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UV-induced T→C transition at a TT photoproduct site is dependent on *Saccharomyces cerevisiae* polymerase η *in vivo*

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ABSTRACT

UV-induced reversion of the *arg4-17 ochre* allele in *Saccharomyces cerevisiae* is largely dependent on translesion polymerase η (Rad30p), known to bypass cyclobutane-type TT dimers in an error-free fashion. *arg4-17* locus reversion was predominantly due to T→C transition of T127, the 3′ T of a TT photoproduct site. This event was at least 20-fold reduced in a *rad30* deletion mutant, irrespective of the status of nucleotide excision repair. These data correlate with known properties of 6–4 TT photoproducts and *in vitro* characteristics of polymerase η and suggest that polymerase η plays an important *in vivo* role in inserting G opposite the 3′ T of 6–4 TT photoproducts at this site. Alternatively, an unprecedented error-prone processing of cyclobutane-type photoproducts at this site by polymerase η must be assumed as the critical mechanism. Whereas photoreactivation results indeed hint at the latter possibility, a possible regulatory influence of reducing the overall UV damage load on the bypass probability of non-cyclobutane-type pyrimidine dimer photoproducts should not be dismissed.

INTRODUCTION

Apart from various ways of repairing DNA damage, prokaryotic and eukaryotic cells have developed mechanisms to tolerate base damage in order to restore replication competence and to increase the time window available for bona fide DNA damage repair (1). In recent years, several eukaryotic DNA polymerases have been characterized that are capable of translesion synthesis past replication-arresting DNA damage in the template strand, such as UV-induced dipyrimidine lesions (for recent reviews see 2–5). These include cyclobutane-type pyrimidine dimers (CPDs) and the less frequent pyrimidine (6–4) pyrimidone adducts (1). In yeast, polymerase ζ, a complex of proteins Rev3 and Rev7, is required for virtually all damage-induced mutagenesis and thus is considered an error-prone bypass polymerase. *In vitro*, polymerase ζ is indeed capable of bypassing cyclobutane-type *cis-syn* thymidine dimers (6,7). However, given the low bypass efficiency of polymerase ζ alone, it has been suggested that polymerase ζ is primarily required for extension of an imperfectly paired primer following the initial step of error-prone base insertion opposite a thymidine dimer, catalyzed by another as yet uncharacterized polymerase (8). In contrast, polymerase η alone can efficiently bypass a TT cyclobutane-type dimer *in vitro* in an error-free mode, by inserting AA as frequently as with an undamaged template (9,10). Both polymerases are evolutionarily conserved. As predicted, compromising human polymerase ζ does indeed reduce UV mutability (11). Inactivation of polymerase η is the basis for the xeroderma pigmentosum variant (XP-V) syndrome (12–14), characterized by UV sensitivity, altered UV dimer bypass, cancer susceptibility and cellular hypermutability (15–18). Yeast, human and *Drosophila* polymerases η have been extensively characterized *in vitro* and the picture of a low fidelity polymerase emerges whose active site can accommodate base pairs of imperfect Watson–Crick geometry while its base insertion preference is determined by remaining capabilities for formation of hydrogen bonds (10,19–29). Recent structure determination of yeast polymerase η has supported this assumption (30).

Given the characterized error-free bypass of UV-induced TT dimers *in vitro*, hypermutability of a yeast *rad30* deletion mutant is predicted. However, the available *in vivo* studies indicate a dependency of the mutational outcome on the system used. Expected UV hypermutability has indeed been demonstrated in a forward mutation system (*can1*) (31) and in reversion systems (*ura3-210* and *ura3-364*) that detect exchanges opposite TC and CC photoproduct sites (32). On the other hand, UV reversion of *amber* allele *trp1-1* was only enhanced in a *rad30* mutant if another error-free bypass pathway was inactivated at the same time (33). Even more surprising, our own data indicated that a deletion of *RAD30* lowered UV-induced mutation frequencies for locus-specific reversion of the *arg4-17 ochre* allele (34). In this study, we present a molecular analysis of UV-induced mutations at the *arg4-17* locus and correlate these findings with recent *in vitro* results on the bypass of photoproducts by polymerases η and ζ.

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MATERIALS AND METHODS

Yeast strains

The wild-type strain used was WS3002/5 (MAT**a** *ade2-1 arg4-17 his3-Δ200 trp1-289 ura3-52 leu2-3,112*). An isogenic *rad30Δ::KanMX4* derivative was constructed by recombination-mediated transplacement using a *RAD30* deletion fragment. The latter was isolated by PCR from an existing *rad30Δ* strain obtained from a yeast strain repository (Euroscarf). Replacement of *RAD14* by *HIS3* has been described elsewhere (35). Yeast transformation was performed by the lithium acetate method (36).

UV treatment

Cells were grown for 48 h to stationary phase in YPD (1% yeast extract, 2% peptone, 2% dextrose) at 30°C, washed with deionized water, briefly sonicated and plated on synthetic medium. All medium ingredients were from US Biological (Swampscott, MA) except where indicated. Solid medium contained 0.67% yeast nitrogen base without amino acids, 4% dextrose and 1.4% agar, supplemented with 740 mg/l SD-Arg mix (Bio101, Vista, CA). If required, arginine sulfate was added to a final concentration of 10 μg/ml for survival plates or 1 μg/ml for selection plates allowing a limited number of cell divisions (37). Aliquots of 8×10^6 cells were plated per mutant selection plate and appropriate dilutions of the same cell suspension were spread on plates used for determination of colony survival. Plates were irradiated with a germicidal 254 nm UV lamp and incubated for 7–10 days at 30°C to allow for red pigmentation of colonies. Only red mutant colonies were counted. For photoreactivation, plates were treated immediately after UVC treatment with 6 kJ/m^2 at 360 nm (Sylvania, Blacklite Blue). Data shown represent the average of four independent experiments.

Sequence analysis

UV-induced mutant colonies were expanded in YPD and chromosomal DNA was prepared from zirconium bead lysates (38). PCR of the *ARG4* region was perfomed using primers CAGA-ACTCTGAGATAAGACTACC (forward) and GCTTCAAT-ACAGCAGATTTAGCG (reverse). Purified PCR products were sequenced by the Emory University Sequencing Core Facility using the listed forward primer as a sequencing primer.

RESULTS

Influence of *RAD30* on UV-induced *arg4-17* reversion

We compared UV-induced locus-specific *arg4-17* reversion in a *rad30* deletion mutant to an isogenic wild-type. Significantly reduced reversion frequencies were found and we confirmed our initial observations of an extended dose range (Fig. 1A and B) (34). At low doses of UV, the difference appears to be less pronounced. We observed a quantitatively similar effect of *RAD30* on *arg4-17* reversion in cells deleted for the nucleotide excision repair (NER) gene *RAD14*, involved in damage recognition (1). As compared to the *rad14Δ* single mutant, a *rad14Δrad30Δ* double mutant exhibited notably enhanced UV sensitivity of colony formation (Fig. 1C). *arg4-17* reversion frequencies were determined under two conditions: on selection medium without any arginine or augmented with 1 μg/l arginine. In contrast to the wild-type, the recovery of UV-induced *ARG*$^+$ revertants in NER-deficient cells is increased if selection plates are supplemented with a small amount of arginine, thus permitting a few rounds of replication on the plate (37). While confirming the published effect of enabling post-irradiation replication, a similar degree of reduced mutability in *rad30Δrad14Δ* versus *rad14Δ* was evident both on completely arginine-free and on supplemented plates (Fig. 1D).

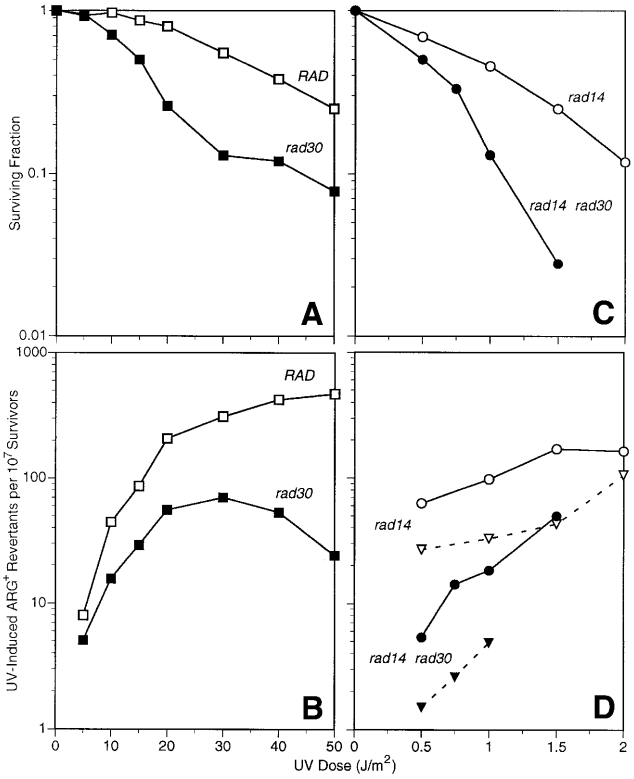

Figure 1. Survival (**A** and **C**) and induced *arg4-17* locus-specific reversion frequencies (**B** and **D**) in UV-irradiated stationary phase cells of *RAD* (open squares), *rad30Δ* (closed squares), *rad14Δ* (open circles and open triangles) and *rad14Δrad30Δ* (closed circles and closed triangles) genotype. Mutant frequencies in NER-deficient cells (D) were measured on plates without any arginine (open and closed triangles) or with a trace amount of arginine (open and closed circles).

Influence of photoreactivation on UV-induced *arg4-17* reversion

When treated with photoreactivating light after UV irradiation, survival and mutability of the *arg4-17* locus were subject to substantial reactivation (Fig. 2A and B). When mutation frequencies were plotted against survival, mutation frequency values of photoreactivated and non-photoreactivated cultures seem to follow an identical curve and thus the effect of photoreactivation on *arg4-17* reversion and survival appears to be quantitatively the same (Fig. 2C). No significant photoreactivation was determined in identically treated cells if the strain had been deleted for the gene *PHR1* encoding yeast photolyase (not shown).

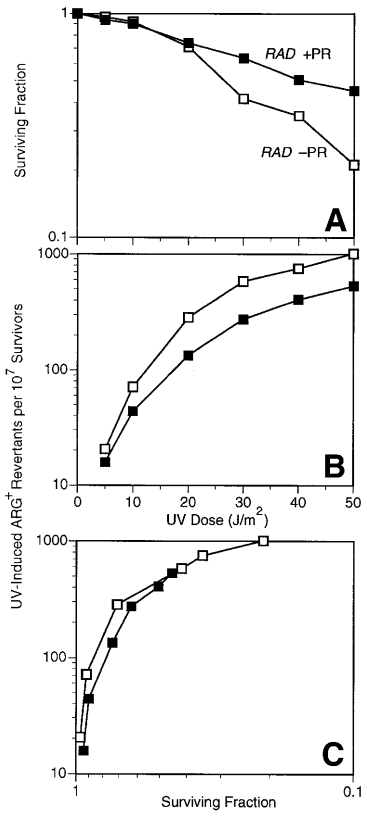

Figure 2. Survival (**A**) and induced *arg4-17* locus-specific reversion frequencies (**B**) in UV-irradiated stationary phase wild-type cells with (closed squares) or without (open squares) photoreactivation (PR). To compare the relative effectiveness of PR on survival versus mutagenesis, induced mutation frequencies were plotted against corresponding survival levels (**C**).

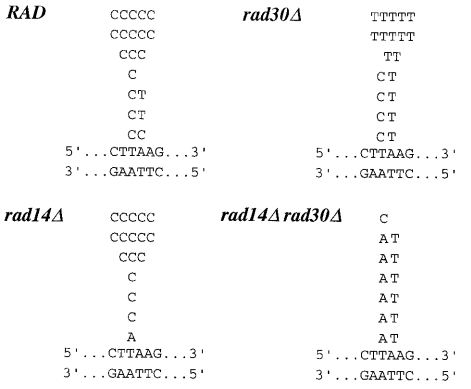

Figure 3. Identified base substitutions at the *arg4-17* locus in UV-induced *ARG*$^+$ clones isolated following treatment with 30 (*RAD* or *rad30*Δ) or 1 J/m^2 (*rad14* or *rad14*Δ*rad30*Δ, isolated from plates with arginine supplementation). Letters above the double-stranded *arg4-17* sequence reflect the types and numbers of detected substitutions. Originally, the *arg4-17* mutation resulted from an AAA (Lys)→TAA (*ochre*) exchange, with TAA corresponding to bases 127–129 of the *ARG4* open reading frame.

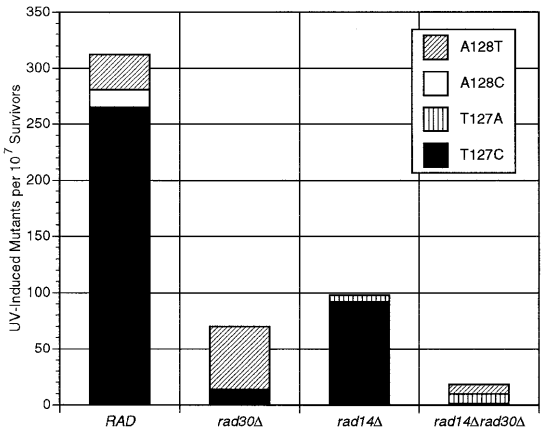

Figure 4. Frequency of UV-induced substitutions at T127 and A128 in *RAD* and *rad30*Δ cells at 30 J/m^2 or in *rad14* and *rad14*Δ*rad30*Δ cells at 1 J/m^2, calculated from overall reversion frequencies (Fig. 1B), and the relative proportions of different misinsertion events (deduced from the sequenced sample shown in Fig. 3).

Sequence of UV-induced base substitutions

We identified the previously uncharacterized chromosomal *arg4-17 ochre* mutation by sequencing of a PCR product encompassing the *ARG4* open reading frame. The *arg4-17* mutation was mapped to codon 43 and was found to originate from an AAA→TAA exchange (lysine to *ochre*). In order to determine the base pair exchanges in UV-induced mutants, we sequenced this region in 20 independent mutant clones each from the wild-type and the *rad30* deletion strain, induced by 30 J/m^2 UV (Fig. 3). In the wild-type, by far the most frequent UV-induced base substitution was identified as a T127→C transition, the only type of event recovered at this position. Additional substitutions occurred at A128, where transversions were found. Both positions (on opposite strands) correlate with potential sites of TT photoproduct formation. The percentages of UV-induced T127 and A128 substitutions in the wild-type were 85 and 15%, respectively. In striking contrast, we found a reversed bias in the *rad30* deletion mutant, with 20% T127→C transitions and 80% A128→T transversions. If one considers the different frequency levels, UV-induced base substitutions at T127 occured at an ~20-fold lower frequency in the *rad30* mutant than in the wild-type (Fig. 4). In contrast, the probability of substitutions at A128 remained essentially unchanged or may even be slightly increased.

Essentially the same effect of *RAD30* on base insertion preference was found in a NER-deficient background (*rad14*Δ versus *rad14*Δ*rad30*Δ) (Fig. 3). In *rad14*Δ, 16 of 17 sequenced mutations recovered at 1 J/m^2 UV were T127→C transitions. In *rad14*Δ*rad30*Δ, this event was found only once among 13 sequenced mutations, whereas six were A128→T transversions. Interestingly, the remaining six mutations were identified as T127→A transversions, events we have not recovered in the NER-proficient background. Again, if different frequency levels are considered, UV-induced T127→C events are specifically and drastically reduced in a *rad30* mutant background (Fig. 4).

DISCUSSION

Reversion of the chromosomal *ochre* allele *arg4-17* has been frequently used in the past for *in vivo* studies of damage-induced mutagenesis in the yeast *Saccharomyces cerevisiae* (39,40). One reason lies in the convenience of identifying

tRNA suppressor mutations that constitute virtually all of the (moderate) spontaneous background and are only weakly induced by 254 nm UV treatment. These can be eliminated from analysis by use of *ade2-1* as a second *ochre* marker in the same strain. Suppressor mutants will most likely suppress both markers and form white colonies, while locus-specific revertants develop red pigmentation due to the unsuppressed *ade2-1* allele. Indeed, we never recovered red colonies that carried mutations outside the *arg4-17* locus.

While we have not extensively studied the selection bias intrinsic to this mutational system, certain conclusions can be drawn from our sequence analysis. The amino acid residue position in question can apparently accommodate a variety of different amino acids and restoration of the original wild-type sequence (Lys43 = AAA) was rarely found among the sequenced mutations. We could not correlate growth characteristics on selective medium with a particular type of mutation. Importantly, an A128→G transition will result in another termination codon and will thus remain undetectable. The third base of codon 43 (A129), opposite the 5′ T of a potential TT photoproduct site, was never found to be mutated.

Inactivation of polymerase η (Rad30p) reduces UV mutability at the *arg4-17* locus significantly and over a wide range of doses (Fig. 1). The apparently less pronounced difference at low UV doses may correlate with the UV inducibility of the *RAD30* transcript (33,34). The mutagenic effect of *RAD30* is specific for UV damage, since a similar influence was not observed for various chemical agents in the same mutational system (34; unpublished data). The influence of *RAD30* is even more striking if one considers the molecular nature of the underlying base substitutions (Fig. 3). Sequence analysis indicated UV-induced misinsertion opposite the 3′ residues of two potential TT dimer sites on opposite strands. G insertion leading to a T127→C transition, by far the most frequent event in the wild-type, is reduced ~20-fold in the *rad30* deletion mutant (Fig. 4).

The notion that overall UV mutability in yeast is critically determined by CPDs is primarily supported by experiments involving photoreactivation that reduces the amount of CPDs specifically. For example, photoreactivation can dramatically lower UV-induced mutation frequencies in a forward mutation system involving multiple photodimer sites, such as the plasmid-borne *SUP4o* system (41). This does not preclude a locally critical importance of other photoproducts. Although by far the most frequent UV base damage, studies with defined single UV photoproducts in plasmid substrates have revealed that *cis-syn* cyclobutane-type TT dimers in yeast are only weakly mutagenic and are easily bypassed if compared to 6–4 dipyrimidine photoproducts (42,43). Interestingly, the event observed here, preferential G insertion opposite the 3′ T of a TT photoproduct, has been observed specifically for the bypass of 6–4 TT photoproducts in *Escherichia coli* (44) and in yeast (45). Recent nuclear magnetic resonance analysis has explained such insertion preference by mispair stabilization due to hydrogen bonding (46). In another yeast chromosomal forward mutation system, a substantial fraction of UV-induced substitutions were indeed T→C transitions involving the 3′ T of a TT site (47).

The observed mutational specificity and influence of bypass polymerases at the preferred *arg4-17* reversion site are in excellent agreement with *in vitro* studies on the bypass of 6–4 TT photoproducts by purified polymerases η and ζ (19,24). Rad30p has been shown to preferentially insert G opposite the 3′ T of a 6–4 TT photoproduct and thus appears to be guided by the base pairing properties of this residue. Thus, our *in vivo* results appear to directly reflect these *in vitro* properties of purified Rad30p if 6–4 TT photoproducts at T126/T127 are assumed as the primary mutagenic lesions. G insertion opposite the 3′ T residue of the potential 6–4 TT product site is indeed largely (but not completely) dependent on *RAD30*, whereas other types of misinsertion that are detected at the neighboring TT site on the opposite strand are clearly not diminished.

However, Rad30p is incapable of extending from the inserted base, which can be accomplished by polymerase ζ (19,48). According to one study, polymerase ζ is incapable of base insertion opposite the 3′ residue (19). Another study suggested that this event is possible, but without a preference for G insertion (48). Indeed, all UV-induced *arg4-17* reversion events are strictly dependent on Rev3p (40) and thus, as predicted by the *in vitro* study, on polymerase ζ, due to its role in extension. Interestingly, Rev1p also plays a major role in UV-induced *arg4-17* reversion (40) and both Rev1p and Rev3p were indeed required for 6–4 TT photoproduct bypass if single photoproducts were assayed in duplex plasmid vectors (43).

Surprisingly, the photoreactivation experiments did not confirm this interpretation. Whereas the yeast photolyase acts specifically on CPDs, UV mutability at the *arg4-17* locus is subject to substantial photoreactivation in NER-proficient (Fig. 2) and NER-deficient cells (37; data not shown) and the effect is not different from the effect on overall survival. We suggest two possible explanations. (i) Whereas relevant premutagenic lesions are indeed (mostly) 6–4 TT photoproducts, the reason for reduced mutagenicity following photoreactivation treatment is a consequence of cellular regulation responding to a lowered amount of overall UV damage. For instance, photoreactivation could conceivably reduce the UV-inducible *RAD30* transcript level (33,34) and thus the amount of enzyme available for mutagenic bypass. One might also want to consider that UV mutagenicity is positively influenced by checkpoint proteins (49), and their activity could be influenced by the overall damage level. (ii) CPDs constitute the relevant premutagenic lesions at T126/T127 that for some unknown reason are subject to a predominantly error-prone bypass by polymerase η. G insertion opposite the 3′ site of a *cis-syn* TT dimer can indeed be catalyzed *in vitro* (10). However, one would have to postulate that either the error-free bypass activity of polymerase η is non-functional or less active at this locus or that other normally active error-prone bypass polymerases are compromised. We noted a high potential for secondary structure within this region (Fig. 5) which may constitute an unusual environment for translesion synthesis. However, Rev3p clearly plays a major role and we also find a significant pro-mutagenic role of polymerase δ by analysis of a *pol32* mutant (data not shown) (50).

The second dimer site in our system gives rise to different types of misinsertions (Fig. 2) and the fact that a deletion of *rad30* leads to a slight increase in mutagenicity at this site is consistent with a role of CPDs. (However, it should be noted that G insertion opposite the 3′ T and thus the most frequent consequence of 6–4 TT photoproduct bypass is not detectable at this position, since another stop codon is created.)

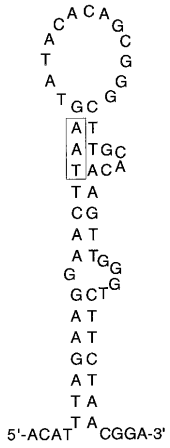

Figure 5. Hypothetical secondary structure following DNA strand separation surrounding the *arg4-17* locus (indicated as boxed TAA).

As expected for repair proteins functioning in separate competing pathways, a *rad30*Δ*rad14*Δ double mutant exhibited synergistically enhanced UV sensitivity as compared to *rad14*Δ (Fig. 1C). Again, a lack of UV-induced T127→C transitions was observed if *RAD30* was deleted in the NER-deficient strain (Fig. 3). Thus, the influence of Rad30p on mutational frequency and specificity appears to be largely independent of NER, the major error-free pathway of UV damage repair, and seems to reflect primarily the initial damage type and incidence. It is worth noting that NER is known to have a profound influence on timing of mutational fixation at the *arg4-17* locus. The dependency of efficient UV-induced reversion on post-irradiation replication in NER-deficient cells has been interpreted as S phase-dependent mutational fixation, in contrast to a largely pre-S phase fixation in the wild-type, most likely during repair synthesis (37). The influence of Rad30p remains essentially the same in both situations and, in spite of different timing, the same basic molecular mechanism may be operating.

However, NER does seem to have an effect on the processes that lead to UV-induced substitution of T127, especially in the absence of polymerase η (Fig. 3). Misinsertion of T leading to a T127→A transversion is detected in NER-deficient but not NER-proficient cells. This could indicate that different polymerases are engaged in translesion synthesis that occurs in the absence of Rad30p.

In summary, the initially puzzling observation of a mutagenic activity of the 'error-free' bypass polymerase η in a specific reversion system could be readily explained by the known mispairing properties of 6–4 TT photoproducts and their effect on translesion synthesis in yeast. Alternatively, an unprecedented predominantly error-prone bypass of a TT CPD by polymerase η at this specific dimer site must be assumed. Whereas photoreactivation ability indeed hints at the latter possibility, we caution that an overall reduced photoproduct level may influence regulatory responses and thus indirectly reduce mutagenic bypass probabilities for non-CPD products. Further experimentation is clearly required and may even result in a general re-evaluation of photoreactivation effects in UV mutation studies in yeast.

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