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Genetics

MMS2, encoding a ubiquitin-conjugating-enzyme-like protein, is a member of the yeast error-free postreplication repair pathway

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ABSTRACT **Among the three *Saccharomyces cerevisiae* DNA repair epistasis groups, the *RAD6* group is the most complicated and least characterized, primarily because it consists of two separate repair pathways: an error-free postreplication repair pathway, and a mutagenesis pathway. The *rad6* and *rad18* mutants are defective in both pathways, and the *rev3* mutant affects only the mutagenesis pathway, but a yeast gene that is involved only in error-free postreplication repair has not been reported. We cloned the *MMS2* gene from a yeast genomic library by functional complementation of the *mms2-1* mutant [Prakash, L. & Prakash, S. (1977) *Genetics* 86, 33–55]. *MMS2* encodes a 137-amino acid, 15.2-kDa protein with significant sequence homology to a conserved family of ubiquitin-conjugating (Ubc) proteins. However, Mms2 does not appear to possess Ubc activity. Genetic analyses indicate that the *mms2* mutation is hypostatic to *rad6* and *rad18* but is synergistic with the *rev3* mutation, and the *mms2* mutant is proficient in UV-induced mutagenesis. These phenotypes are reminiscent of a *pol30-46* mutant known to be impaired in postreplication repair. The *mms2* mutant also displayed a *REV3*-dependent mutator phenotype, strongly suggesting that the *MMS2* gene functions in the error-free postreplication repair pathway, parallel to the *REV3* mutagenesis pathway. Furthermore, with respect to UV sensitivity, *mms2* was found to be hypostatic to the $rad6_{\Delta 1–9}$ mutation, which results in the absence of the first nine amino acids of Rad6. On the basis of these collective results, we propose that the *mms2* null mutation and two other allele-specific mutations, $rad6_{\Delta 1–9}$ and *pol30-46,* define the error-free mode of DNA postreplication repair, and that these mutations may enhance both spontaneous and DNA damage-induced mutagenesis.**

Ubiquitin (Ub) is a highly conserved 76-residue protein, often found covalently joined to other proteins. Ub conjugation has been shown to participate in many eukaryotic metabolic processes, including ribosome biogenesis (1), mating type regulation (2), cell cycle control (3), DNA repair (4), and other responses (5). Ub is bound to the ubiquitin-activating enzyme (E1), which activates Ub and enables it to bind to the ubiquitin-conjugating enzyme (Ubc or E2). A single cysteine residue in the proper consensus sequence of a Ubc is absolutely required to bind Ub by a thioester bond and attach it to the target molecule. In some instances a third protein, a ubiquitin ligase enzyme (E3), is required to help the Ubc specify the target protein according to an N-terminal rule (for review, see refs. 6 and 7).

The *RAD6* gene in *Saccharomyces cerevisiae* encodes a 172-amino acid, 20-kDa E2 enzyme, Ubc2 (4, 8). Rad6 appears to be a multifunctional protein—-not only do *rad6* mutants display a slow-growth phenotype, but they are defective in DNA damage-induced mutagenesis and are sensitive to killing by a variety of DNA-damaging agents, including UV, ionizing radiations, and alkylating agents; as well, *rad6* diploids are deficient in sporulation (for review, see ref. 9). It is well established that the Ubc activity of Rad6 is required for all of its functions, because *rad6* mutants carrying amino acid substitutions at the sole cysteine residue (C88) displayed phenotypes indistinguishable from the *rad6*Δ null mutant (10). The N-terminal 15 amino acids are almost identical among Rad6 homologs from *S. cerevisiae, Schizosaccharomyces pombe, Drosophila,* and humans (8, 11–13). Deletion of the first 9 amino acids from Rad6 ($rad6_{\Delta 1–9}$) results in partial sensitivity to UV-induced killing, increased mutagenesis, and the loss of sporulation (14). The Rad6 N terminus is also required for physical interaction with an E3 enzyme Ubr1, and for N-end rule protein degradation (14–16). The C-terminal tail of Rad6 is not required for DNA repair but is essential for sporulation (17); it is also required for *in vitro* multi-ubiquitination of histones (18). It is thus conceivable that Rad6 possesses both Ubr1-dependent and Ubr1-independent E2 activities, and that these different E2 activities may define its different roles.

Many lines of genetic evidence support the role of Rad6 and Rad18 in both the error-free postreplication repair (PRR) and error-prone mutagenesis pathways (9). When lethal replication-blocking lesions occur within the cell, the stalled replication machinery at the lesion must be resolved to allow for subsequent repair synthesis. Rad6 is targeted to stalled replication machinery, presumably by its interaction with the single-stranded DNA binding protein Rad18 (19). *rad18* and *rad6* mutants share many phenotypes, including similar levels of sensitivity to killing by a variety of DNA-damaging agents, and defects in UV-induced mutagenesis (9). Indeed, Rad18 and Rad6 belong to the same epistasis group, and they form a stable complex distinct from the Rad6–Ubr1 complex (19, 20). It was thus proposed that the single-stranded DNA-binding and ATP-hydrolytic activities of Rad18 and the E2 activity of Rad6 direct the complex (21) to the stalled replication machinery for initiation of both the PRR and mutagenesis pathways.

Within the yeast Rad6–Rad18 repair pathway, error-prone mutagenesis is mediated by a nonessential DNA polymerase ζ (Polζ), which has been extensively characterized. *REV3* encodes the catalytic subunit of Polζ (22). Purified Polζ, consisting of Rev3 and Rev7, has been shown to replicate past a thymine dimer much more efficiently than does Polα (23). Polζ-mediated bypass of replication blocks may result in an elevated number of mutations, because *rev3* mutants reduced both spontaneous and DNA damage-induced mutations (for review, see ref. 24). However, because of the lack of mutants defective in PRR but not mutagenesis, it is not clear how the error-free PRR pathway oper-

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Abbreviations: MMS, methyl methanesulfonate; PCNA, proliferating cell nuclear antigen; Pol, DNA polymerase; PRR, postreplication repair; Ub, ubiquitin; Ubc, ubiquitin-conjugating enzyme.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U66724).

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ates. Recently, yeast proliferating cell nuclear antigen (PCNA) (25) and Pol δ (26) have been implicated in the PRR pathway, likely at a late stage of DNA synthesis. We report here the molecular cloning and characterization of a newly identified yeast gene *MMS2*, encoding a Ubc-like protein. We further demonstrate that the *mms2* mutant shares remarkably similar phenotypes with *pol30-46* and $rad6_{\Delta 1-9}$, and that the *mms2* mutation is hypostatic to $rad6_{\Delta 1-9}$ but synergistic with *rev3*. We propose that the above three mutations are all defective in PRR but proficient in mutagenesis.

MATERIALS AND METHODS

Yeast Strains and Cell Culture. The yeast strains used in this study are listed in Table 1. WX17–4a was isolated from MD-2/FY86 diploid segregants to combine *mms2-1* with *ura3* for library screening. The isogenic *rad4*Δ, *rad6*Δ, *rad18*Δ, *rad50*Δ, and *rev3*Δ mutants were described previously (27). The *URA3* selectable marker of WXY9221 was removed by selection on a 5-fluoroorotic acid plate (28) to obtain WXY9579. The haploid strain T43 bearing an *mms2*Δ mutation and its corresponding wild type BY448 were a gift from S. Bacchetti and L. Ma (McMaster University, Hamilton, ON, Canada). Yeast cells were grown at 30°C either in a rich yeast extract/peptone/dextrose (YPD) medium or in a synthetic dextrose (SD) medium supplemented with amino acids and bases at recommended concentrations (29).

Screening a Genomic Library. A YCp50-based yeast genomic library (30) was obtained from M. Rose (Princeton University, Princeton, NJ) and used to transform WX17–4a. A two-step screening protocol was followed. First, uracil-independent (Ura^+) transformants were obtained on SD − uracil plates. A total of approximately 10,000 Ura^+ colonies were then streaked onto YPD and YPD + 0.04% methyl methanesulfonate (MMS). The MMS-resistant clones were subjected to a plasmid cosegregation test and the YCp50-based plasmids were recovered by transforming *Escherichia coli* with total yeast DNA.

DNA Sequencing and Sequence Analysis. Nucleotide sequences of the *MMS2* open reading frame and its surrounding regions were determined by a dideoxynucleotide chain-terminating method (31) using a T7 DNA polymerase sequencing kit (Pharmacia LKB). The *MMS2* sequence (GenBank accession no. U66724) was analyzed to search for intron sequences, and the deduced amino acid sequence was used to perform homology searches and multiple sequence alignments.

The *mms2-1* mutant allele was PCR-amplified by using the MD-2 yeast genomic DNA as a template, and was cloned as a 1.1-kb *Bgl*II fragment (see Fig. 1*A*) into the general-purpose plasmid pTZ18R (Pharmacia). The entire *mms2-1* sequences of three independent clones were determined.

Table 1. *Saccharomyces cerevisiae* strains

Strain	Genotype
B635	**a** *cyc1-115 lys2 his1 trp2*
MD-2	B635 with *mms2-1*
FY86	*α his3-Δ200 ura3-52 leu2-Δ1 GAL*$^+$
WX17-4a	**a** *his3-Δ200 ura3-52 lys2 mms2-1*
DBY747	**a** *his3-Δ1 leu2-3,112 trp1-289 ura3-52*
SBU	DBY747 with *mms2*::*URA3*
SBL	DBY747 with *mms2*::*LEU2*
WXY9579	DBY747 with *rad50*Δ::*hisG*
SBU50h	DBY747 with *rad50*Δ::*hisG mms2*::*URA3*
WXY9394	DBY747 with *rad4*Δ::*hisG*::*URA3*::*hisG*
SBL4U	DBY747 with *rad4*Δ::*hisG*::*URA3*::*hisG mms2*::*LEU2*
WXY9376	DBY747 with *rad6*Δ::*LEU2*
SBU6L	DBY747 with *rad6*Δ::*LEU2 mms2*::*URA3*
WXY9326	DBY747 with *rad18*Δ::*TRP1*
SBU18T	DBY747 with *rad18*Δ::*TRP1 mms2*::*URA3*
WXY9382	DBY747 with *rev3*Δ::*LEU2*
SBUr3L	DBY747 with *rev3*Δ::*LEU2 mms2*::*URA3*
BY448	*α leu2*Δ *his3*Δ*ura3-52 trp1*Δ *ade2 lys2*
T43	BY448 with *mms2*Δ::*TRP1*

Plasmid pSCW-$rad6_{\Delta 1-9}$ was constructed by PCR amplification of the *RAD6* gene with a mutation primer 5′-TCGAATTCAAGTCCACACCAGCTAG-3′, where an *Eco*RI site was created, followed by a mutated translation initiation codon (AAG instead of ATG), and a 3′ end specific primer to generate a *Bgl*II site 3′ to the *RAD6* translation stop codon. The *Eco*RI–*Bgl*II fragment was inserted into the *Eco*RI–*Bam*HI sites of pSCW231 (14) to place the $rad6_{\Delta 1-9}$ under the control of an *ADH1* promoter. This mutation results in translation initiation of the *rad6* gene at the second ATG, which encodes the 10th amino acid of Rad6 (14). It has been determined that the pSCW-$rad6_{\Delta 1-9}$ transformants will produce >10-fold more Rad6 protein than do wild-type cells (14).

Plasmid Manipulation. A series of deletions was made within the library clone YCpM2 insert, and the resulting plasmids were used to transform WX17–4a to map the *MMS2* gene. The *MMS2* gene was subcloned as a 3-kb *Xba*I–*Hin*dIII fragment from YCpM2 into YCplac33 (32) to form YCp-MMS2, and into pTZ18R to form pTZ-MMS2. A linker was inserted into the unique *Nco*I site of pTZ-MMS2 to convert it to a *Bam*HI site, which was then used to clone either the 1.8-kb *LEU2* gene from YDp-L (33) or the 1.2-kb *URA3* gene from YDp-U (33), resulting in pmms2::LEU2 and pmms2::URA3, respectively (Fig. 1*A*).

Cell Killing and Mutagenesis Assays. MMS-induced liquid killing was performed at 30°C in YPD as previously described (27). For UV treatment, cells were plated at different dilutions and then exposed to 254-nm UV light, either in a UV crosslinker (Fisher model FB-UVXL-1000 at ≈2,400 $\mu W/cm^2$) or with a UV lamp (Ultraviolet Products model UVGL-25 at 40 $\mu W/cm^2$) at given doses in the dark. Cells were plated in duplicate either on YPD to score cell survival or on SD − tryptophan to score tryptophan-independent (Trp^+) revertants. DBY747 bears a *trp1-289* amber mutation that can be reverted to Trp^+ by several different mutational events. The plates were incubated at 30°C in a dark chamber for 4 days to prevent photoactivation.

Spontaneous Trp^+ reversion rates of DBY747 derivatives were measured by a modified Luria and Delbruck fluctuation test as described (34). An overnight yeast culture was used to inoculate five tubes, each containing 10 ml of fresh YPD, to a final titer of 20 cells per ml. Incubation was continued until a titer of 2×10^8 cells per ml was reached. Cells were collected, washed, resuspended, and plated. Each set of experiments contained five independent cultures of each strain; each culture was plated onto YPD in duplicate to score total survivors, and onto SD − tryptophan plates to score Trp^+ revertants. Spontaneous mutation rates (number of revertants per cell per generation) were calculated as previously described (35).

RESULTS

***MMS2* Encodes a Ubc-like Protein.** The *mms2-1* mutant was originally isolated by Prakash and Prakash (36) by its enhanced sensitivity to MMS. The *MMS2* gene was cloned by screening a single-copy yeast genomic library for the functional complementation of the *mms2-1* MMS-sensitive phenotype. The initial *MMS2* clone, YCpM2, contains a 10-kb insert. By a combination of deletion mapping and DNA sequencing, a small *MMS2* open reading frame was discovered that conferred an MMS-resistant phenotype in the *mms2-1* strain (Fig. 1*A*). The *MMS2* gene is located on chromosome 7 and resides between *MAD1* and *SNR10* (Fig. 1 *A* and *C*). It encodes a predicted 137-amino acid, 15.5-kDa protein with a single intron. The splicing sequences located within *S. cerevisiae* introns are highly conserved. We confirmed that the first exon encoding three amino acids is required for *MMS2* function, because *MMS2* clones using the second ATG codon at nucleotide 218 as the translational start were unable to complement the *mms2-1* mutation, whereas an intronless *MMS2* clone is

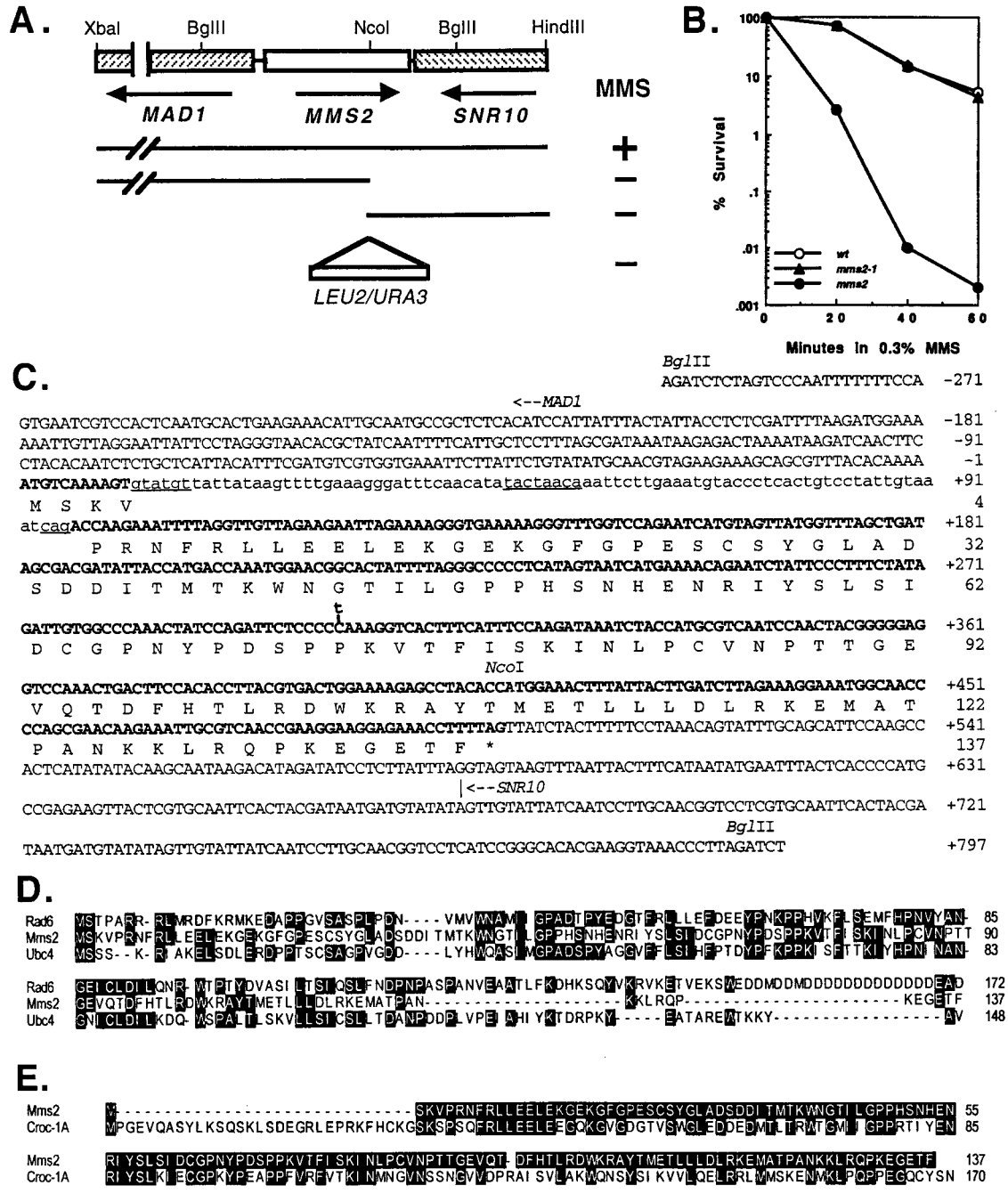

FIG. 1. Physical characterization of the *S. cerevisiae MMS2* gene. (*A*) Mapping and disruption of the *MMS2* gene. A subclone containing the 3-kb *Xba*I–*Hin*dIII fragment enables (+) the *mms2-1* mutant to grow on YPD plates containing 0.4% MMS. Further deletions to the *Nco*I site from either end abolished (−) the *MMS2* function. Either a *URA3* or a *LEU2* fragment was inserted at the *Nco*I site to construct the *mms2*::*URA3* and *mms2*::*LEU2* disruption cassettes. (*B*) Killing of DBY747 (wt), WX17–4a (*mms2-1*), and SBU (*mms2*) in a liquid culture containing 0.3% MMS. (*C*) The nucleotide and deduced amino acid sequences of the *MMS2* gene (GenBank accession no. U66724). Exons are in boldface. Lowercase indicates intron sequences. Consensus sequences within the intron are underlined. The translation initiation site for *MAD1* and the transcriptional termination site for *SNR10* are indicated with an arrow for direction. A C-to-T transition found in the *mms2-1* mutation at nucleotide 303 is marked. (*D*) Amino acid sequence alignments of Mms2 with two yeast Ubc proteins, Rad6 (Ubc2) and Ubc4. Residues shared by two or more proteins are highlighted. (*E*) Amino acid sequence alignment of Mms2 with Croc1. Residues in Croc1 identical to Mms2 are highlighted.

able to complement the MMS-sensitive phenotype of *mms2-1* (data not shown).

Two pieces of evidence strongly suggest that the cloned *MMS2* gene is allelic to *mms2-1*. First, the cloned *MMS2* was used to disrupt the chromosomal *MMS2* gene and the mutant became sensitive to MMS; this *mms2* mutation was unable to complement the *mms2-1* mutation in a diploid. Secondly, the *mms2-1* allele from MD-2 was cloned by PCR amplification and a C-to-T transition at nucleotide 303, which results in a single amino acid substitution, P73L (Fig. 1C), was identified from all three independent *mms2-1* clones. *mms2-1* in a single-copy plasmid was unable to complement MMS sensitivity of the *mms2* mutant (data not shown).

The C-terminal two-thirds of the deduced Mms2 shares significant homology with almost all known Ubc proteins, with *P* values ranging from 10^{-11} to 10^{-3}. The middle third of Mms2 shares up to 40% identity and 60% similarity with some Ubc proteins (Fig. 1*D*), suggesting an evolutionary conservation. Like Ubcs, the deduced Mms2 is rich in proline residues (12/137), particularly in the middle one-third region (8/41),

indicating that it may form a globular protein. Despite the high overall degree of homology between Mms2 and Ubcs, Mms2 lacks a critical consensus sequence that all Ubc proteins display around their active Cys residue (Fig. 1*D*). The absence of this consensus sequence suggests that Mms2 does not possess direct Ub-conjugating ability. Partially purified Mms2 from an *E. coli* expression system was unable to bind Ub and lacked E2 activity (V. Chau, Wayne State University, personal communication). As a matter of fact, Mms2 has even stronger homology ($P = 10^{-39}$) with Croc1, a recently identified transactivator of the *c-fos* enhancer (37), which also lacks E2 activity (38). The two proteins share 49% amino acid identity throughout the entire length of Mms2 (Fig. 1*E*).

The *mms2* Null Mutant Is Sensitive to Both MMS and UV. The original *mms2-1* mutant was characterized as sensitive to MMS- but not to UV-induced killing (36). We found that the *mms2-1* mutant is sensitive to MMS by a plate assay, but not in a liquid assay (Fig. 1 *A* and *B*) and that the *mms2-1* mutation results in only partial loss of function, since a single copy of *mms2-1* was unable to rescue the *mms2* mutant from killing by MMS, yet *mms2-1* in a multicopy plasmid was able to complement the *mms2* mutant (data not shown). It should be noted that P73 mutated in *mms2-1* is conserved in all the corresponding Ubcs as well as in Croc1 (Fig. 1 *D* and *E*), and that it is adjacent to another highly conserved proline residue, which, when mutated (e.g., P64S in Rad6 and P71S in Ubc3), results in temperature-sensitive mutants (39). In contrast to the *mms2-1* mutant, the *mms2* mutant was sensitive to MMS in both plate and liquid assays (Fig. 1*B*). In addition, compared with an isogenic wild-type strain, the *mms2* mutant is more sensitive to UV (see Fig. 2) and a UV-mimetic agent, 4-nitroquinoline 1-oxide (data not shown). Thus the role of the *MMS2* gene appears not to be limited to the protection of cells from DNA methylation damage. Our *mms2* disruption mutant

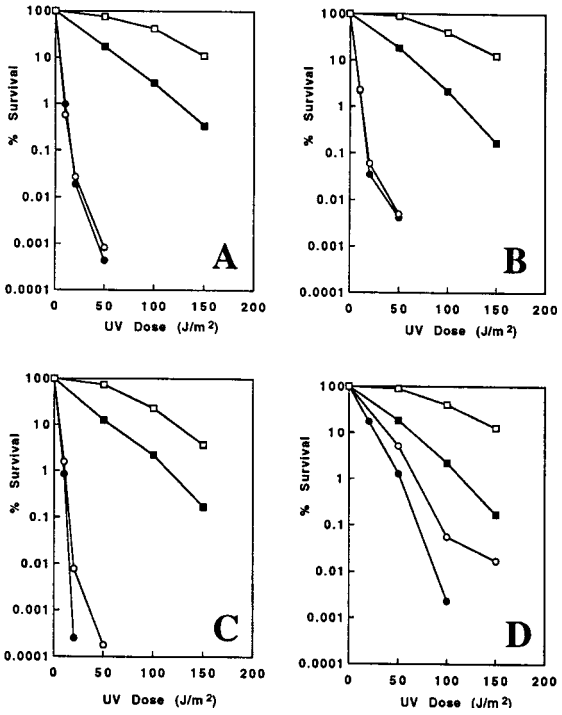

FIG. 2. Epistatic analyses of *mms2* with radiation repair pathway mutations with respect to UV sensitivity. (*A–D*) □, DBY747 (wt); ■, SBU (*mms2*). (*A*) *mms2* and *rad6*. ○, WXY9376 (*rad6*Δ); ●, SBU6L (*rad6*Δ *mms2*). (*B*) *mms2* and *rad18*Δ. ○, WXY9326 (*rad18*Δ); ●, SBU18T (*rad18*Δ *mms2*). (*C*) *mms2* and *rad4*Δ. ○, WXY9394 (*rad4*Δ); ●, SBL4U (*rad4*Δ *mms2*). (*D*) *mms2* and *rad50*Δ. ○, WXY9579 (*rad50*Δ); ●, SBU50h (*rad50*Δ *mms2*). The results are an average of two to four independent experiments.

Table 2. UV-induced mutagenesis of *S. cerevisiae* strains

Strain (genotype)	UV dose, J/m²	Viability, %	Reversion frequency* per 10^7 viable cells
DBY747 (wild type)	0	100	4.8 ± 0.8
	2	100	20.2 ± 3.4
	4	100	29.2 ± 2.6
	6	96	36.0 ± 8.4
	10	92	65.8 ± 1.3
SBU (*mms2*)	0	100	18.6 ± 0.4
	2	93	45.5 ± 8.0
	4	92	65.0 ± 5.0
	6	82	72.5 ± 3.6
	10	80	119.2 ± 24.4

*DBY747 and SBU are isogenic strains carrying the *trp1-289* mutation. Trp^+ revertants were scored as the mean ± SD of at least two independent experiments.

is likely a complete loss-of-function mutant because it was as sensitive to MMS and UV as was the *mms2*Δ strain T43, in which the entire *MMS2* gene is deleted (data not shown).

***MMS2* Belongs to the *RAD6* Epistasis Group.** The relative level of MMS and UV sensitivity of the *mms2* null mutant, as well as the Ubc-like sequence of the Mms2 protein, led us to speculate that *MMS2* may function in the *RAD6* pathway. To test this hypothesis, we created *mms2 rad6*Δ and *mms2 rad18*Δ double mutants and found that, as expected, the double mutants were no more sensitive to UV (Fig. 2 *A* and *B*) and MMS (data not shown) than were the respective *rad* single mutants. Thus *rad6* and *rad18* are epistatic to *mms2*, indicating that *MMS2* belongs to the *RAD6* group. We also performed epistatic analysis of *mms2* with nucleotide excision repair (*rad4*Δ) and recombination repair (*rad50*Δ) mutations. The *mms2 rad4*Δ (Fig. 2*C*) and *mms2 rad50*Δ (Fig. 2*D*) double mutants were found to be more sensitive to UV than either of the corresponding single mutants, and the killing effects appeared to be additive. Thus the *MMS2* gene does not belong to the *RAD3* or *RAD52* epistasis groups; it is specific for the *RAD6* pathway. However, unlike *rad6, rad18,* and other *rev* mutants, *mms2* does not impair UV-induced mutagenesis, and the mutation frequency before and after low-dose UV-treatment actually increased to a certain degree (Table 2). This result indicates that *MMS2* does not affect the mutagenesis pathway.

***MMS2* and *REV3* Mutagenesis.** To investigate the role of *MMS2* within the *RAD6* pathway, we measured UV- and MMS-induced killing of the *mms2 rev3*Δ double mutant. To our surprise, while each of the *mms2* and *rev3*Δ single mutants was only moderately sensitive to the DNA-damaging agents, the *mms2 rev3*Δ double mutant was extremely sensitive to both UV (Fig. 3*A*) and MMS (Fig. 3*B*); the levels of *mms2 rev3*Δ sensitivity to killing by UV and MMS were actually comparable to those of an isogenic *rad18*Δ single mutant (Fig. 2*B*, Fig. 3, and data not

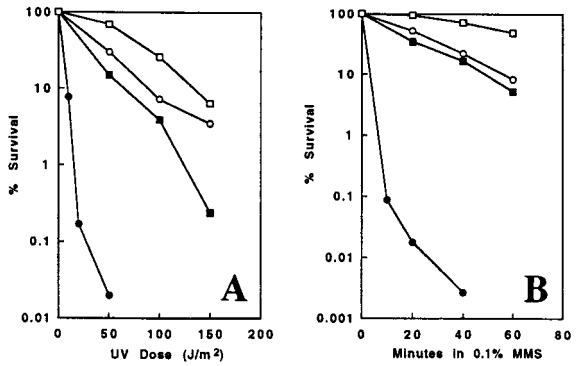

FIG. 3. *mms2* is synergistic to *rev3* with respect to both UV (*A*) and MMS (*B*) sensitivity. □, DBY747 (wild type); ■, SBU (*mms2*); ○, WXY9382 (*rev3*Δ); ●, SBUr3L (*rev3*Δ*mms2*). The results are an average of two independent experiments.

Table 3. Spontaneous mutation rates of *S. cerevisiae* strains

Strain	Key alleles	Mutation rate* $\times 10^9$	Relative rate†
DBY747	Wild type	7.0 ± 4.4	1
SBU	*mms2*	216.6 ± 50.0	31.0
WXY9382	*rev3*Δ	8.7 ± 5.0	1.2
SBUr3L	*mms2 rev3*Δ	8.7 ± 5.0	1.2

*All strains are isogenic and carry the revertable *trp1-289* amber mutation. Rates are expressed as number of revertants per cell per generation. The results shown are the mean ± SD of five (DBY747 and SBU) or three (WXY9382 and SBUr3L) sets of experiments.
†Relative to the wild-type strain.

shown). The effect of the two mutations was clearly synergistic, because the fractions of *mms2 rev3*Δ surviving after the highest dose of UV (50 J/m^2) or 40-min treatment with 0.1% MMS were 1,000-fold lower than expected if the *mms2* and *rev3*Δ effects were simply additive (Fig. 3). This result led us to speculate that *MMS2* represents a repair pathway that is an alternative to *REV3* mutagenesis –namely, the error-free PRR pathway.

We further predicted that if the *mms2* mutation affects only error-free PRR, it may increase the potential for mutagenesis by channeling lesions to the error-prone pathway. The spontaneous mutation rates of *mms2* and *rev3*Δ single mutants and the *mms2 rev3*Δ double mutant were measured. The *mms2* single mutant increased the spontaneous mutation rate by >30-fold at the *trp1-289* allele, and this increase is completely dependent on the functional *REV3* gene, because the spontaneous mutation rate of the *mms2 rev3*Δ double mutant is as low as the *rev3*Δ single mutant (Table 3). This result is consistent with a role for *MMS2* in PRR apart from *REV3* mutagenesis.

***mms2* Is Hypostatic to *rad6*$_{\Delta 1-9}$.** Some of the *mms2* mutant phenotypes, including the intermediate level of UV sensitivity and increased mutagenesis, are remarkably similar to the *rad6* mutant with an N-terminal deletion, *rad6*$_{\Delta 1-9}$, described by the Prakash laboratories (14). We reasoned that the *rad6*$_{\Delta 1-9}$ mutation may also affect the error-free PRR but not the mutagenesis pathway. A critical experiment would be to see whether *rad6*$_{\Delta 1-9}$ and *mms2* mutations show an epistatic interaction. We constructed a *rad6*$_{\Delta 1-9}$ allele in a pSCW231 plasmid as previously described (14). The resulting plasmid, pSCW-rad6$_{\Delta 1-9}$, was used to transform *rad6*Δ and *mms2 rad6*Δ mutants to create *rad6*$_{\Delta 1-9}$ and *mms2 rad6*$_{\Delta 1-9}$ strains, respectively. While *rad6*$_{\Delta 1-9}$ partially rescued *rad6* sensitivity (cf. Fig. 2*A* and Fig. 4*A*), epistatic analysis (Fig. 4) showed that the *mms2 rad6*$_{\Delta 1-9}$ double mutant was as sensitive to killing by both UV (Fig. 4*A*) and MMS (Fig. 4*B*) as was the *rad6*$_{\Delta 1-9}$ single mutant. Thus, *mms2* not only is hypostatic to *rad6*Δ it is also hypostatic to the *rad6*$_{\Delta 1-9}$ deletion with respect to both UV and MMS sensitivity.

DISCUSSION

Several lines of direct and indirect evidence support the assertion that the *MMS2* gene belongs to the *RAD6* epistasis group. First, the *mms2* null mutant is sensitive to killing by both MMS and UV at the level characteristic of a *RAD6*-pathway mutant. This is in contrast to the *rad3*-pathway mutants, which are especially sensitive to UV and chemicals that produce structurally distorting lesions, but are marginally sensitive to MMS; and to the *rad52*-pathway mutants, which are extremely sensitive to ionizing radiations and MMS, but less sensitive to UV compared with mutants belonging to the other two pathways (9, 24, 27). Second, the deduced Mms2 is homologous to all Ubcs, indicating its possible involvement in ubiquitination, an activity required for the *RAD6* DNA repair pathway. Finally, the *mms2* mutation is hypostatic to both *rad6* and *rad18*, but is additive to *rad4* and *rad50*, suggesting that it is defective in the *RAD6* pathway. Within the *RAD6* pathway, *mms2* is hypostatic to the N-terminal deletion *rad6*$_{\Delta 1-9}$ but is synergistic with the *rev3* mutation. These seemingly controversial results are reconcilable, taking into account the unique ability of *RAD6* to control two parallel subpathways, namely, error-free PRR and mutagenesis. While the mutagenesis pathway is known to be mediated by *REV3*, encoding the catalytic subunit of Polζ, as well as other genes such as *REV1* (40, 41) and *REV7* (23), little is known about the error-free PRR pathway. Our declaration that *MMS2* functions in an error-free PRR pathway alternative to the error-prone pathway is based on the combined evidence that *MMS2* belongs to the *RAD6* epistasis group, that the *mms2* mutant does not affect *REV3*-mediated spontaneous and UV-induced mutagenesis, and that the *mms2* mutation is synergistic with the *rev3* mutation. Our analysis of the *rad6*$_{\Delta 1-9}$ mutation, along with the results of others (14), also demonstrated that, like *MMS2*, the N terminus of Rad6 is required for error-free PRR but not for mutagenesis. The DNA repair phenotypes of *mms2* are also strikingly similar to the phenotype of the *pol30-46* mutant, including the level of UV sensitivity, epistasis to *rad6* and *rad18*, and synergism with *rev3* (25). It is interesting to note that a *pol3-3* (polδ) mutant at a restrictive temperature impairs PRR (26) and that a *pol3-13* mutant has been characterized as having a defect in the *RAD6* pathway (42). Very recently, an *S. cerevisiae RAD30* gene, encoding an *E. coli* DinB and UmuC homolog, was reported to function in the error-free PRR pathway (43). However, unlike *mms2*, the *rad30* mutation does not seem to increase spontaneous mutation rate, nor does it show a synergistic interaction with *rev3* with respect to UV sensitivity (43). Nevertheless, it will be of great interest to extend epistatic analysis to encompass *mms2, rad6*$_{\Delta 1-9}$, *pol30-46*, *pol3-13,* and *rad30* to determine whether they do in fact belong to the same epistasis subgroup.

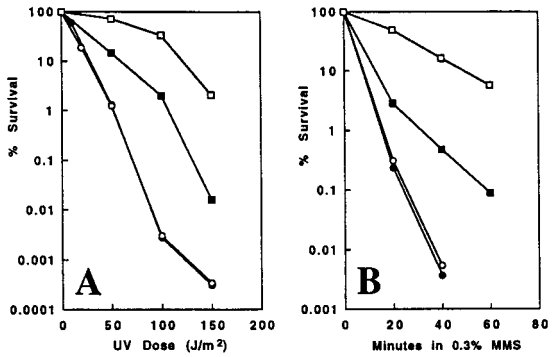

FIG. 4. *mms2* is hypostatic to *rad6*$_{\Delta 1-9}$ with respect to both UV (*A*) and MMS (*B*) sensitivity. □, DBY747 (wild type); ■, SBU (*mms2*); ○, WXY9376/pSCW-rad6$_{\Delta 1-9}$ (*rad6*$_{\Delta 1-9}$); ●, SBU6L/pSCW-rad6$_{\Delta 1-9}$ (*mms2 rad6*$_{\Delta 1-9}$). The results are an average of two independent experiments.

Although the *mms2* single mutant is only moderately sensitive to MMS and UV, we argue that the *mms2* mutation, and probably *rad6*$_{\Delta 1-9}$ and *pol30-46* as well, may be completely defective in the error-free PRR pathway. This argument is derived from results demonstrating that the *mms2 rev3* and *pol30-46 rev3* double mutants are as sensitive to killing by UV as their respective *rad18* single mutants. In other words, simultaneous defects in both error-free PRR (*mms2* or *pol30-46*) and mutagenesis (*rev3*) branches are equivalent to a mutation (*rad18*) known to be defective in both pathways. Furthermore, we have shown that the increased spontaneous mutation rate observed in the *mms2* mutant is completely dependent on the functional *REV3*, indicating that when error-free PRR is dysfunctional, all the spontaneous lesions could be channeled to the mutagenesis pathway. A working model is presented in Fig. 5 to illustrate our current understanding of the *RAD6* DNA repair pathway. PCNA and Polδ likely function in the late stage of PRR for DNA synthesis. Considering that Ubcs may form either a heterodimer (44, 45) or a homodimer (46), and that Ubc-like proteins are similar to Ubcs not only in sequence but also in predicted secondary and tertiary structure (38), it is possible that Mms2 physically interacts with Rad6 to direct the Rad6–Rad18 complex to PRR, and that this interaction may require the Rad6 N terminus. The N terminus of Rad6 is also essential for its interaction with Ubr1 and for sporulation (14, 15). Indeed, we found that *mms2* mutant strains are partially defective in both N-end-rule protein degradation and

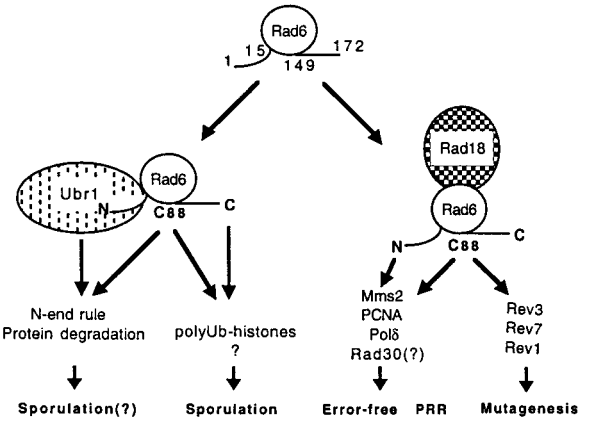

FIG. 5. Schematic diagram of possible Rad6-mediated metabolic pathways. Rad6 forms distinct complexes with either Ubr1 or Rad18; the Rad6–Rad18 complex is proposed to be responsible for DNA repair (19–21). The DNA repair component consists of an N-terminal-dependent error-free PRR pathway and an N-terminal-independent error-prone mutagenesis pathway. Mms2, PCNA, Polδ, and Rad30 are proposed to participate in the error-free PRR, whereas Rev1, -3, and -7 are responsible for mutagenesis.

sporulation (unpublished observations), suggesting that Mms2 may physically interact with Rad6.

We have consistently observed a >30-fold increase in spontaneous mutation rate of the *mms2* mutant. This is compared with a fewfold increase in other DNA repair mutants (e.g., *rad1*Δ, *rad6*Δ, *rad18*Δ, *apn1*Δ, and *mgt1*Δ; data not shown), and an approximately 20-fold increase with the *msh2* DNA mismatch repair mutant (47), in an otherwise isogenic background. Hence, *mms2* is probably one of the most prominent mutators reported in budding yeast. The $rad6_{\Delta 1-9}$ (14) and *pol30-46* (25) mutations also appear to increase the spontaneous mutation frequency, albeit to a lesser degree than *mms2*, a difference due either to experimental systems, including strain backgrounds, different reversion alleles, and experimental protocols, or to a possibility that $rad6_{\Delta 1-9}$ and *pol30-46* mutations affect other cellular processes in addition to error-free PRR.

Genetic instability of the human genome contributes to cancer and other diseases (24, 48). Like other DNA repair pathways (24), the DNA PRR and mutagenesis pathways also appear to be highly conserved from yeast to human. For example, two human Rad6 homologs have been identified (13); inactivation of *HR6B* from mice results in phenotypes parallel to the yeast *rad6* mutant (49). In addition, a *hREV3* cDNA encoding a putative human Polζ has been identified (50). The human *MMS2* homolog, *CROC1,* initially isolated by its ability to transactivate the *c-fos* enhancer element (37) was also found to be down-regulated in human colon carcinoma cells upon chemical-induced differentiation (38) and to be up-regulated when simian virus 40-transformed human embryonic kidney cells became immortal (L. Ma, S.B., C. Lavery, S. Lin, W.X., and S. Bacchetti, unpublished results), suggesting that *CROC1* may play a role in tumorigenesis and carcinogenesis. We have also isolated a cDNA encoding another *MMS2* homolog that is highly related but distinct from *CROC1* (unpublished data). Hence, understanding the role of *MMS2* in error-free PRR and its implication in *REV3* mutagenesis may prove to be relevant to public health.

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