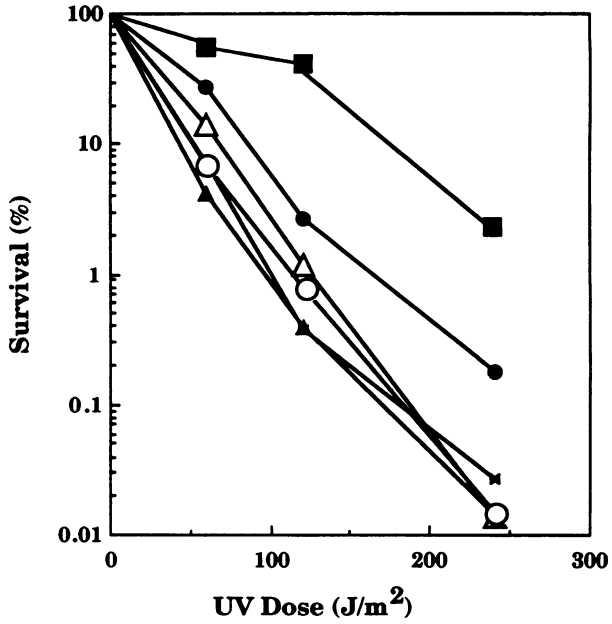

FIG. 4. DNA repair proficiency of *rec2* alleles. Cultures (10 ml) of isogenic *rec2* strains were grown to mid-log phase and harvested by centrifugation. After washing, cells were plated on YEPS medium and irradiated with 254-nm UV light. Survivors were counted after 3 days. *REC2* alleles are indicated as follows: closed squares, *REC2*; closed circles, *rec2-2*; open circles, *rec2-3*; crosses, *rec2-5*; closed triangles, *rec2-6*; open triangles, Δ*rec2*::*HPH*. *rec2-4*, *rec2-7*, and *rec2-8* were not different from *REC2* and are not shown.

the homologous sequence that flanks the *ade1-1* lesion. Since the plasmid does not replicate autonomously, only those recombination events that restore a functional chromosomal gene are selected. Recombinants can arise by a single-crossover event, by gene conversion, by double-crossover events, or by multiple rounds of single-crossover events, but these can be easily identified by Southern blot hybridization and the unwanted classes can be eliminated. A single crossover between the plasmid-borne and the genomic *ade1* loci results in integration of the plasmid, yielding a duplication in which the reconstructed *ADE1* allele lies in tandem with the *ade1-1* allele. Connecting the two alleles is the plasmid vector sequence, which includes a unique *Bam*HI site.

For construction of the desired strains, the mutagenized versions of the *REC2* gene were inserted into this unique *Bam*HI site of plasmid pCM441. After transformation of a Δ*rec2*::*HPH ade1-1* strain to Ade$^+$, transformants were analyzed by Southern hybridization and strains for further analysis were chosen with the *REC2* allele of interest integrated between the two *ade1* alleles (Fig. 4). The efficiency of integration was markedly reduced in the Δ*rec2* mutant (see below), but it was possible to obtain the desired strains. All of the mutated *REC2* strains constructed for this study contained single-copy integrants, as verified by Southern blot hybridization analysis. It should be noted that the sequence of RecA homology in the mutated *REC2* gene is present in the haploid state in the strains constructed, since the resident *rec2* allele, namely Δ*rec2*::*HPH*, is deleted for this sequence. Thus, any observed activity of the introduced *rec2* allele in question would necessarily be an inherent feature of that allele and would not arise through ectopic gene conversion directed by the endogenous locus.

DNA repair proficiency of the *rec2* alleles. The mutants generated were tested for DNA repair proficiency by examination of sensitivity to killing by ultraviolet light (Fig. 4). Several were found to be as defective in repair as the *rec2-1* (originally isolated by Holliday) and Δ*rec2*::*HPH* null mutants. These included the *rec2-3*(K257A) mutant, in which the conserved lysine at position 257 in the Walker A-type nucleotide-binding motif is changed to alanine, but not the *rec2-8*(K257R) mutant. Other strains with mutations in the RecA homology region with a null phenotype included the *rec2-5*(D234A) and *rec2-6*(G239A) mutants, although the mutant with the same change in the adjacent residue, *rec2-7*(G240A), remained completely proficient in repair. The mutant with the *rec2-2*(L237F) mutation, found also in the *recA13* mutant, was slightly less sensitive than the *rec2* null control strain.

Recombination proficiency of the *rec2* alleles. The recombination proficiency of the different mutants was assessed with an assay that measures recombination of a cloned DNA fragment on a plasmid with its homologous sequence in the genome during transformation. This assay utilizes the nonreplicating plasmid pCM381, which carries an allele of the *LEU1* gene missing an essential sequence (Fig. 5). This plasmid does not complement the *leu1-1* mutant and cannot restore leucine prototrophy by illegitimate recombination, since it contains only a fragment of the *LEU1* gene. Leucine prototrophy can be restored only by homologous recombination between the crippled *leu1* allele on the plasmid and the corresponding locus in the genome. Thus, transformation to leucine prototrophy is a direct measurement of homologous recombination and of recombination proficiency. When the frequency of recombination was measured in a *REC2* strain, there was a dose-dependent increase with increasing input DNA (Fig. 6). A basal level of recombination was detected in the *rec2-1* mutant, but this increased little in response to input DNA. To control for variation in DNA uptake and cellular competence, a comparison between the frequencies of Leu$^+$ transformants obtained with pCM381 and with pCM216, an autonomously replicating plasmid bearing the fully complementing *LEU1* gene, was made. Transformation by the latter occurs at high efficiency and does not depend on recombination (10).

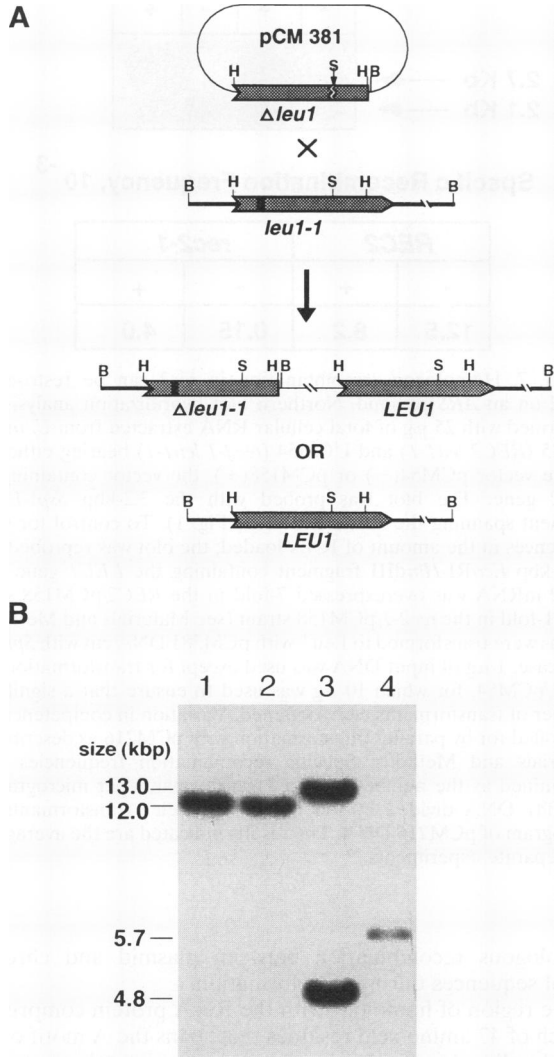

FIG. 5. Homologous recombination assay. (A) Schematic representation of homologous recombination events between plasmid pCM381, which contains a noncomplementing fragment of the *U. maydis LEU1* gene deleted at the 3′ end of 300 bp, and the *leu1-1* mutant, which contains a lesion in the gene at the approximate position indicated. Transformation to leucine prototrophy can take place by crossing over, which results in integration of the plasmid to yield a tandem repeat of *leu1* sequences separated by vector sequence, or else by replacement, which results in no change in the genomic DNA structure. Restriction endonuclease recognition sites are B, *Bam*HI; H, *Hin*dIII; and S, *Sph*I. All transformation experiments were performed with pCM381 DNA cut to the linear form with *Sph*I. (B) A representational Southern blot illustrating the modes of homologous recombination is shown. Genomic DNA (ca. 2 μg) extracted from *U. maydis* UCM5 transformed to leucine prototrophy with the 5.7-kbp pCM381 plasmid was digested with *Bam*HI and processed for Southern hybridization analysis as described in Materials and Methods by use as a probe of a 0.7-kbp *Nco*I fragment from the *LEU1* gene that spans the *Sph*I site. The endogenous *leu1* locus resides on a 12.0-kbp *Bam*HI fragment. Crossover events result in generation of fragments of 13.0 and 4.8 kbp. Lanes: 1, untransformed control; 2, Leu$^+$ transformants from gene replacement; 3, Leu$^+$ transformant from crossing over; 4, pCM381 plasmid DNA cut with *Sph*I.

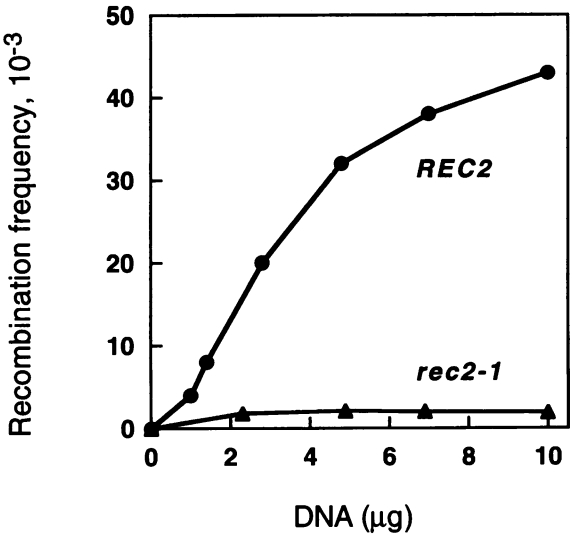

FIG. 6. Homologous recombination during plasmid transformation is defective in *rec2*. *U. maydis* strains UCM5 (*REC2 leu1-1*) and UCM174 (*rec2-1 leu1-1*) were transformed to leucine prototrophy with the indicated amounts of pCM381 DNA cut to linear form with *Sph*I. Recombination frequencies were calculated by dividing the number of Leu$^+$ transformants obtained with the indicated amount of pCM381 DNA by the number of Leu$^+$ transformants per microgram of pCM216 DNA. The latter determination was made with 30 ng of pCM216 DNA, an amount within the linear range of response (10).

The mode of recombination was determined by Southern hybridization. Genomic DNA extracted from transformants was digested with *Bam*HI, which cleaves the plasmid once in the vector sequence but does not cut within the endogenous chromosomal *leu1* allele. The single *Bam*HI fragment containing the *leu1* locus detected in the untransformed control disappears and is replaced by two different fragments in transformants resulting from crossing over. In Leu$^+$ transformants arising as a result of gene conversion or double crossovers, collectively termed replacements, this *Bam*HI fragment is not altered (Fig. 5). Of 20 Leu$^+$ recombinants examined after transformation of the *REC2* control, 7 resulted from crossing over and 13 resulted from gene replacement. In *rec2-1*, the spectrum of events was not significantly different, although the overall frequency of recombination was reduced as mentioned above.

When the *rec2* site-directed mutants were assayed to test their effects in the recombination assay, it was found that the strains defective in DNA repair were also defective in homologous recombination (Table 3). The range of difference in frequencies between the *REC2* strains and the mutant strains was about 40-fold in this particular assay. The site directed mutants could be grouped as either proficient or deficient, but no intermediate phenotype such as that seen in the UV sensitivity of *rec2-2*(L237F) mutants was observed. Some scatter in frequency was noted with the presumed *rec2* null mutants, due simply to the low number of Leu$^+$ transformants that arose. For this reason, transformations were performed with 10 μg of DNA, although with this amount the response was slightly out of the range of linearity (Fig. 6). Accordingly, there could be some inaccuracy and exaggeration of the difference in recombination frequencies between wild-type and *rec2* strains.

None of the mutant alleles was found to interfere with the

TABLE 3. Recombination frequency of site-directed mutants

Allele	Specific recombination frequency[a] (10^{-3})
REC2	6.4
Δrec2::HPH	0.5
rec2-2(L237F)	0.15
rec2-3(K257A)	0.24
rec2-4(S228A)	11.0
rec2-5(D234A)	0.17
rec2-6(G239A)	0.16
rec2-7(G240A)	6.0
rec2-8(K257R)	4.3

[a] Transformation to Leu$^+$ was carried out with 1 μg (for recombination-proficient strains) or 10 μg (for recombination-deficient strains) of pCM381 DNA cut with *Sph*I, except in the case of *Δrec2::HPH*, for which only 1 μg was used. Specific transformation frequency was evaluated as described in Materials and Methods by parallel transformation with 30 ng of pCM216. Specific recombination frequency = number of Leu$^+$ transformants per microgram of pCM381 DNA/number of Leu$^+$ transformants per microgram of pCM216 DNA.

functioning of the *REC2* gene. This was determined by introducing the mutated genes on a multicopy autonomously replicating vector into a *REC2* test strain. No decrease in DNA repair proficiency was observed with any *rec2* allele.

Recombination between plasmid and chromosomal sequences is not limited by the *REC2* gene. It was of interest to determine whether or not the *REC2* gene might be rate limiting in recombination during plasmid transformation. To answer this question, the frequency of recombination between the plasmid and chromosomal *leu1* alleles was measured for strains harboring a multicopy vector expressing the *REC2* gene. This plasmid vector was pCM158, which had been shown previously to rescue the radiation sensitivity as well as the defects in allelic recombination and meiosis of the *rec2-1* mutant (4). The level of the 2.7-kb *REC2* message was about 10-fold higher in the *REC2* strain transformed with pCM158 than in the strain transformed with the vector alone, as determined by Northern blot hybridization (Fig. 7). A comparable level of 2.7-kb *REC2* message was present in the *rec2-1* strain transformed with pCM158. The 2.1-kb *rec2-1* transcript recognized by the hybridization probe was also evident. When recombination was measured, it was found that the frequency was not any higher in cells with multiple copies of *REC2* than in cells with a single copy of *REC2*. It is concluded that neither the *REC2* copy number nor the mRNA level limits recombination between plasmid and chromosomal sequences.

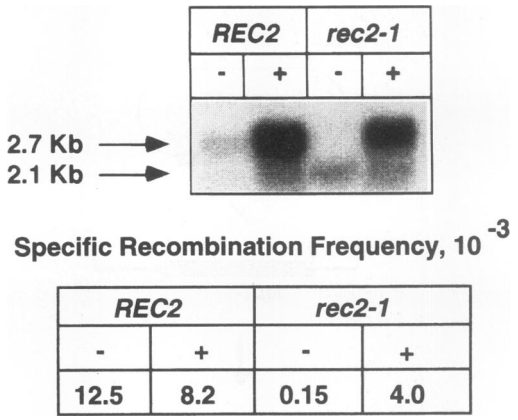

FIG. 7. Homologous recombination in *rec2* can be restored by *REC2* on an *ARS* plasmid. Northern blot hybridization analysis was performed with 25 μg of total cellular RNA extracted from *U. maydis* UCM5 (*REC2 leu1-1*) and UCM54 (*rec2-1 leu1-1*) bearing either the shuttle vector pCM54(−) or pCM158(+), the vector containing the *REC2* gene. The blot was probed with the 3.2-kbp *Ssp*I-*Eco*RI fragment spanning the *REC2* gene (see Fig. 1). To control for slight differences in the amount of RNA loaded, the blot was reprobed with a 3.0-kbp *Eco*RI-*Hin*dIII fragment containing the *LEU1* gene (31). *REC2* mRNA was overexpressed 7-fold in the *REC2*/pCM158 strain and 11-fold in the *rec2-1*/pCM158 strain (see Materials and Methods). Strains were transformed to Leu$^+$ with pCM381 DNA cut with *Sph*I. In each case, 1 μg of input DNA was used except for transformation into *rec2-1*/pCM54, for which 10 μg was used to ensure that a significant number of transformants were obtained. Variation in competence was controlled for by parallel transformation with pCM216 as described in Materials and Methods. Specific recombination frequencies were determined as the number of Leu$^+$ transformants per microgram of pCM381 DNA divided by the number of Leu$^+$ transformants per microgram of pCM216 DNA. The results indicated are the averages of two separate experiments.

DISCUSSION

Genetic studies on the *rec2* mutant of *U. maydis* are consistent with the interpretation that the *REC2* gene product plays a direct role in homologous recombination. The mutant is defective in DNA repair and mitotic crossing over, shows little radiation-induced allelic recombination, and is completely blocked in meiosis. These are all properties to be expected for a mutant altered in a gene that plays a direct role in homologous recombination (16). Furthermore, in studies on interchromosomal recombination between autonomously replicating plasmids, it was deduced that the defect in recombination observed in the *rec2* mutant was likely a result of a failure in a step comprising homologous pairing and strand exchange. The two principal findings from the work reported here support the conclusion that *REC2* plays a direct role in homologous recombination. First, sequence analysis indicates that the *REC2* gene product shares an important structural feature with the *E. coli* RecA protein. Second, the *REC2* gene governs homologous recombination between plasmid and chromosomal sequences during transformation.

The region of homology with the RecA protein comprises a stretch of 47 amino acid residues that spans the A motif of the ATP-binding domain. It is evident from the crystal structure of RecA that this stretch forms an unusual region of the protein featuring tightly packed residues that are highly constrained by neighboring regions (35). The region includes residues in the loop connecting helix B and β-sheet 1 which, in turn, connect to a loop leading to the ATP-binding site A motif. This stretch extends away from the ATP-binding site towards the surface of the protein filament. The region is essential for biological activity of RecA in recombination and for biochemical activity in homologous pairing reactions carried out in vitro. The *recA13* and *recA56* mutants, resulting from missense mutations in this region (L-51 to F and R-60 to C, respectively) are deficient in recombinational activity as measured in vivo and lack strand exchange and DNA-dependent ATP hydrolytic activity in vitro (19). The pair of glycine residues in the region, G-54 and G-55, contain unusual ϕ and ψ backbone torsion angles and are in a region with little space for a side chain. This entire region is also highly conserved in the yeast RecA homologs Dmc1, Rad51, and Rad57 (34).

This homologous region in the *U. maydis* Rec2 protein was found to be functionally sensitive to mutation. Site-directed changes in the region which abolish DNA repair and recombination proficiency are in general coincident with residues essential for the RecA family protein structure and function in

accordance with a model for RecA-ATP interactions. In the structural analysis of RecA and related yeast and bacteriophage recombination proteins reported by Story et al. (34), residues T-42, D-48, G-54, and G-55 were of particular interest because of their evolutionary invariance and their clustered location in the structurally unusual loop. From the crystal structure of RecA protein, it is apparent that the hydroxyl moiety in the side chain of T-42 hydrogen bonds to the acidic group of D-48, that the G-54–G-55 peptide stacks on the D-48 side chain, and that the backbone N-H of G-54 forms a hydrogen bond with the backbone carbonyl moiety of D-48. These prominent structural features prompted our choosing the homologous residues in Rec2 protein for targeted mutagenesis, although the contribution of any of these four residues to the activity of RecA protein is not known. The results from the studies with Rec2 make it clear that the aspartate and the glycine residues in Rec2 corresponding to D-48 and G-54 of RecA are essential for Rec2 function, whereas the other two homologous and highly conserved residues are not. One other notable amino acid change in Rec2 in this region of conservation was L237F, corresponding to the missense mutation in RecA L51F, which gives rise to the *recA13* allele. This mutation in Rec2 also cripples gene function but does not completely abolish DNA repair capacity as is the case in *recA13*.

Substitution of alanine for lysine within the nucleoside phosphate binding loop A motif eliminates recombination and repair activity, indicating the likely importance of nucleoside triphosphate hydrolysis in Rec2 function. An interesting difference from RecA that is shared to some degree by both Rec2 and Rad51 of *S. cerevisiae* (33) is the biological activity remaining in mutants containing arginine in place of lysine at this position. The RecA K72R mutated protein is attenuated in nucleoside triphosphate hydrolysis (28), but the mutant is completely defective in DNA repair and in the ability to support plaque formation of *red*$^-$ *gam*$^-$ phage λ (23). In summary, the data gathered on site-directed mutagenesis of Rec2 are consistent with the hypothesis that Rec2 has a functional RecA-like domain which is essential for activity in recombination and repair.

In prokaryotes, RecA protein is highly conserved as a multifunctional enzyme dedicated to two very different processes, namely homologous recombination and proteolytic cleavage of repressors (see reference 19 for a review). Other members of the RecA family have maintained conservation in protein structure but have diversified in biological function. For instance, the bacteriophage T4 UvsX protein is highly conserved in structure in comparison with RecA protein but is functionally dedicated to DNA replication rather than recombination (7). Mechanistically, it lacks the ability to cleave repressors (46) but possesses a homologous pairing function. In *S. cerevisiae*, it seems apparent that Dmc1, Rad51, and Rad57 could have evolved from an ancestral RecA, but the distinct phenotypes and modes of expression indicate how the three proteins have become diversified and specialized in the particular recombinational processes needed in mitosis versus meiosis. Rec2 bears structural similarity to RecA in a region of the protein that is essential for activity in genetic recombination. Beyond this region, the structural resemblance to RecA and the other members of the RecA family ends, yet a function for Rec2 in recombination remains conserved. In recognition of the similar and divergent structural and functional features, it might be supposed that the conserved structural element is a signature sequence for homologous recombination function.

The reduction in homologous recombination between plasmid DNA and the genome in the *rec2* mutant indicates that the normal cellular functions operating in recombinational repair and meiosis in *U. maydis* also take part in the more artificial situation of recombination after DNA transformation. This observation could be relevant to the issue of altering the genomes of higher organisms by external manipulations, since it is possible that insight into the control of gene targeting in eukaryotes might be realized from analysis of genes controlling recombination in fungi. Several important findings are apparent from these experiments. Homologous recombination of plasmid DNA with the genome is significantly decreased but is not completely abolished in the *rec2* mutant. This could indicate that an alternative pathway for recombination remains in operation in the absence of *REC2* gene function. Nevertheless, the spectrum of recombination events apparent in the collection of infrequent transformants that did arise is similar to that in the wild type. Given the redundancy of RecA-like functions in yeast, if a second pathway is at work in *U. maydis*, it would seem more likely to result from a second RecA-like activity involved in promoting similar types of recombination reactions than from a nonconservative process exemplified by the single-strand annealing pathway (22). Perhaps more interesting from the perspective of utility for gene targeting is the observation that no improvement in targeting frequency results after an increase of the copy number of *REC2*. Thus, the supply of RecA-like recombination function might not be rate limiting in gene-targeting experiments, although the caveat that no correlation between the level of *REC2* mRNA and Rec2 protein has been established as yet must be raised. Further analysis of the Rec2 protein will no doubt lead to greater knowledge of the mechanism of homologous pairing in recombination.

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