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Requirement of DNA Polymerase η for Error-Free Bypass of UV-Induced CC and TC Photoproducts

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The yeast *RAD30*-encoded DNA polymerase η (Polη) bypasses a *cis-syn* thymine-thymine dimer efficiently and accurately. Human DNA polymerase η functions similarly in the bypass of this lesion, and mutations in human Polη result in the cancer prone syndrome, the variant form of xeroderma pigmentosum. UV light, however, also elicits the formation of *cis-syn* cyclobutane dimers and (6-4) photoproducts at 5′-CC-3′ and 5′-TC-3′ sites, and in both yeast and human DNA, UV-induced mutations occur primarily by 3′ C to T transitions. Genetic studies presented here reveal a role for yeast Polη in the error-free bypass of cyclobutane dimers and (6-4) photoproducts formed at CC and TC sites. Thus, by preventing UV mutagenesis at a wide spectrum of dipyrimidine sites, Polη plays a pivotal role in minimizing the incidence of sunlight-induced skin cancers in humans.

The UV component of sunlight is a major epidemiological risk factor for skin cancers that include melanomas, basal cell carcinomas, and squamous cell carcinomas. In the United States, the frequency of skin cancers approaches that of all other cancers combined and is on the rise because of the depletion of the ozone layer (10, 21, 23). UV-induced DNA lesions are removed by nucleotide excision repair, but if left unrepaired, they present a block to normal DNA replication. The yeast *RAD30* and human *RAD30A* genes encode a DNA polymerase, polymerase η (Polη), which has the unique ability to efficiently and accurately replicate through a UV-induced *cis-syn* thymine-thymine (TT) dimer, and defects in *hRAD30A* cause the variant form of xeroderma pigmentosum (12, 13, 16, 22, 27). Xeroderma pigmentosum XPV patients suffer from highly elevated levels of sunlight-induced skin cancers.

Because of the efficient insertion of As opposite the TT dimer, it has been suggested that Polη is an A rule polymerase (8). In addition to cyclobutane dimers at two adjacent thymines, UV also induces the formation of lesions at dipyrimidine sites that involve a cytosine, most commonly at 5′-TC-3′ and 5′-CC-3′ sequences. In fact, the 3′ cytosine in both sequence contexts is highly mutagenic, and in both yeast and humans, UV-induced mutations occur primarily by a C→T transition that would result from the insertion of an A opposite the 3′ damaged C residue during DNA replication (1, 3, 6). If Polη were an A rule polymerase which inserts an A residue by default opposite the various lesions, then the bypass of a CC or a TC cyclobutane dimer by Polη would be mutagenic, not error free as for the TT dimer.

In addition to *cis-syn* cyclobutane pyrimidine dimers, UV induces the formation of pyrimidine (6-4) pyrimidinone photoproducts. The (6-4) photoproduct is formed most frequently at a TC site, whereas the dimer is formed more frequently than the (6-4) photoproduct at a CC site (2, 4, 5). The C of a *cis-syn* cyclobutane dimer, however, is quite unstable, and in vitro it deaminates to U (24, 25), thus making in vitro bypass studies with TC or CC dimers difficult. Here, we utilize a genetic system designed to test for the role of yeast Polη in the bypass of UV-induced lesions at 5′-TC-3′ and 5′-CC-3′ sites. We find that UV-induced mutations occur at the 3′ C of TC and CC sequences, and importantly, the incidence of these mutations is about fivefold higher in the *rad30*Δ strain than in the wild-type strain. These studies provide evidence for the requirement of Polη in error-free bypass of cyclobutane dimers and (6-4) photoproducts formed at TC and CC sites.

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MATERIALS AND METHODS

Generation of deletions of yeast genes. All yeast strains were derived from EMY74.7 (*MAT***a** *his3*Δ*-100 leu2-3,112 trp1*Δ *ura3-52*). The *rad30*Δ mutation was generated as described previously (14). To generate the *rev3*Δ mutation, a 4.6-kb DNA fragment containing the entire *REV3* gene was first cloned into pUC19. The internal 3.5-kb *Bst*11075 fragment of *REV3* was then replaced by the *URA3* gene blaster fragment, generating plasmid pPM292. This plasmid, when cut with *Eco*RI and *Bam*HI and transformed into yeast, deletes genomic *REV3* from nucleotide +436 to +3928 of the 4,152-bp open reading frame (ORF). To generate the *ura3*Δ mutation, 889-bp and 747-bp PCR products corresponding to the 5′ and 3′ regions, respectively, of the *URA3* gene were amplified from yeast genomic DNA and directionally cloned into pUC19. The yeast *HIS3* gene was then inserted between these two PCR products, generating plasmid pPM1048. This plasmid, when digested with the restriction enzymes *Asp*718 and *Sal*I and transformed into yeast, deletes from nucleotide +24 to +774 of the 804-bp *URA3* ORF. Deletions were confirmed by PCR analysis of yeast genomic DNA. Loss of the *URA3* gene derived from the gene blaster fragment in the various yeast strains was selected for by plating on medium containing 5-fluoro-orotic acid. To determine UV-induced reversion frequencies, yeast strains were transformed to TRP^+ with plasmids pPM1020 and pPM1021, harboring the *ura3-210* and *ura3-364* mutant alleles, respectively, which were constructed as described below.

Construction of the *ura3-210* and *ura3-364* mutations. Mutations were introduced into the *URA3* gene in pUC19-based plasmid YIplac211 (9) using the MORPH system site-specific mutagenesis kit (5 Prime → 3 Prime, Inc., Boulder, Colo.). For the *ura3-210* mutation, the oligonucleotide N4675 (5′-GAAGCATTAGGTCCCAAAATTCGTTTACTAAAAACACATGTGG-3′) was used, and for the *ura3-364* mutation, the oligonucleotide N4676 (5′-CCGCCAAGTACAATTTTTTACCGTTCGAAGACAGAAAATTTGCTG-3′) was used. The *ura3-210* mutation is a T→C missense mutation at position +166 in the *URA3* ORF that creates a Cys_{56}→Arg_{56} change in the encoded protein. The *ura3-364* mutation is a T→C missense mutation at position 263 in the *URA3* gene. In this case, the mutation results in a Leu_{88}→Pro_{88} change in Ura3 protein. The *ura3-364* allele also contains a C→G silent mutation at position 264 within the proline

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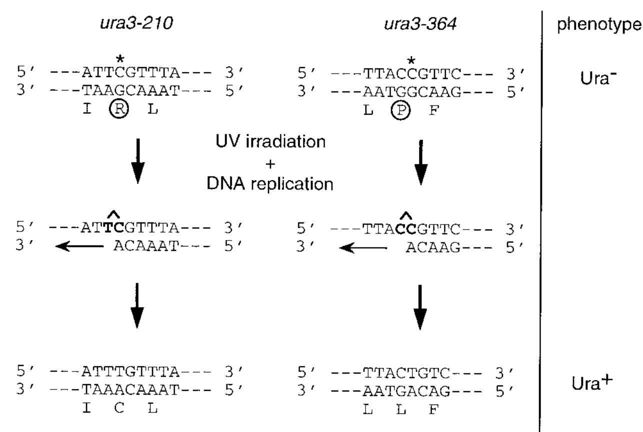

FIG. 1. Reversion assay for *ura3-210* and *ura3-364*. The region of the *URA3* gene corresponding to the *ura3-210* and *ura3-364* mutations is shown. The asterisk indicates the position of a T→C mutation. The amino acid substitution in each mutant is circled. Following UV irradiation, a TC photoproduct in *ura3-210* or a CC photoproduct in *ura3-364* can be formed and is shown in bold with a caret over it. Replication across either lesion by the insertion of an A residue opposite the 3′ C results in reversion of the alleles to a Ura^+ phenotype.

codon that eliminates a second potential CC site. Both alleles were sequenced and found not to contain any other mutations. Subsequently, the *ura3-210* and *ura3-364* alleles were used to replace the wild-type *URA3* allele in the yeast *CEN/ARS* plasmid YCplac33 (9) containing the *TRP1* gene as a selectable marker, generating plasmids pPM1020 and pPM1021, respectively. The phenotype of the *ura3-210* and *ura3-364* alleles was confirmed by the inability of yeast strains harboring either pPM1020 or pPM1021 to grow on media lacking uracil and by resistance to 5-fluoro-orotic acid.

UV mutagenesis and sequence analysis of *URA3* revertants. Yeast strains lacking the genomic *URA3* gene (*ura3Δ::HIS3*) and harboring plasmid pPM1020 or pPM1021 were grown overnight at 30°C in synthetic complete medium lacking tryptophan (SC−trp). Cells were harvested by centrifugation, washed, and resuspended to a density of 10^8 cells/ml in sterile H_2O, and dilutions were plated on the appropriate medium. Plates were irradiated with UV light at a dose of 1 $J/m^2/s$ under yellow light and incubated for 3 to 5 days at 30°C in the dark. For UV-induced mutagenesis, cells were plated on SC−trp for viability determinations and plated on SC−trp that also lacked uracil (SC−trp−ura) to determine reversion frequencies of *ura3-210* and *ura3-364* alleles. The frequency of spontaneous *URA3* revertants was subtracted from the frequency of revertants observed following UV irradiation. To determine the sequence of revertants induced by UV light, plasmid DNA was isolated from UV-induced $URA3^+$ yeast colonies, and the *URA3* gene was amplified by PCR. PCR products were then sequenced in the regions corresponding to the respective mutations using the thermosequenase kit from Amersham and α-^{33}P-labeled dideoxynucleoside triphosphates.

RESULTS

To determine whether Polη is involved in the error-free bypass of UV-induced TC and CC photoproducts, we designed a genetic assay for *Saccharomyces cerevisiae* that utilizes the *ura3-210* and *ura3-364* mutant alleles, which completely inactivate *URA3* function (19). The *ura3-210* allele has a T to C mutation at position 166 in the *URA3* gene, and this mutation is in a 5′-TC-3′ sequence. The resulting mutant protein contains an arginine at position 56 instead of the normal cysteine residue (Fig. 1). Reversion of this mutant allele to the wild-type *URA3* gene occurs by the incorporation of an A residue opposite the 3′ C of the TC UV-induced lesion, restoring the cysteine codon. The *ura3-364* allele is also a T to C mutation, but at position 263 in the *URA3* gene, and this mutation is in a CC sequence. The mutant protein contains a proline at position 88 instead of leucine (Fig. 1). The *ura3-364* allele also reverts to the wild type by the incorporation of an A opposite the 3′ C of the CC UV-induced lesion, but reversion of this allele can also occur by the incorporation of two As opposite the two Cs in the CC sequence.

The *ura3-210* and *ura3-364* alleles were incorporated into a low-copy-number yeast plasmid containing the *TRP1* gene for selection. Wild-type, *rad30Δ*, *rev3Δ*, and *rad30Δ rev3Δ* yeast strains lacking the genomic *URA3* gene but harboring the mutant *ura3* gene on a plasmid were irradiated with UV light, and the frequency of *URA3* revertants was determined. As shown in Fig. 2, UV sensitivity was increased in *rev3Δ* and *rad30Δ* single mutants, and the UV sensitivity of the *rad30Δ rev3Δ* double mutant was greater than that of the single mutants, consistent with the involvement of the *RAD30* and *REV3* genes in alternate pathways for bypassing UV lesions. UV irradiation induces the reversion of the *ura3-210* allele in both the wild-type and *rad30Δ* strains. However, at all UV doses, the reversion frequency was about fivefold higher in the *rad30Δ* strain than in the wild type (Fig. 3), indicating a role for Polη in the incorporation of the correct G residue opposite the 3′ C residue in TC photoproducts. These UV-induced mutations depend upon the *REV3* gene, since few or no mutations occurred in the *rad30Δ rev3Δ* strain (see the legend to Fig. 3). Sequence analysis of 32 independent revertants from the wild-type and *rad30Δ* strains revealed that all UV-induced mutations occur by a 3′ C to T change (Table 1), indicating the incorporation of an A opposite the 3′ C.

The *ura3-364* allele containing the CC sequence also reverts in a UV dose-dependent manner (Fig. 4). In this case, however, the frequency of revertants was about fivefold lower than that for the TC sequence in *ura3-210*. In both yeast and mammalian cells, UV-induced TC photoproducts are often much

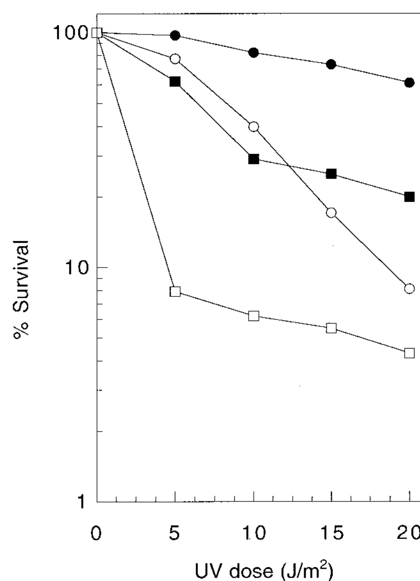

FIG. 2. Survival after UV irradiation of various yeast strains lacking the genomic *URA3* gene and harboring the plasmid pPM1020, which carries the *ura3-210* mutation. Cells were UV irradiated and plated on SC−trp for viability determinations. Each curve represents the average of three independent experiments. Symbols: ●, wild type; ■, *rev3Δ*; ○, *rad30Δ*; □, *rad30Δ rev3Δ*.

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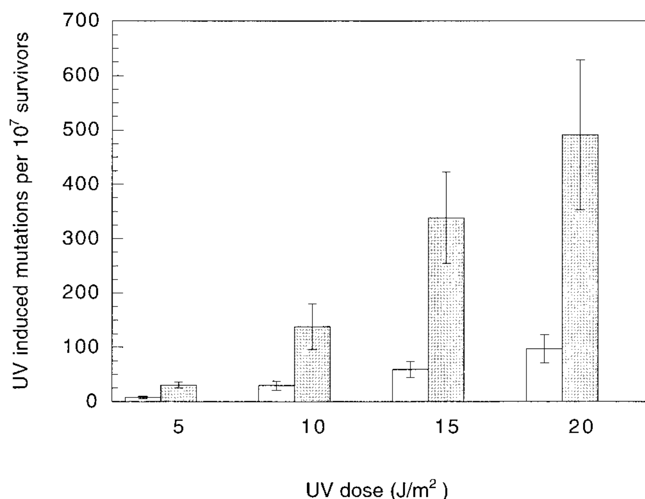

FIG. 3. UV-induced reversion of the *ura3-210* allele in wild-type (white bar), *rad30*Δ (gray bar), and *rad30*Δ *rev3*Δ yeast strains. Yeast strains lacking the genomic *URA3* gene and harboring plasmid pPM1020, which carries the *ura3-210* mutation, were UV irradiated, and the average frequency of *URA3* revertants was determined from results of three independent experiments. Spontaneous mutation frequencies ranged from 0.1×10^{-7} to 1×10^{-7} for the various experiments and were similar for the different strains. The standard error is shown for each determination. The frequency of UV-induced revertants at 5, 10, 15, and 20 J/m^2 for the *rad30*Δ *rev3*Δ strain was ≤1 in 10^7. Also, few or no UV-induced revertants were observed for the *rev3*Δ strain.

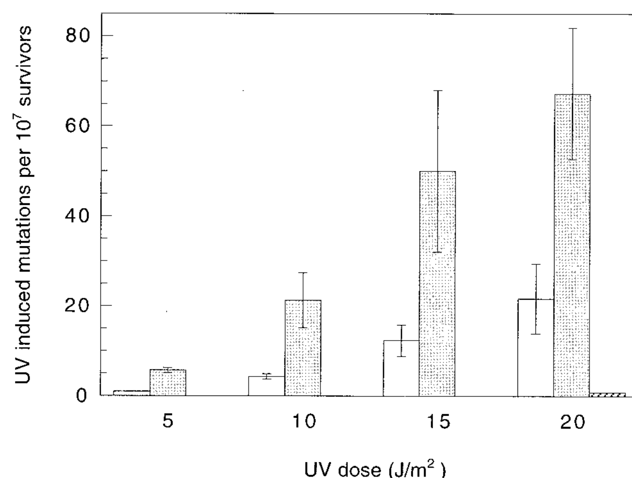

FIG. 4. UV-induced reversion of the *ura3-364* allele in wild-type (white bar), *rad30*Δ (gray bar), and *rad30*Δ *rev3*Δ (hatched bar) strains. Yeast strains lacking the genomic *URA3* gene and harboring plasmid pPM1021, which carries the *ura3-364* allele, were UV irradiated, and the average frequency of *URA3* revertants was determined from results of three independent experiments. Spontaneous mutation frequencies ranged from 0.05×10^{-7} to 0.2×10^{-7} for the various experiments and were similar for the different strains. The standard error is shown for each determination. No UV-induced revertants were observed for the *rad30*Δ *rev3*Δ strain at 5, 10, and 15 J/m^2. Also, few or no UV-induced revertants were observed for the *rev3*Δ strain.

more mutagenic than the CC photoproducts (1, 3, 6). This may be due to the more frequent formation of (6-4) photoproduct at the TC site than at the CC site (2, 4, 5). UV-induced reversion of *ura3-364* was four- to fivefold higher in the *rad30*Δ strain than in the wild-type strain (Fig. 4), indicating a role for Polη in the accurate bypass of CC photoproducts. Again, few or no UV-induced revertants were formed in the *rad30*Δ *rev3*Δ strain (Fig. 4). In both the wild-type and *rad30*Δ strains, as was observed for the TC sequence, reversion of the CC sequence also occurs by a 3′ C→T change, indicating the incorporation of an A opposite the 3′ C (Table 1). In two cases for the wild-type strain, however, a C→T mutation was additionally observed at the 5′ C in the CC sequence. The occurrence of a tandem CC→TT mutation was also observed for the *rad30*Δ strain (Table 1).

DISCUSSION

We have developed a genetic system to measure the frequency of UV-induced mutations at the 3′ C of 5′-TC-3′ and 5′-CC-3′ sequences and find that the incidence of these mutations is about fivefold higher for the *rad30*Δ strain than for the wild-type strain. These data provide strong evidence for a role of Polη in the error-free bypass of UV lesions at TC and CC sites. The (6-4) photoproduct is formed more frequently at the TC site, and the *cis-syn* cyclobutane dimer is formed more frequently at the CC site (2, 4, 5); thus, these results indicate a role for Polη in the error-free bypass of cyclobutane dimers and of (6-4) photoproducts at these sites. Hence, the ability of Polη for error-free bypass of UV lesions is not restricted to the TT dimer.

These observations also have a bearing on the role of cytosine deamination in UV mutagenesis of CC and TC sites in eukaryotic cells. In *Escherichia coli*, such deamination has been proposed to account for the C→T transitions which then occur because of accurate insertion of adenines opposite uracils resulting from cytosine deamination (26). If Polη were to replicate through uracil-containing dimers by inserting an A opposite a U, as it does so efficiently for the TT dimer, in that case, inactivation of Polη should reduce the frequency of UV-induced mutations at the TC and CC sites. The fact that 3′ C→T transitions rise approximately fivefold in the *rad30*Δ strain indicates that Polη bypasses UV lesions at these sites by inserting the correct nucleotide G opposite the Cs. Also, the almost absolute requirement of *REV3*-encoded Polζ for C→T transitions at these sites points to the active involvement of this polymerase in the mutagenic bypass of TC and CC photoproducts wherein an A is incorporated opposite the 3′ C. Thus, in eukaryotic cells, cytosines in dimers must persist long enough for Polη- and Polζ-dependent damage bypass to occur.

A number of physical studies have indicated that a *cis-syn* TT dimer has only a modest effect on DNA structure, and this distortion does not affect the ability of the two Ts in the dimer to base pair with As (7, 11, 18). By contrast, a (6-4) TT photoproduct induces a large structural distortion in the DNA helix, and the 3′ T in this lesion is perpendicular to the 5′ T (17,

TABLE 1. Sequence analysis of UV-induced (20 J/m^2) revertants for wild-type (RAD^+) and *rad30*Δ yeast strains

Strain	Genotype	*ura3* allele	Reversion type	No. of revertants/total
YSL903	Wild type	*ura3-210*	C→T	32/32
YSL905	*rad30*Δ	*ura3-210*	C→T	32/32
YSL911	Wild type	*ura3-364*	CC→CT	30/32
			CC→TT	2/32
YSL913	*rad30*Δ	*ura3-364*	CC→CT	27/28
			CC→TT	1/28

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20). Nuclear magnetic resonance studies have also indicated that the O2 carbonyl of the 3′ T in the (6-4) TT lesion can form a stable hydrogen bond with the imino and amino protons of an opposed G residue (20). The (6-4) lesion at the TC site is expected to be structurally very similar to that at the TT site, and the O2 carbonyl of the 3′ C in the TC (6-4) lesion is also predicted to form a hydrogen bond with the G residue. Incorporation of a G opposite the 3′ C by Polη would result in error-free bypass of a TC (6-4) lesion. In support of this premise, our steady-state kinetic studies with purified yeast and human Polη indicate that they both insert a G opposite the 3′ T of the (6-4) TT photoproduct fairly efficiently, but Polη is unable to insert the subsequent nucleotide; Polζ then inserts an A opposite the 5′ T of the lesion, thereby completing the bypass process (R. E. Johnson, L. Haracska, S. Prakash, and L. Prakash, unpublished observations). Similarly, opposite a (6-4) TC photoproduct, we expect Polη to insert a G opposite the 3′ C and Polζ to extend by inserting an A opposite the 5′ T of this lesion. The sequential action of Polη and Polζ will then coordinate the error-free bypass of (6-4) TC photoproducts. Thus, although Polη would function in an error-prone manner in the bypass of the rare (6-4) TT photoproduct, it would promote error-free bypass of the more frequently formed (6-4) photoproducts at the TC site and those formed at the CC site. The insertion of a nucleotide by Polη opposite the 3′ site in the (6-4) photoproduct suggests that its active site can conform to the severe distortion conferred upon DNA by this lesion.

By analogy to the efficient and accurate bypass of a *cis-syn* TT dimer, we expect Polη to bypass a *cis-syn* CC or TC cyclobutane dimer by inserting the correct nucleotides opposite the two residues of the dimer. The ability of Polη to promote accurate bypass of a *cis-syn* cyclobutane dimer or a (6-4) photoproduct at the TC and CC sites shows that Polη is not an A rule polymerase, inserting an A opposite UV lesions by default. Further, our observation that UV-induced reversion at TC and CC sites in both the wild-type and *rad30*Δ strains occurs by a 3′ C→T mutation indicates that a DNA polymerase other than Polη is responsible for the incorporation of an A residue opposite the 3′ C of photoproducts. Although, and as expected, UV-induced mutagenesis at both the TC and CC sites is nearly abolished in the *rev3*Δ or the *rad30*Δ *rev3*Δ strains, *REV3*-encoded DNA polymerase ζ may not be the enzyme which inserts the A residue opposite the 3′ C in these photoproducts, because Polζ is highly inefficient at inserting nucleotides opposite the 3′ site of these lesions (15). Instead, Polζ functions in the subsequent step of elongating from the base incorporated opposite the lesion by another DNA polymerase (15).

Yeast and human Polη resemble each other in structure and function, and steady-state kinetic analyses have indicated that they both bypass a *cis-syn* TT dimer with the same efficiency and fidelity as through undamaged Ts (16, 27). Genetic studies presented here support a role for yeast Polη in the error-free bypass of TC and CC photoproducts, and they suggest a similar role for human Polη. Thus, by preventing UV mutagenesis at a wide spectrum of dipyrimidine sites, Polη plays an indispensable role in minimizing the incidence of sunlight-induced skin cancers in humans.

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S.-L.Y. and R.E.J. contributed equally to this work.
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