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The *Saccharomyces cerevisiae RAD6* Group Is Composed of an Error-Prone and Two Error-Free Postreplication Repair Pathways

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ABSTRACT

The *RAD6* postreplication repair and mutagenesis pathway is the only major radiation repair pathway yet to be extensively characterized. It has been previously speculated that the *RAD6* pathway consists of two parallel subpathways, one error free and another error prone (mutagenic). Here we show that the *RAD6* group genes can be exclusively divided into three rather than two independent subpathways represented by the *RAD5*, *POL30*, and *REV3* genes; the *REV3* pathway is largely mutagenic, whereas the *RAD5* and the *POL30* pathways are deemed error free. Mutants carrying characteristic mutations in each of the three subpathways are phenotypically indistinguishable from a single mutant such as *rad18*, which is defective in the entire *RAD6* postreplication repair/tolerance pathway. Furthermore, the *rad18* mutation is epistatic to all single or combined mutations in any of the above three subpathways. Our data also suggest that *MMS2* and *UBC13* play a key role in coordinating the response of the error-free subpathways; Mms2 and Ubc13 form a complex required for a novel polyubiquitin chain assembly, which probably serves as a signal transducer to promote both *RAD5* and *POL30* error-free postreplication repair pathways. The model established by this study will facilitate further research into the molecular mechanisms of postreplication repair and translesion DNA synthesis. In view of the high degree of sequence conservation of the *RAD6* pathway genes among all eukaryotes, the model presented in this study may also apply to mammalian cells and predicts links to human diseases.

THE *Saccharomyces cerevisiae RAD6* DNA postreplication repair (PRR) and mutagenesis pathway consists of *RAD5*(*REV2*), *RAD6*(*UBC2*), *RAD18*, *REV1*, *REV3*, and *REV7* (Lawrence 1994; Friedberg *et al.* 1995). It is now generally agreed that the Rad18 single-stranded DNA-binding protein (Bailly *et al.* 1994) and the Rad6 ubiquitin-conjugating enzyme (Jentsch *et al.* 1987) form a stable complex (Bailly *et al.* 1994, 1997a,b), which is required for both PRR and mutagenesis. The mutagenesis pathway (*rev*) mutants were initially isolated by their reduced mutations after UV treatment (Lemontt 1971, 1972). *REV1* encodes a deoxycytidyl transferase (Nelson *et al.* 1996a) with a stretch of amino acid sequence homologous to *Escherichia coli* UmuC (Larimer *et al.* 1989). *rev2* did not reduce mutation frequency in most mutagenesis assays and is allelic to *RAD5*, encoding a protein with DNA helicase and zinc-binding domains (Johnson *et al.* 1992) and DNA-dependent ATPase activity (Johnson *et al.* 1994). *REV3* encodes the catalytic subunit of a nonessential DNA polymerase ζ (Morrison *et al.* 1989; Nelson *et al.* 1996b). Purified Polζ (consisting of Rev3 and Rev7) is capable of bypassing thymine dimers more efficiently than Polα (Nelson *et al.* 1996b). Thus, the yeast mutagenesis pathway appears to rely on a specific DNA polymerase (Polζ) to bypass DNA replication blocks at the cost of increased mutations.

A large body of evidence argues for the existence of an error-free PRR pathway distinct from mutagenesis. The repair pathway mediated by the *RAD5* gene is referred to as error free, since deletion of *RAD5* does not strongly interfere with UV-induced mutagenesis; however, the *rad5* mutation limits instability of simple repetitive sequences (Johnson *et al.* 1992) and enhances nonhomologous end-joining of double-strand breaks (Ahne *et al.* 1997). In addition, several yeast genes have been recently reported to belong to the *RAD6* pathway and participate in error-free PRR. First, an allele-specific *POL30* mutation, *pol30-46*, is epistatic to *rad6* and *rad18*, but is synergistic with *rev3.* The *pol30-46* mutant is normal in UV-induced mutagenesis and DNA synthesis but displays significantly reduced PRR activity (Torres-Ramos *et al.* 1996). *POL30* is essential and encodes proliferating cell nuclear antigen (PCNA) required for both Polδ and Polε DNA synthesis (Prelich *et al.* 1987; Lee *et al.* 1991; Ayyagari *et al.* 1995). Inactivation of Polδ, but not Polε, results in impaired PRR activity (Torres-Ramos *et al.* 1997). Hence, PCNA and Polδ may be required in the later stages of error-free PRR. Second, the *RAD30* gene is placed into the error-free PRR path-

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way on the basis of genetic analysis of the *rad30* mutant (McDonald *et al.* 1997). *RAD30* encodes a novel DNA polymerase (Polη), which is homologous to the *E. coli* DinB, UmuC, and *S. cerevisiae* Rev1, and can efficiently bypass a thymine-thymine dimer *in vitro* with high fidelity (Johnson *et al.* 1999b). Mutations in its human homolog *hRAD30* were found in all XP-V patients (Johnson *et al.* 1999a; Masutani *et al.* 1999b) whose cells display defective Polη activity (Masutani *et al.* 1999a). Third, strains with a mutation in a newly identified *MMS2* gene encoding a Ubc-like protein were found to share many phenotypes with *pol30-46* (Broomfield *et al.* 1998). In addition, the *mms2* mutant exhibited significantly increased spontaneous mutation rates in a *REV3*-dependent manner (Broomfield *et al.* 1998; Xiao *et al.* 1999), which would be expected if *MMS2* plays a role in error-free PRR parallel to the *REV3* mutagenesis pathway. More recently, Mms2 and Ubc13 have been shown to form a complex, which is responsible for *in vitro* Lys-63 ubiquitin chain assembly (Hofmann and Pickart 1999). It has been proposed that this unique Lys-63 polyubiquitination on target protein(s) may be part of a novel signal transduction mechanism to recruit PRR proteins to the site of DNA damage (Hofmann and Pickart 1999). To understand how the error-free PRR pathway is constituted, whether or not the above error-free PRR genes belong to the same pathway, and how enzymatic activities associated with these gene products contribute to error-free PRR, we conducted extensive genetic analysis in the hope of defining subpathways within the *RAD6* group. Our results support a model in which the *RAD6/RAD18* PRR/mutagenesis pathway consists of three rather independent subpathways represented by *REV3*, *RAD5*, and *POL30*. In addition, *MMS2* and *UBC13* may be required for both *RAD5* and *POL30* error-free PRR pathways. In contrast, the *RAD30* gene plays a rather minor and specific role in the protection of yeast cells from UV damage and does not appear to belong to any of the above subpathways.

MATERIALS AND METHODS

Yeast strains and cell culture: Haploid *S. cerevisiae* strains used in this study are listed in Table 1. Three parental strains used in this study are DBY747, originally obtained from Dr. D. Botstein (Stanford University); BY448, from Dr. B. Andrews (University of Toronto, Canada); and PY39-0, from Dr. Burgers (Washington University, St. Louis). Other strains are all isogenic derivatives of the above strains created by targeted gene disruption. Yeast cells were cultured at 30° in either a rich YPD medium or a synthetic SD medium supplemented with various nutrients (Sherman *et al.* 1983). Intact yeast cells were transformed by a modified lithium acetate method. For one-step targeted gene disruption (Rothstein 1983), plasmid DNA containing the desired disruption cassette was cleaved with restriction enzymes prior to yeast transformation. All targeted gene disruption mutants were confirmed by Southern hybridization prior to phenotypic analysis.

Plasmids and plasmid construction: A plasmid containing the *rev3Δ::LEU2* cassette was obtained from Dr. A. Morrison (National Institute of Environmental Health Sciences). The *rev3Δ::LEU2* cassette contains the *REV3* coding region (Morrison *et al.* 1989) with an internal 1.7-kb *Sna*BI fragment replaced by a 2.1-kb fragment containing the *LEU2* gene (A. Morrison, personal communication). Plasmid pBJ22 containing the *rad5Δ::hisG-URA3-hisG* (Johnson *et al.* 1992) was received from Dr. L. Prakash (University of Texas Medical Branch, Galveston). Plasmid prad18Δ1 containing the *rad18Δ::LEU2* cassette (Fabre *et al.* 1989) was obtained from Dr. B. Kunz (Deakin University, Geelong, Victoria, Australia). Strategies for creating *mms2::LEU2* (Broomfield *et al.* 1998) and *mms2Δ::HIS3* (Xiao *et al.* 1999) mutations are as previously described.

The *ubc13* disruption cassettes were made as follows. A 1.7-kb yeast genomic DNA at the *UBC13* coding region was PCR amplified with oligonucleotides UBC13-1(5′-CTTGGGCATG CTGACAATG-3′) and UBC13-2 (5′-CGGAATTAAACGTG GACCC-3′). After *Sph*I-*Xho*I digestion, the DNA fragment was cloned into *Sph*I-*Sal*I sites of pTZ18R (Pharmacia, Piscataway, NJ). A 0.8-kb *Bss*HII-*Nru*I fragment containing essentially the entire *UBC13* coding region from the resulting pTZ-UBC13 was deleted and converted into a *Bgl*II site with a *Bgl*II linker to form pubc13ΔBg. *Bgl*II-linearized pubc13ΔBg was used as a vector to clone either a 1.16-kb *Bam*HI fragment from YDp-H or a 1.6-kb *Bam*HI fragment from YDp-L (Berben *et al.* 1991) to form pubc13Δ::HIS3 and pubc13Δ::LEU2, respectively. The *ubc13Δ::HIS3* and *ubc13Δ::LEU2* cassettes were released by *Xba*I-*Mlu*I digestion.

The *rad30* disruption cassettes were made as follows. Plasmid pJM80 (McDonald *et al.* 1997) was a gift from Dr. R. Woodgate (National Institute of Child Health and Human Development, National Institutes of Health). The 2.46-kb *RAD30* PCR product was isolated from pJM80 as an *Spe*I fragment and cloned into the *Spe*I site of pBlueScript (Strategene, La Jolla, CA). A 1.0-kb *Afl*II fragment within *RAD30* was deleted and replaced by a *Bgl*II linker to form prad30ΔBg. *Bgl*II-linearized prad30ΔBg was used as a vector to clone either the 1.6-kb *Bam*HI fragment from YDp-L (Berben *et al.* 1991) or the 3.8-kb *Bam*HI-*Bgl*II fragment from pNKY51 (Alani *et al.* 1987) to form prad30Δ::LEU2 and prad30Δ::hisG-URA3-hisG, respectively. The *rad30Δ::LEU2* disruption cassette was released by *Stu*I-*Nar*I digestion and the *rad30Δ::hisG-URA3-hisG* disruption cassette was released by *Ssp*I digestion.

Cell killing by DNA-damaging agents: Methyl methanesulfonate (MMS) and UV-induced quantitative killing experiments were performed at 30° in YPD. Overnight yeast cultures were used to inoculate fresh YPD at a 10-fold dilution and cells were allowed to grow for another 4–6 hr. For MMS treatment, MMS was added to the culture at a final concentration as specified and aliquots were taken at given intervals. Cells from each sample were collected via centrifugation, washed, diluted, and plated in duplicate on YPD. For UV treatment, cells were plated in duplicate at different dilutions and then exposed to 254 nm UV light in a UV crosslinker (Fisher Science model FB-UVXL-1000 at $\sim$2400 $\mu W/cm^2$) at given doses in the dark. The colonies were counted after a 3-day incubation. Untreated cells were also plated and scored as 100% survival.

MMS-induced killing was also measured by a gradient plate assay. Thirty milliliters of molten YPD agar were mixed with the appropriate concentration of MMS to form the bottom layer; the gradient was created by pouring the media into tilted square petri dishes. After brief solidification, the petri dish was returned flat and 30 ml of the same molten agar without MMS was poured to form the top layer. A 0.1-ml sample was taken from an overnight culture, mixed with 0.9 ml of molten 1% agar, and immediately imprinted onto freshly

TABLE 1

***Saccharomyces cerevisiae* strains**

Strain	Genotype	Source
DBY747	*MAT***a** *his3-Δ1 leu2-3,112 ura3-52 trp1-289*	D. Botstein
WXY326	DBY747 with *rad18Δ::LEU2*	Lab stock
WXY376	DBY747 with *rad6Δ::LEU2*	Lab stock
WXY382	DBY747 with *rev3Δ::LEU2*	Lab stock
WXY642	DBY747 with *mms2Δ::HIS3*	Lab stock
WXY850	DBY747 with *ubc13Δ::LEU2*	This study
WXY861	DBY747 with *mms2Δ::HIS3 ubc13Δ::LEU2*	This study
WXY862	DBY747 with *rev3Δ::LEU2 ubc13Δ::HIS3*	This study
WXY731	DBY747 with *rad5Δ::hisG-URA3-hisG*	This study
WXY732	DBY747 with *mms2::LEU2 rad5Δ::hisG-URA3-hisG*	This study
WXY736	DBY747 with *rev3Δ::LEU2 rad5Δ::hisG-URA3-hisG*	This study
BY448	*MATα leu2-Δ1 his3Δ200 ura3-52 trp1Δ ade2-107 lys2-1*	B. Andrews
T43	BY448 with *mms2Δ::TRP1*	Lab stock
WXY724	BY448 with *rad30Δ::HIS3*	This study
WXY725	BY448 with *mms2Δ::TRP1 rad30Δ::HIS3*	This study
PY39-0	*MATα ura3-52 trp1-Δ901 leu2-3,112 can1 pol30-Δ1* [pBL230(*POL30 TRP1*)]	P. Burgers
PY39-46	PY39 with pBL230-46 (*pol30-46 TRP1*) instead of pBL230	P. Burgers
WXY857	PY39-0 with *rad5Δ::hisG-URA3-hisG*	This study
WXY858	PY39-46 with *rad5Δ:: hisG-URA3-hisG*	This study
WXY859	PY39-0 with *mms2::LEU2*	This study
WXY860	PY39-46 with *mms2::LEU2*	This study
WXY880	PY39-46 with *rad5Δ:: hisG-URA3-hisG mms2::LEU2*	This study
WXY876	PY39-0 with *rad18Δ::LEU2*	This study
WXY879	PY39-46 with *rad5Δ:: hisG-URA3-hisG rad18Δ::LEU2*	This study
WXY887	PY39-46 with *rad5Δ:: hisG-URA3-hisG rev3Δ::LEU2*	This study
WXY1004	PY39-0 with *rad30Δ::hisG-URA3-hisG*	This study
WXY1005	PY39-46 with *rad30Δ::hisG-URA3-hisG*	This study
WXY1006	PY39-0 with *rad5Δ::hisG-URA3-hisG rad30Δ::LEU2*	This study

made gradient plates via a microscope slide. Gradient plates were incubated at 30° for the time indicated before taking photographs.

RESULTS

***MMS2* and *UBC13* belong to the same error-free PRR pathway:** It was recently reported (Hofmann and Pickart 1999) that Ubc13 and Mms2 form a complex *in vitro*, which is involved in the ubiquitin chain assembly through lysine 63. Epistatic analyses of yeast *ubc13* and *mms2* mutations also suggest that these two genes belong to the same pathway (Hofmann and Pickart 1999). We deleted *UBC13* from various mutant strains to further characterize the *UBC13* gene function using the same criteria that defined *MMS2.* The *ubc13* deletion mutant was indeed moderately sensitive to UV (data not shown) and to MMS (Figure 1A). Like *mms2*, the *ubc13* mutation is synergistic with *rev3* (Figure 1A) and belongs to the *RAD6* pathway (Brusky *et al.* 2000). On a 0.005% MMS gradient plate, both *ubc13* and *rev3* single mutants grow to full length, whereas the *ubc13 rev3* double mutant does not grow at all. These results are consistent with a recent report (Brusky *et al.* 2000) placing *UBC13* within the error-free PRR pathway. The *ubc13* mutant appears to be slightly more sensitive to MMS than either the *mms2* single mutant or the *mms2 ubc13* double mutant by a gradient plate assay (Figure 1A) and this result is reproducible, suggesting that *mms2* is epistatic to *ubc13.* A similar result was also observed by UV killing (Hofmann and Pickart 1999). These results would indicate that *MMS2* acts upstream of *UBC13*, which is inconsistent with the model (Hofmann and Pickart 1999) in which the Ubc13-Mms2 complex formation is required for its function(s). Furthermore, *ubc13*, *mms2*, and *ubc13 mms2* mutants are indistinguishable in an MMS-induced liquid killing experiment (Brusky *et al.* 2000). Hence, the significance of the observed difference and genetic interactions between *UBC13* and *MMS2* remains to be elucidated. It should be borne in mind that although the *ubc13 rev3* double mutant is strikingly more sensitive to DNA-damaging agents than its respective single mutants, it is still less sensitive than the *rad6* or *rad18* single mutant to killing by UV (Brusky *et al.* 2000) and MMS (Figure 1B). Under conditions of extremely low concentration of MMS (0.001%) and extended time of incubation, the *ubc13 rev3* double mutant grows to full length, while *rad6* and *rad18* mutants only grow partially. The same phenomenon was also

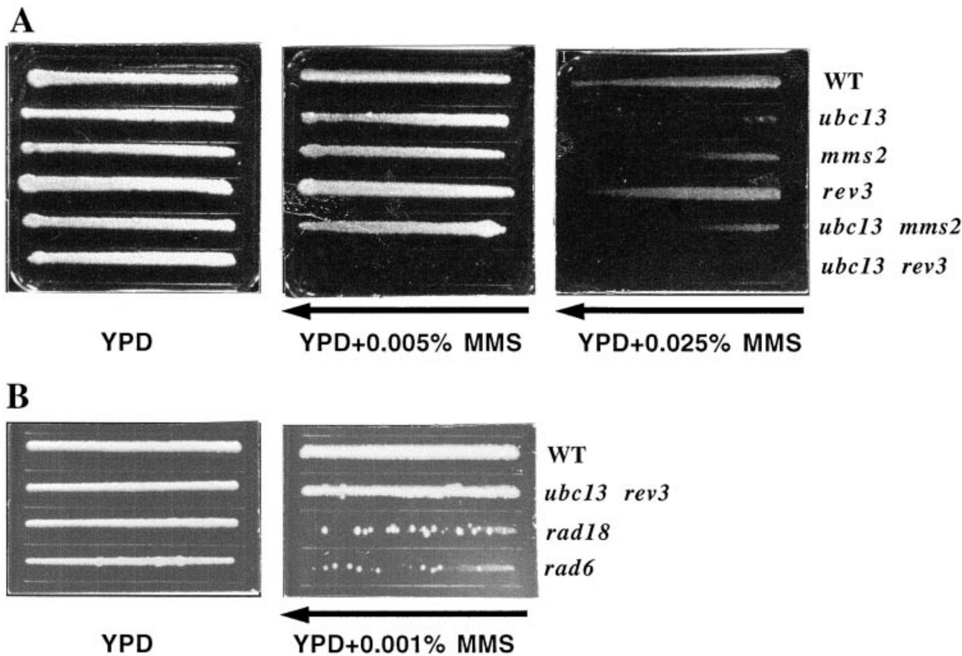

Figure 1.—Phenotypes of the *ubc13* mutants by a gradient plate assay. Yeast cells were printed onto YPD or YPD gradient plates containing different concentrations of MMS as indicated, and the plates were photographed after (A) 42 hr or (B) 63 hr incubation at 30°. Strain genotypes are indicated. All the strains are isogenic derivatives of DBY747. The arrow points toward higher MMS concentration. DBY747 (WT); WXY850 (*ubc13*Δ); WXY642 (*mms2*Δ); WXY382 (*rev3*Δ); WXY861 (*mms2*Δ *ubc13*Δ); WXY862 (*rev3*Δ *ubc13*Δ); WXY326 (*rad18*Δ) and WXY376 (*rad6*Δ). Individual colonies along the length of the MMS plate in (B) are revertants in the *rad6* and *rad18* mutants and have been repeatedly observed; these revertants are probably derived from *srs2/radH* mutations that suppress *rad6* and *rad18* sensitivity to DNA-damaging agents (Lawrence and Christensen 1979; Aboussekhra *et al.* 1989).

observed for the *mms2 rev3* double mutant (Broomfield *et al.* 1998; Xiao *et al.* 1999).

***RAD5* and *POL30* represent two distinct error-free PRR pathways:** Both *rad5* (Johnson *et al.* 1992) and *pol30-46* (Torres-Ramos *et al.* 1996) mutations are additive to *rad3* and *rad52* group mutations and synergistic with *rev3* and both genes belong to the *RAD6/RAD18* pathway. In addition, *rad5* and *pol30-46* mutants are normal in UV-induced mutagenesis. These observations place *RAD5* and *POL30* within the error-free PRR pathway. To see if these two genes act in the same PRR pathway, we created isogenic single and double mutant strains and found that when the *rad5* and *pol30-46* mutations are combined, the double mutant is extremely sensitive to killing by either UV (Figure 2A) or MMS (Figure 2B), and the effect is considered to be highly additive (UV) or synergistic (MMS). This result would agree with the notion that *RAD5* and *POL30* constitute two parallel error-free PRR pathways within the *RAD6/RAD18* pathway.

***MMS2* is common to the *RAD5* and *POL30* pathways:** To see if either *RAD5* or *POL30* acts in the same pathway as *MMS2/UBC13*, we performed epistatic analyses with respect to killing by either UV or MMS. The *rad5* mutant is significantly more sensitive to UV (Figure 3A) and to MMS (Figure 3B) than its isogenic *mms2* mutant; nevertheless, the *rad5 mms2* double mutant is more sensitive than either of the corresponding single mutants, and the killing effect appears to be simply additive. This result indicates that *MMS2* and *RAD5* act in related but distinct pathways, although it does not rule out the possibility of overlapping functions. Similarly, inactivation of the *mms2* gene enhances *pol30-46* mutant sensitivity to either UV (Figure 3C) or MMS (Figure 3D) to a comparable extent as it does to the *rad5* mutant, suggesting that *MMS2* and *POL30* act in different or overlapping error-free PRR pathways. It should be noted that the *POL30* gene is essential for cell survival and that *pol30-46* may be a partial loss-of-function mutation with respect to error-free PRR. On the other hand, Pol30/PCNA also physically interacts with factors in-

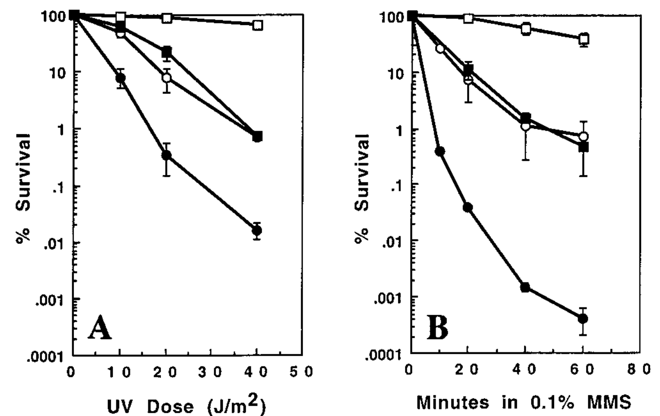

Figure 2.—*RAD5* and *POL30* belong to different DNA repair pathways. (A) UV-induced killing; (B) MMS-induced killing. (□) PY39-0 (wt); (■) PY39-46 (*pol30-46*); (○) WXY857 (*rad5*Δ); (●) WXY858 (*pol30-46 rad5*Δ). All the results are the average of at least three independent experiments with standard deviations.

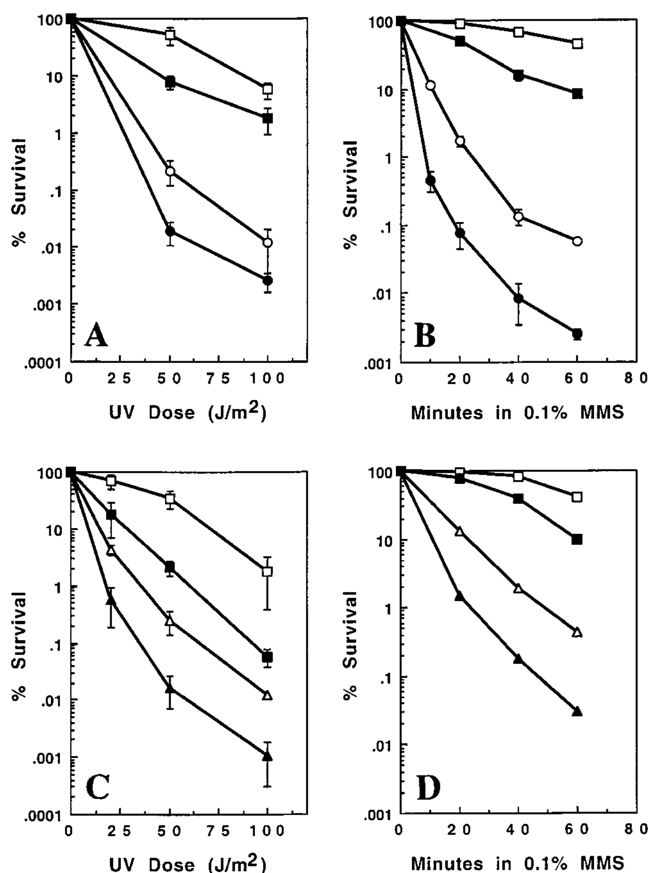

Figure 3.—Genetic interactions of *MMS2* with *RAD5* and *POL30* pathways. (A and B) *rad5 vs. mms2*. (□) DBY747 (wt); (■) WXY642 (*mms2*Δ); (○) WXY731 (*rad5*Δ); and (●) WXY732 (*rad5*Δ *mms2*Δ). (C and D) *pol30-46 vs. mms2*. (□) PY39-0 (wt); (■) WXY859 (*mms2*); (△) PY39-46 (*pol30-46*); (▲) WXY860 (*pol30-46 mms2*). All the results are the average of at least three independent experiments with standard deviations except D, which was from two sets of experiments.

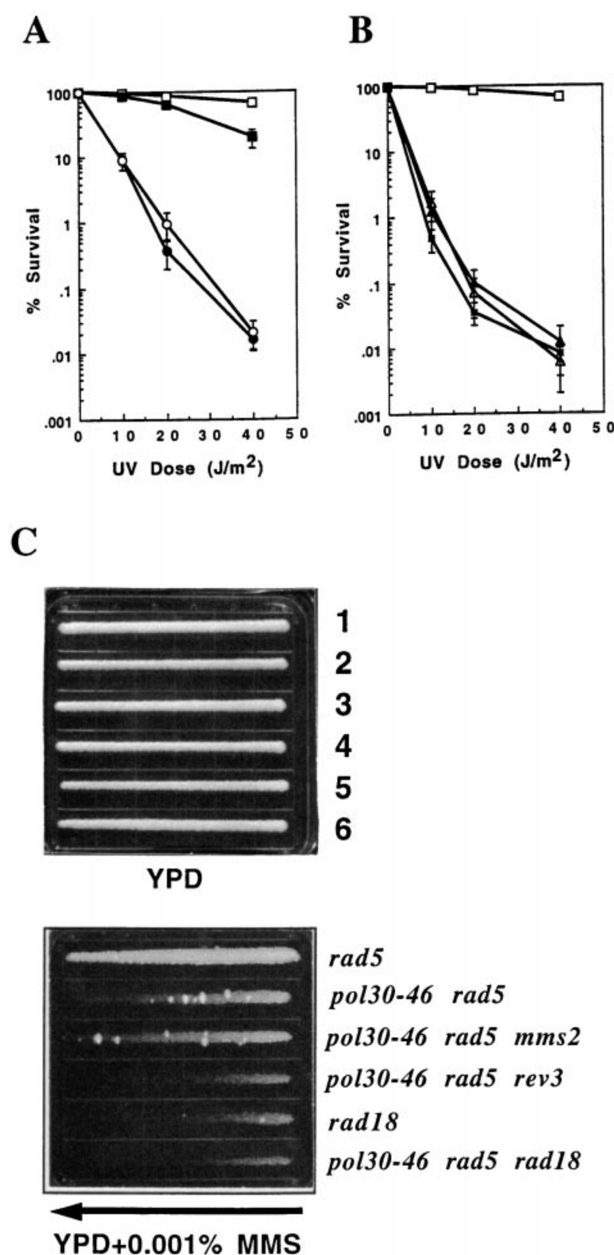

Figure 4.—Epistatic analyses with the *rad18* mutation. (A and C) *mms2* is epistatic to the *rad5 pol30-46* double mutations. (B and C), *rad5*, *pol30-46*, and *rev3* are epistatic to *rad18*. (A and B) UV-induced killing. (□) PY39-0 (wt); (■) WXY859 (*mms2*); (●) WXY858 (*pol30-46 rad5*Δ); (○) WXY880 (*pol30-46 rad5*Δ *mms2*); (▲) WXY876 (*rad18*Δ); (✻) WXY879 (*pol30-46 rad5*Δ *rad18*Δ); and (△) WXY887 (*pol30-46 rad5*Δ *rev3*Δ). All the strains are isogenic to PY39-0, and results presented in A and B are from the same sets of experiments with standard deviations. (C) MMS-induced killing by a gradient plate assay. The gradient plates were photographed after 70 hr incubation at 30°. Lane 1, WXY857; lane 2, WXY858; lane 3, WXY880; lane 4, WXY887; lane 5, WXY876; and lane 6, WXY879. Strain genotypes are indicated at the bottom.

volved in nucleotide excision repair (Gary *et al.* 1997), mismatch repair (Johnson *et al.* 1996; Umar *et al.* 1996), and base excision repair (Li *et al.* 1995; Wu *et al.* 1996), which may further complicate the above epistatic analyses.

Although *MMS2* is not assigned to either the *RAD5* or the *POL30* pathway, it may belong to both error-free PRR pathways. This hypothesis is consistent with the observed additive effects between *mms2* and *rad5* or *pol30-46* single mutations (Figure 3). Indeed, the *rad5 pol30-46 mms2* triple mutant is no more sensitive than the *rad5 pol30-46* double mutant to either UV (Figure 4A) or MMS (Figure 4C). We therefore propose that the Ubc13/Mms2 complex promotes both error-free PRR pathways represented by Rad5 and PCNA. In this model, Ubc13/Mms2 may act as a signal transducer to sense DNA damage or stalled replication, but is not absolutely required for the PRR activity via either Rad5 or PCNA.

***RAD30* is specific for UV damage and is distinct from all PRR pathways:** *RAD30* encodes a novel DNA polymerase, Polη, which is able to synthesize DNA *in vitro* past thymine-thymine dimers in an error-free manner (Johnson *et al.* 1999c). Previous epistatic analyses

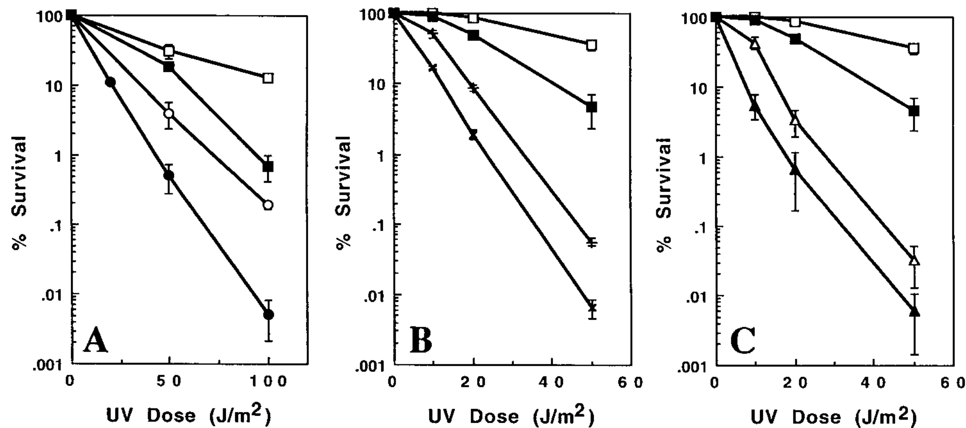

Figure 5.—The *rad30* mutation is additive to *mms2* (A), *rad5* (B), and *pol30-46* (C) mutations with respect to killing by UV. (A) BY448 derivatives. (□) BY448 (wt); (○) T43 (*mms2*Δ); (■) WXY724 (*rad30*Δ); and (●) WXY725 (*mms2*Δ *rad30*Δ). (B and C) PY39-0 derivatives. (□) PY39-0 (wt); (△) PY39-46 (*pol30-46*); (+) WXY858 (*rad5*Δ); (■) WXY1004 (*rad30*Δ); (▲) WXY1005 (*pol30-46 rad30*Δ); and (X) WXY1006 (*rad5*Δ *rad30*Δ). All the results are the average of three independent experiments with standard deviations.

placed *RAD30* within the error-free branch of *RAD6* pathway (McDonald *et al.* 1997). However, unlike *mms2*, *ubc13*, *rad5*, and *pol30-46*, which are synergistic with the *rev3* mutation, the *rad30* mutation is only slightly additive to *rev* mutations (McDonald *et al.* 1997 and our unpublished data). Furthermore, the *rad30* mutants are only sensitive to killing by UV, but not to a variety of other DNA-damaging agents including MMS, ionizing radiations, and a UV-mimetic agent 4-nitroquinoline-N-oxide, and do not display an increased spontaneous mutation rate (Roush *et al.* 1998 and our unpublished data). These results suggest that *RAD30* differs from all other *RAD6* pathway genes. Indeed, the *rad30* mutation appears to be simply additive to *mms2* (Figure 5A), *rad5* (Figure 5B), or *pol30-46* (Figure 5C) with respect to killing by UV. We note that McDonald *et al.* (1997) reported a strong synergistic interaction between *rad5* and *rad30* at low UV doses. Our results with 10 J/m^2 UV treatment (Figure 5B) also suggest a synergistic interaction between *rad30* and *rad5.* However, at higher doses, the interaction is apparently additive.

Reconstitution of the *RAD6/RAD18* pathway by three distinct PRR/mutagenesis subpathways: Having established a working hypothesis of two separate error-free PRR pathways, we attempted to construct a comprehensive model for *RAD6/RAD18* PRR and mutagenesis. Both *rad6* and *rad18* mutants are extremely sensitive to killing by a variety of DNA-damaging agents and share other phenotypes such as increased spontaneous mutation rates but decreased UV-induced mutagenesis (Lawrence 1994; Friedberg *et al.* 1995). However, the *RAD6* gene is also involved in functions other than DNA postreplication repair, such as sporulation (Montelone *et al.* 1981), N-end rule protein degradation (Dohmen *et al.* 1991; Sung *et al.* 1991), polyubiquitination of histone H2B (Watkins *et al.* 1993; Robzyk *et al.* 2000), and telomere silencing (Huang *et al.* 1997). The *rad6* mutation also confers a slow-growth phenotype not shared by *rad18* (Lawrence 1994; Friedberg *et al.* 1995). Thus, the *rad18* mutation instead of *rad6* was used to represent complete defects in the PRR and mutagenesis. We have previously shown that yeast cells carrying both *mms2* and *rev3* mutations, although extremely sensitive to either UV or MMS, are still not as sensitive as the *rad18* single mutant (Xiao *et al.* 1999). In the present study, we also found that the *rad18* mutant is more sensitive than the *ubc13 rev3* (Figure 1B) and *rad5 pol30-46* (Figure 4) double mutants. If the *RAD6/RAD18* PRR pathway consists of three subpathways represented by *RAD5*, *POL30*, and *REV3*, the *rad5 pol30-46 rev3* triple mutant would be phenotypically equivalent to the *rad18* single mutant, and the combination of any subpathway mutations with *rad18* will be no more sensitive than the *rad18* single mutant. Indeed, both UV (Figure 4B) and MMS (Figure 4C) killing experiments show that the *pol30-46 rad5 rev3* triple, *pol30-46 rad5 rad18* triple, and *rad18* single mutants are indistinguishable, providing key support to our three-subpathway hypothesis.

DISCUSSION

The yeast *S. cerevisiae* has proved to be a paradigm for the study of DNA repair and mutagenesis in eukaryotes. Of three major DNA radiation damage repair pathways, namely, the *RAD3* nucleotide excision repair, the *RAD6* PRR and mutagenesis, and the *RAD52* recombinational repair pathways, the *RAD6* pathway is the most complicated and least characterized (Friedberg *et al.* 1995). Historically, the *RAD6* pathway has included all *RAD* genes that do not belong to either of the well-defined *RAD3* and *RAD52* groups. However, unlike the *RAD3* pathway mutants, which are extremely sensitive to UV but less sensitive to MMS and ionizing radiation, and *RAD52* pathway mutants, which are extremely sensitive to MMS and ionizing radiation but are less sensitive to UV, the *RAD6* pathway mutants are sensitive to a broad range of DNA-damaging agents that probably share a common feature that inhibits DNA synthesis. It has been proposed that the *RAD6* group consists of more than one subpathway (McKee and Lawrence 1979; Friedberg 1988); however, these subpathways

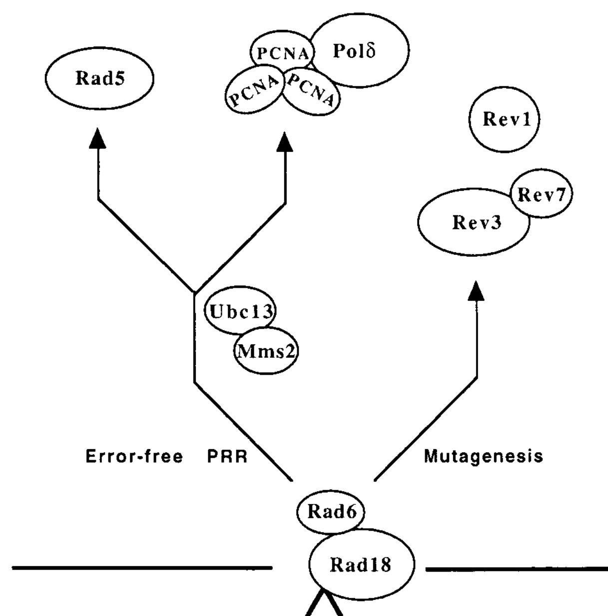

Figure 6.—A model of the error-free postreplication repair and mutagenesis pathways in yeast.

have not been exclusively defined, especially in the branch of error-free PRR. On the basis of previous reports and results obtained from this study, we present a comprehensive model of yeast *RAD6/RAD18* DNA PRR and mutagenesis pathway, which is illustrated in Figure 6. In this model, we propose that the *RAD6* group genes are responsible for the cellular tolerance to a variety of DNA replication-blocking lesions. The *REV1,3,7* genes constitute a well-defined translesion synthesis pathway that replicates bypass lesions with low fidelity (Lawrence and Hinkle 1996). Mutations in the error-free PRR pathway genes are synergistic with *rev* mutations with respect to killing by DNA-damaging agents and are proficient in UV-induced mutagenesis. *RAD5* and *POL30* are assigned to two distinct PRR pathways based on the synergistic interaction between *rad5* and *pol30-46.* Polδ is included along with PCNA on the basis of reports that certain *pol3* (*e.g.*, *pol3-13*) mutations are epistatic to *rad6* (Giot *et al.* 1997) and that Polδ is required for PRR, while Polε is not (Torres-Ramos *et al.* 1997).

Probably the most significant finding of this study is that the *RAD6/RAD18* PRR and mutagenesis pathway can be exclusively defined by three subpathways represented by *REV3*, *RAD5*, and *POL30.* This conclusion is primarily based on the fact that *REV3*, *RAD5*, and *POL30* all belong to the *RAD6* epistasis group (Johnson *et al.* 1992; Lawrence 1994; Friedberg *et al.* 1995; Torres-Ramos *et al.* 1996) and on our observations that *rev3 rad5 pol30-46* and *rad5 pol30-46 rad18* triple mutants and the *rad18* single mutant are indistinguishable in response to killing by either UV or MMS.

Although *RAD30* has been placed in the *RAD6* group (McDonald *et al.* 1997), we are unable to assign it into any of the three existing subpathways. We argue that *RAD30* is probably not a typical *RAD6* pathway gene, since it only protects cells from a specific type of DNA damage. Although *RAD30* functions in an error-free manner, the *rad30* mutation is not synergistic with *rev* mutations (McDonald *et al.* 1997). However, since an allele-specific *pol30-46* mutation instead of the *pol30* null mutation was used in our epistatic analysis, we are unable to rule out the possibility that *RAD30* belongs to the *POL30* subpathway. It is of interest to note that Rad30, like other recently discovered UmuC DNA polymerase superfamily proteins (Nelson *et al.* 1996a; Johnson *et al.* 1999a; Masutani *et al.* 1999b; Tang *et al.* 1999; Wagner *et al.* 1999), synthesizes DNA in a distributive manner (Johnson *et al.* 1999b) and consequently its *in vivo* function may be related to a cognate non-UmuC family DNA polymerase. For instance, UmuC mutagenesis requires PolIII (Friedberg *et al.* 1995) and the Rev1 function is dependent on Rev3 (Wagner *et al.* 1999). Hence, Rad30 may indeed require Polδ for its *in vivo* function.

The PRR and mutagenesis pathway appears to be highly conserved within eukaryotes; thus a model established in budding yeast likely applies to other eukaryotic organisms. Numerous homologs of the *RAD6* pathway genes have been identified in various organisms. In particular, *RAD6*, *POL30*, *MMS2*, *UBC13*, and *REV3* homologs have been reported (Koken *et al.* 1991; Yamaguchi *et al.* 1996; Gibbs *et al.* 1998; Xiao *et al.* 1998a,b; Johnson *et al.* 1999a; Masutani *et al.* 1999b), some of which are able to functionally complement the corresponding yeast defects (Koken *et al.* 1991; Xiao *et al.* 1998b). Furthermore, human cells or animals compromised for the yeast *RAD6* group genes display phenotypes reminiscent of the corresponding yeast mutants (Roest *et al.* 1996; Gibbs *et al.* 1998; Johnson *et al.* 1999a; Masutani *et al.* 1999b). It is of great interest to note that while human diseases have been linked to mutations in both nucleotide excision repair genes (Friedberg *et al.* 1995) and recombination repair genes (Carney *et al.* 1998; Varon *et al.* 1998), as well as a UV-specific PRR gene, *hRAD30* (Johnson *et al.* 1999a; Masutani *et al.* 1999b), there is yet no disease linked to mutations within other PRR pathway genes. This is not to say that the PRR pathway fails to contribute significantly to the protection of cells against DNA damage; on the contrary, the *rad6* and *rad18* mutants are as sensitive to different DNA-damaging agents as any of the other severe DNA repair mutants (Lawrence 1994; Friedberg *et al.* 1995). It is possible that the PRR pathway is of such vital importance that mammalian cells may have developed additional mechanisms to prevent loss of such gene functions. For example, each of the *RAD6* (Koken *et al.* 1991) and *MMS2* (Xiao *et al.* 1998b) genes has two mammalian homologs with >90% amino acid sequence identity and functional redundancy. Furthermore, the important

DNA repair/tolerance genes may have been rendered essential by playing additional roles in mammalian cells. Such examples have been found with members involved in nucleotide excision repair (*e.g.*, *XPB* and *XPD*, de Laat *et al.* 1999), recombination repair (*e.g.*, *hMRE11*, *hRAD50*, and *hRAD51*, Paques and Haber 1999), and base excision repair (*e.g.*, *REF1*, Xanthoudakis *et al.* 1996) pathways and will probably be demonstrated with some PRR pathway genes as well. The elucidation of the yeast PRR and mutagenesis pathways will greatly facilitate the full understanding of this most challenging DNA damage tolerance pathway in eukaryotic cells and shed light on cancer and genetic diseases related to the genetic defects of this pathway.

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